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SA-4-1BBL AS A PLATFORM TO DEVELOP ADJUVANT SYSTEMS FOR
PROPHYLACTIC AND THERAPEUTIC VACCINES

By

Gunes Dinc
B.S., Uludag University, 2007
M.S., University of Louisville, 2011

A Dissertation
Submitted to the Faculty of the
School of Medicine 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, KY

May 2014

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A Dissertation Approved on

March 13, 2014

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DEDICATION

This dissertation is dedicated to my parents

Mrs. Ozcan Dinc and Mr. Kerim Dinc

and my fiancé Mr. Onur Akbulut

who have given me invaluable support, understanding, and eternal love from overseas,

and

waited for me with patience.

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ABSTRACT

SA-4-1BBL AS A PLATFORM TO DEVELOP ADJUVANT SYSTEMS FOR PROPHYLACTIC AND THERAPEUTIC VACCINES

Gunes Dinc

March 13, 2014

Vaccines against infectious diseases are one of the most critical accomplishments in modern medicine. Despite significant progress in vaccinology, there is still a dire need for developing vaccines against various acute and chronic infections and cancer. In general, vaccines are categorized as prophylactic, given to healthy individuals to prevent disease, and therapeutic, administered to people who already have disease. As such, the nature, quality, and quantity of immune responses required for the efficacy of these two types vaccines are different. Prophylactic vaccines against infectious diseases primarily rely on the generation of neutralizing high titers of antibody for their efficacy. These vaccines are generally effective because they target a host with an unaltered and competent immune system. In marked contrast, the efficacy of therapeutic vaccines has been a major challenge since they are administered into a host with a compromised immune system. Therapeutic vaccines need not only to generate effective adaptive cellular, particularly CD8⁺ T cell, immune responses to chronic infection and cancer, but they also need to overcome various immune evasion mechanisms employed by infection

and progressing tumor. For both types of vaccines, the generation of a long-lasting adaptive immunity is the key.

Historically, prophylactic vaccines against infections were made from live-attenuated or inactivated forms of the microbes, but there were concerns about stability, side effects and safety of such vaccines. Advancements in molecular biology and DNA technologies led to the development of recombinant subunit vaccine with well-defined antigens. In particular, vaccines based on recombinant proteins present an attractive approach because of their ease of production, storage, distribution, and safety profiles. However, recombinant protein based subunit vaccines are poorly immunogenic and require adjuvants for efficacy. Most of the adjuvants that have been approved for clinical use, and those under development primarily target innate arm of the immune system for the generation of subsequent adaptive immunity. Key to the initiation of adaptive immune responses is the interactions between an APC and T cells and acquisition of 3 distinct signals by T cells. Signal 1 is delivered by the interaction of TCR on T cells with an MHC/peptide complex on APC. This signal is then qualified by costimulatory receptor ligand interaction on the APC and T cells, providing signal 2. Signal 3 is provided by various cytokines elaborated by activated APCs and T cells and critical for the expansion of the immune response. The lack of costimulation during these interactions results in T cell anergy or apoptosis. Costimulation is not only important for the generation of adaptive immunity, but also is involved in the regulation of the various immune evasion mechanisms employed by cancer and chronic infections. Therefore, we hypothesized that costimulatory ligands may serve as the preferred adjuvants for generating effective and long-lasting adaptive immunity. We particularly focused on the natural costimulatory

ligands of tumor necrosis factor (TNF) family given their pleiotropic function on cells of innate, adaptive, and regulatory immunity. The TNF family represents a critical group of costimulatory molecules since their receptors (TNFR) are inducibly expressed on activated cells and may serve as preferred targets for antigen specific responses through induction of expansion, survival of T cells and establishment of long term memory. Among these family members, 4-1BB/4-1BBL interaction has received the most attention as signaling through 4-1BB provides essential signals for CD8⁺ T cell expansion, effector function, and survival. Importantly, this signaling also endows effector CD8⁺ T cells resistant to suppression by regulatory T cells that are the predominant mechanism of immune evasion used by cancer and chronic infections.

Since 4-1BBL has costimulatory function as a cell surface membrane-bound protein and has no function in soluble form, our laboratory has previously generated a novel form of this molecule chimeric with streptavidin, SA-4-1BBL. This molecule was demonstrated to have robust costimulatory activity with a Th₁ bias as a soluble protein. The main premise of this PhD thesis is to use SA-4-1BBL as an adjuvant platform to develop adjuvant systems for subunit vaccines with desired immune activities for targeted indications. We particularly focused on subunit vaccines against two indications; *Y. pestis* and breast cancer for the development of prophylactic and therapeutic vaccines, respectively. First, we tested if SA-4-1BBL can improve the immune efficacy of a lead subunit vaccine, rF1-V (a recombinant *Y. pestis* fusion protein), adjuvanted with alum with a Th₂ bias against plague. Inasmuch as the lead candidate vaccine generates a Th₂ response, and Th₁ cellular responses have been shown to be important in protection against *Y. pestis* infection, we hypothesize that SA-4-1BBL as a Th₁ adjuvant will

improve the immune efficacy of the lead candidate vaccine. Single immunization with a vaccine formulation containing rF1-V as antigen and SA-4-1BBL as single adjuvant generated increased TNF α and IFN γ signature cytokines for Th₁ responses in both CD4⁺ and CD8⁺ T cells without detectable antibody titers against rF1-V. This vaccine formulation protected 20% of mice against bubonic plague. However, in a prime-boost setting, SA-4-1BBL and rF1-V generated long lasting high titers of antibodies and protected all mice from bubonic *Y. pestis* infection. Alum adjuvanted rF1-V vaccine generated high titers of antibodies against rF1-V without a significant Th₁ response, and protected 80% of mice against bubonic plague. A combination of SA-4-1BBL and alum as an adjuvant system generated a balanced Th₁ cellular and humoral responses that resulted in 100% protection in bubonic plague model.

Next, we tested if SA-4-1BBL has efficacy as adjuvant component of a Her-2/neu protein-based subunit vaccine against breast cancer and if the therapeutic efficacy of this subunit vaccine can further be improved by using toll-like receptor 4 (TLR4) agonist monophosphoryl lipid A (MPL) as adjuvant system. We hypothesize that MPL will work in synergy with SA-4-1BBL by targeting antigen presenting cell for activation, antigen presentation to T cells, leading to T cell activation and up regulation of 4-1BB receptor. Activated T cells will then serve as a direct target of SA-4-1BBL for expansion, acquisition of effector function, and establishment of long-term memory. A prime-boost immunization with extracellular domain of the rat Her-2/neu protein and SA-4-1BBL resulted in eradication of established Her-2/neu expressing A2L2 tumors in 10% of BALB/c mice. In contrast, MPL monotherapy did not have a therapeutic effect. However, vaccination with combined adjuvants resulted in eradication of established tumors in 30%

of BALB/c mice, and showed better therapeutic efficacy over individual therapies. Furthermore, immunization with combined adjuvants resulted in eradication A2L2 tumors in 20% of tolerogenic BALB/neuT mice. Depletion of Tregs prior to tumor challenge increased the efficacy of combined adjuvants to 40%. The therapeutic efficacy of combined adjuvant platform correlated with increased tumor specific killing response and pro-inflammatory cytokine IFN γ production. The combination of SA-4-1BBL and MPL achieved therapeutic efficacy in the absence of detectable toxicity as assessed by various indicators of toxicity, including liver enzymes, total number of various lymphocyte populations in several lymphoid tissues, vaccine-induced organ damage, and histological analysis of the liver.

Taken together, these data provide scientific rationale for using SA-4-1BBL as a novel adjuvant platform with other adjuvants having synergistic immune activities for the development of subunit vaccines against intracellular infections and cancer.

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CHAPTER 1

INTRODUCTION

Successful vaccine development has significantly reduced the rate of highly infectious diseases and human mortality. As such, viral vaccinations against measles, rubella, and poliomyelitis dramatically reduced the incidence of these diseases. Further, vaccination for small-pox successfully eradicated small-pox, one of the most deadly diseases in the world, while poliomyelitis is in the verge of eradication [1]. These critical accomplishments in medicine demonstrate the importance of immune therapy. The tremendous impact of vaccination on humans was greatly demonstrated by vaccinologist Stanley Plotkin when he stated: “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” [2]. While the development and worldwide use of such vaccines have had a dramatic impact on human health, still vaccines are not available for many infectious diseases and cancer.

The first human vaccine was developed against smallpox, and introduced by Edward Jenner in 1798 using related but weaker animal virus, cowpox [3]. However, the successful history of vaccination was started with Louis Pasteur when he first discovered

the attenuation of gram negative bacteria, *Pasteurella multocida* in 1881. Subsequently, chemically attenuated rabies vaccine was developed for humans by Louis Pasteur and Emile Roux in 1885 [3], which was followed by the development of numerous attenuated and inactivated viral and bacterial vaccines. While these vaccine strategies were effective, possibly because inherently they contain natural adjuvants, such as particulated proteins, oligonucleotides, and lipids [4], there were concerns about stability during storage, occurrence of adverse effects and safety, especially in immunocompromised individuals [5]. Therefore, in the last several decades, as a result of advancement in molecular biology and genetic engineering, numerous strategies, such as DNA vaccines, viral or bacterial vector based vaccines, and recombinant protein/peptide vaccines have been employed to generate new human vaccines.

Naturally, in the early phase of infection, microbial compounds activate the innate arm of immunity through recognition by pattern recognition receptors (PRRs) on antigen-presenting cells (APCs). This leads to activation of APCs and induces the production of proinflammatory cytokines and upregulation of costimulatory molecules on the cell surface, which in turn primes the long-lasting adaptive immunity mediated by T and B lymphocytes [6]. This process provides T cells with three important signals, signals 1, 2, 3 [7] for their activation, acquisition of effector function, and long-term immune memory. Signal 1 is mediated by T-cell receptor (TCR), recognizing foreign peptides in the peptide binding groove of major histocompatibility complex (MHC) molecules on the surface of cells. Signal 2 is provided by various costimulatory molecules, while signal 3 is mediated by cytokines, such as IL-1 β , and IL-12 produced by activated APCs [8]. Failure to receive the second signal can lead to T cell anergy or apoptosis. As a result, an

ideal vaccine should mimic the natural infection to generate a potent immune response with the induction of long-lived adaptive immune response.

Vaccine formulations should be chosen based on type of the treatment since prophylactic vaccines are given to healthy individuals to prevent infections and virally derived cancers. Generation of the long term immune memory, particularly humoral immunity, is the main goal. Otherwise, induction of an acute effector response may induce undesired side effects, such as inflammation, and may not provide protection for the future infections [9]. As a result, most of the traditional prophylactic vaccines against infections are targeting the generation of long-term antibody response. On the other hand, therapeutic vaccines are given to people with established infections and compromised immune system. Therapeutic vaccines primarily rely on CD8⁺ T cell responses for the elimination of viruses, such as HIV and herpes virus, intracellular bacteria, and cancer. Furthermore, generation of a long term memory is also critical for therapeutic tumor vaccines to control recurrences. For both prophylactic and therapeutic vaccines, induction of adaptive immunity which is mediated by T and B lymphocytes, and activation of innate immunity to shape the long-lasting adaptive immune response are crucial.

In addition to efficacy, some other criteria for new vaccine candidates for human use should be sufficed, such as; (1) safety, (2) stability, and (3) low cost for widespread use, especially for third world or developing countries. Finally, it is desirable that vaccine should not require frequent administrations [10]. Therefore, recombinant protein-based subunit vaccines containing well characterized single or a combination of proteins, fusion proteins, or peptides serving as antigens have been attractive strategies in the field of vaccinology in the last few decades since they can be administered safely and produced

easily in a cost effective manner. However, protein-based subunit vaccines are poorly immunogenic and need to be administered with immune stimulant components known as adjuvants to generate an adequate and efficient appropriate immune response with long-lived immune memory against target antigens [11, 12].

Adjuvants:

The first description of adjuvants (Latin word *adjuvare*, means “to help or aid”) was made by Ramon as “substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone” [13]. Adjuvants are a critical determinant of the success or failure of vaccines and the use of adjuvants as components of protein-based vaccine formulations can also reduce the amount of antigen needed for the generation of effective immunity and the number of vaccine administered. Because of the undefined nature of these adjuvants and their molecular complexity, it has been a challenge to fully understand mechanisms underlying their immune efficacy[5]. As a result, adjuvants have been described as “the immunologists dirty little secret” by Charlie Janeway.

Adjuvants are necessary to boost the immune response to naturally weak antigens. In 1920, the first adjuvants were developed and variety of substances, such as chemicals and microbial components, have been used to boost the immune system [14]. Adjuvant selection is made based on some parameters, including the physical and chemical nature of the antigen, type of desired immune responses, population age, and route of administration [4]. Historically, the importance of the humoral immune response for vaccine effectiveness has put the emphasis on the development of adjuvants, which are

capable of enhancing antibody responses. As a result, many adjuvants are effective in increasing antibody titers, but do not elicit significant Th₁ or cytotoxic T lymphocyte (CTL) responses [15]. However, for some diseases, such as malaria and tuberculosis, it is difficult to generate a broad antibody response to clear primary infection, and both humoral and cellular responses are required for protection from some viruses (e.g. HIV-1) and intracellular bacteria (e.g. *Y. pestis*). Development of therapeutic cancer vaccines face an even greater challenge since their target often consists of tumor associated self-antigens (TAA) which are poorly immunogenic and expressed heterogeneously by tumor cells that undergo mutation. Although tolerance to TAAs is not absolute since Her-2/neu TAA generates antibody and CTL responses in some breast and ovarian cancer patients [16, 17], these responses are not potent enough to eliminate the tumor cells as tumors continue to grow and metastasize. Therefore, induction of potent cellular immunity is critical for the generation of therapeutic immune responses for chronic infections and cancer, and more importantly for the induction of long-term memory response to control tumor recurrences [18].

Importance and role of vaccine adjuvants:

T cell responses are critical for protection from cancer and infectious diseases, elimination of infected and tumor cells, and the induction of long lasting memory response to control recurrences. Moreover, induction of T helper (Th) responses is also important to improve humoral and cellular response durability and quality. Recombinant protein antigens generate modest antibody responses while they induce little to no T cell responses when administered in the absence of adjuvants. As a result, adjuvants which

are capable of inducing protective levels of humoral, CTL and Th cellular responses have been used in recombinant protein based vaccines.

Adjuvants can induce high antibody responses to vaccine antigens by increasing not only overall antibody titers but also functional antibodies [19]. In addition, adjuvants can also broaden antibody response, which is critical for the elimination of many pathogens that are capable of mutating their antigens (antigenic drift), such as malaria, influenza virus, HIV, and HPV. For example, it has been reported that in influenza and HPV vaccines, adjuvants broaden the antibody response to target antigens [20, 21]. Induction of strong CTL responses has also been observed against target antigens using adjuvants [22]. As such, immunization with immunostimulating complexes (ISCOMs) and HIV-1gp160 envelope glycoprotein or influenza haemagglutinin generates long lasting antigen specific CD8⁺ T cell responses [23]. Further, QS-21 (saponin based adjuvant) has also been shown to induce CTL responses to subunit viral antigens such as HIV-1 gp120, and human cytomegalovirus gB, and induced neutralization of the respiratory syncytial virus (RSV) by heightening Th₁ driven IgG_{2a} antibody titers to virus fusion (F) protein [24].

Recombinant protein based sub-unit vaccines may require multiple administrations to elicit sufficient humoral immune response. Recruitment of adjuvants may reduce the number of immunizations or the amount of antigen used in the vaccine formulation. As such, it has been demonstrated that immunization with glucopyranosyl lipid adjuvant–stable emulsion (GLA-SE) and recombinant influenza H5 protein reduced the amount of antigen required for the generation of proper antibody response after one immunization more than 30-fold compared to antigen alone [25].

Currently FDA approved or adjuvants under development

With the development of recombinant vaccines (particularly recombinant protein based vaccines) that are less immunogenic, the investigation and design of new adjuvants have been the object of industry and academic in the last few decades. However, development of new adjuvants has been challenging due to their molecular complexity and lack of significant insight in their mechanisms of action [5]. The idea of using inactivated or live attenuated microbes in vaccine formulations is primarily based on their ability to activate innate immunity through activation of APCs, and presentation of exogenous antigens to T cells. As a result, several adjuvant candidates have been under development, such as oil emulsions, cytokines, or saponin-based adjuvants, and mineral salts such as aluminum hydroxide (alum) and less toxic version of bacterial lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), have already been approved by FDA. The adjuvants target primarily innate immunity through activation of APCs and uptake and presentation of antigens by these cells to T cells [26]. Finally, activation of innate immunity leads to the prime of adaptive immune responses where cellular arm plays an important role for clearance of infected and transformed cancer cells, while humoral arm clears pathogens through antibodies.

Adjuvants can be divided into three major groups according to their mechanisms of action, such as delivery vehicles, immunomodulators, and both delivery and immunomodulatory components [4]. Delivery vehicles do not contain immune stimulatory molecules, and instead they induce the desired immune response through (1) sustained release of antigen by forming an antigen depot and (2) increasing antigen uptake and presentation by APCs to increase the specific immune response to the antigen.

Vehicles include mineral salts, emulsions, liposomes or virosomes. On the other hand, immunomodulators directly act on the immune system by (1) up-regulation of cytokines and chemokines, (2) recruitment of specific cell types to the inoculation site, (3) activation and maturation of APCs by induction of the costimulatory molecule expression and signaling, and (4) activation of inflammasomes [13, 27]. Immunomodulators include saponins, cytokines, TLR ligands, and costimulatory molecules. Vehicles which also deliver immunostimulatory components are in the third group such as mineral salts, emulsions, and immunostimulating complexes (ISCOM) [28].

Delivery vehicles

Aluminum based adjuvants:

In human vaccines, the most widely used adjuvants are aluminum based adjuvants and FDA approved Alhydrogel was the first and only adjuvant until MPL approval in the context of the prophylactic HPV vaccine in 2009. Aluminum hydroxide or phosphate adjuvants are currently being used in Diphtheria, Tetanus, HBV and HPV vaccines. Alum acts by inducing the formation of an antigen depot at the injection site which has been shown to be critical in alum adjuvanticity [13] and slowly releases antigen to stimulate the immune system continuously, and enhance the antigen uptake [29, 30].

Alum is a potent adjuvant for the generation of antibody responses to target antigens and primarily induces Th₂ responses (IgG₁, IgE and the production of IL-4), rather than Th₁ responses in many of the traditional childhood vaccines against bacterial or viral surface molecules [29-31]. Since the induction of cellular immunity is critical for protection from cancer and infection, new vaccine approaches utilize another adjuvant,

MPL, along with alum (e.g., AS04) to induce the generation of cellular immunity, particularly Th₁, for optimal effectiveness [32]. Although alum based adjuvants have been shown to be efficacious, they are associated with severe inflammatory site reactions due to recruitment of blood cells, formation of granulomas, and increased allergenicity due to high IgE titers. Moreover, aluminum based adjuvants cannot be frozen and lyophilised [33, 34], limiting their widespread use.

Oil in water emulsions:

Particulate adjuvants, such as oil-in-water emulsions, are naturally utilized for uptake by APCs to induce potent immune responses. MF59 is the first oil-in-water adjuvant licensed for use in Europe for seasonal and pandemic influenza (H1N1 and H5N1) vaccines. It consists of the oil squalene encircled by non-ionic surfactants. Flud, a subunit influenza vaccine containing MF59 licensed in Europe by Novartis [35], has been distributed as more than 27 million doses since 1997 [36] and has shown to provide strong memory and sustained antibody responses compared to the non-adjuvanted vaccine. Aflunov is another influenza vaccine containing MF59 licensed by Novartis and marketed in 2010 to protect against H5N1 flu. MF59 has also been evaluated as an adjuvant in vaccines for other viral infections, such as HIV, hepatitis C, and cytomegalovirus.

MF59 generates its immune stimulatory activity by the induction of an inflammatory environment at the inoculation site characterized by enhanced cytokine and chemokine responses, which in turn induce the trafficking of CD11b⁺ and MHC II⁺ cells to the muscle [37]. Moreover, it is demonstrated that it targets macrophages and DCs at the inoculation site and draining lymph nodes, enhancing antigen uptake by forming

antigen depot [38], increasing antibody production against target antigen, and favoring a Th₂ response [39]. Although, oil in water emulsions were associated with generation of autoantibodies in non-autoimmune mice [40], MF59 adjuvanted influenza vaccines did not induce autoantibodies. However, emulsion adjuvants may induce side effects, such as inflammatory reactions, granulomas and ulceration at the inoculation site.

Immunomodulators

Saponin based adjuvants:

Saponins are natural glycosides of steroid or triterpene found in plants, lower marine animals and some bacteria [41]. They have the ability to modulate immune responses by different action mechanisms, such as antitumor, antiviral, antifungal, and anti-inflammatory [42]. They can induce not only antibody responses, but also cellular immune responses against target antigen with the advantage of the requirement of low doses needed for adjuvanticity. It has been demonstrated that saponins induce production of Th₁ cytokines (IL-2 and IFN- γ) and antibodies of the IgG_{2a} isotype, and they may interact with APCs to modulate immune responses [22, 43]. Most likely, saponins perform these stimulatory activities through the interaction with APCs. Although it is not known yet, the adjuvant effect of saponins might be related to their ability to induce pore formation. It is also likely that pore formation induces antigen uptake and presentation and subsequent activation of CTL responses [44].

The lead candidate saponin based adjuvants are Quil A and its derivative QS-21. QS-21 has been tested as an adjuvant initially for cancer vaccines (melanoma, prostate, and breast) and subsequently for infectious diseases (malaria, herpes, HIV-1, influenza, or hepatitis B) in clinical trials and showed promise [24]. Further, QS-21 adjuvant is also

being tested in ACC-001 vaccine for mild to moderate Alzheimer's disease in a clinical trial conducted by Pfizer since 2009 and in sialyl Lewis-keyhole limpet hemocyanin conjugate vaccine for metastatic breast cancer by NCI since 2007. The critical advantage of the use of Quil A and QS-21 in vaccine formulations is that they stimulate both Th₁ and CTL responses against vaccine antigens [23, 45, 46]. However, a balance of efficacy versus side effects is the key for these adjuvants since Quil A and QS-21 are highly toxic and have hemolytic activity due to their structure and cause hemolysis of the red blood cells which restricts their use in human vaccines [47].

Cytokines:

Cytokines are naturally derived substances that play an important role in controlling the immune system. They can improve both humoral and cellular immune responses by inducing antibody production, proliferation and differentiation of B and T cells, enhancing cytokine production and improving cytotoxicity activity of T cells [48]. IL-2 has been extensively studied as an adjuvant for vaccines due to its pleiotropic effects and critical role in T-cell responses. IL-2 is the first cytokine administered in clinical trials in cancer vaccines. However, its action mechanism depends on the form of administration. Continuous IL-2 administration increase the proliferation of antigen specific T cells and IL-2 in oil-in-water emulsion induces antibody response [49]. Although IL-2 has been used as an adjuvant in clinical trials for melanoma [50-54], it has been reported by NCI that melanoma vaccines given with IL-2 do not generate better anti-tumor responses than IL-2 alone [55]. However, dosing and timing of IL-2 administration still need to be well determined. Even though high doses or multiple administrations of IL-2 induce better clinical outcome, at high doses, the use of IL-2 is

associated with toxicity [56]. Furthermore, while IL-2 enhances cytotoxic activity of NK cells and CTLs, it can also induce the expansion of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs), which in turn contribute to tumor immune suppressive activity [57].

There are other cytokines, such as IL-7 and IL-21, have been tested in clinical trials against cancer. IL-7 does not stimulate expansion of Tregs or induce toxicity like IL-2, but it did not generate an anti-tumor effect either [58-60]. On the other hand, IL-21 showed anti-tumor activity [61], but it also has some limitations due to the expression of its receptor on multiple tumor cells [62, 63]. IL-15 is another cytokine under development by NCI to be tested in clinical trials [56].

Toll like Receptor (TLR) agonists:

Understanding the recognition of microbial structures by innate immunity and its important role in host defense against infection has showed rapid progress. Due to the critical role of TLR signaling in innate immunity and bridging innate immunity with adaptive immune responses, several well characterized and defined TLR ligands have been developed as adjuvants in human vaccines. TLRs are a family of pattern recognition receptors (PRR) which recognize the pathogen associated molecular patterns (PAMP), such as proteins, lipids, lipoproteins, and nucleic acids of microbes. TLR ligation induces the activation of APCs, and the production and release of proinflammatory cytokines, such as TNF, IL-1, IL-6, which in turn prime and activate antigen specific T cells.

Among all the members in this family, TLR4 agonists are the most developed adjuvants. TLR4 responds to LPS, an outer membrane component of Gram-negative bacteria. MPL, a derivative of LPS from *Salmonella Minnesota*, is the second adjuvant to be approved by FDA in the USA. It is currently used as one of the components of the

adjuvant system in the Cervarix (HPV) and Fendrix (HBV) vaccines from GSK along with L1 recombinant protein and hepatitis B surface antigen (HBsAg).

The TLR7/8 pathway plays an important role against viral infections which recognizes single stranded RNAs. TLR7 stimulation induces production of IFN α , while TLR8 stimulation leads to production of proinflammatory cytokines through NF-kB activation. TLR7 agonist imiquimod has been approved by FDA for topical administrations for the treatment of basal cell carcinoma (BCC) and actinic keratosis (AK) [27]. However, TLR7/8 agonist, resiquimod, has shown inconsistent results in clinical trials for genital herpes simplex virus (HSV)-2 infection [64].

TLR9 also plays an important role for recognition of pathogens and signaling through TLR9 leads to secretion of pro-inflammatory cytokines. TLR9 agonist CpG oligodeoxynucleotide (ODN) 1018 is the adjuvant in the Heplisav (Dynavax) vaccine candidate for HBV, which has completed a phase III clinical trial. It has been shown that Heplisav induces rapid and increased antibody production and sustains the antibody response in healthy adults [65]. It was recently announced that another TLR9 agonist adjuvant called IC31 is currently undergoing Phase I/IIa clinical trial for a candidate tuberculosis vaccine. In preclinical studies, it has been demonstrated that IC31 promotes highly efficient Th₁ responses through MyD88 signaling pathway [66].

Nevertheless, inappropriate TLR signaling may result in acute and chronic inflammation, and systemic autoimmune diseases, even though it is giving promising results in the clinic. Defects in the negative regulation of TLR signaling lead to development of such diseases. Furthermore, endogenous compounds released by dying

cells can stimulate TLR signaling, and contribute to inflammation and autoimmune diseases [67].

Costimulatory molecules:

For the generation of protective immune responses through vaccination, it is critical to activate antigen specific T cells. T cell activation is regulated by successive signaling events from first signal, TCR/MHC interaction; second signal, costimulatory molecules, and third signal, proinflammatory cytokines [8]. Failure to receive second signal can lead to T cell anergy or apoptosis. In early phase of natural infections, microbial compounds are recognized by PRRs on the APCs, which lead to generation of second and third signals for T cell activation [7]. However, these signals can also be provided by vaccines with the recruitment of adjuvants such as costimulatory molecules [68]. Costimulatory receptors could be divided into two super families; immunoglobulin family (IgSF) and tumor necrosis factor receptor family (TNFRSF). It has been proposed that for initial T cell activation signaling from IgSF is important, while TNFRSF costimulation is critical for T cell effector function, survival and memory [69, 70].

Use of costimulatory molecules targeting activation and effector function of T cell responses in vaccines is divided into two approaches, those enhance the antigen specific immune response or shape it for desired immunity, and others to block or remove negative regulatory mechanisms to generate potent immune responses [71]. In the case of poorly immunogenic tumors, such as B16 melanoma, both approaches have been utilized to generate better anti-tumor responses. For example, co-administration of agonistic antibody for 4-1BB receptor in TNFRF and blocking antibody for CTLA-4 in IgF along with B16-Flt3-ligand vaccine (FVAX), promoted B16 melanoma tumor rejection in a

synergistic manner [72]. Moreover, combined treatment with anti-DR5, anti-4-1BB, and anti-CD40 mAbs successfully eradicated established 4T1 mammary tumors [73].

Immunoglobulin superfamily:

In IgSF, perhaps the most well characterized and described members are CD28 and B7 families. The CD28 glycoprotein is constitutively expressed on naive CD4⁺ and CD8⁺ T cells, while its ligands B7-1 and B7-2 are inducibly expressed on the surface of APCs upon activation. CD28 ligation with B7-1 and B7-2 molecules provides essential signal for T cell activation, expansion and differentiation. Therefore, tumor cells transfected with B7 family members have been used in cancer immunotherapy to promote them to act as APCs and induce T cell activation [74, 75]. Moreover, CD28/B7 interaction prevents apoptosis of T cells and sustains their proliferation, which was determined by the upregulation of bcl-x_L gene expression [76].

The inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) expression is induced following T cell activation and subsequent to CTLA-4 upregulation, CD28 expression is downregulated. While the B7/CD28 interaction positively stimulates T cell responses, B7/CTLA-4 ligation negatively regulates these responses. To overcome the inhibitory mechanism of CTLA-4 for T cell responses which is critical for elimination of cancer cells and pathogen infected cells, antibodies targeting CTLA-4 have been developed. As such, Ipilimumab (Yervoy), a humanized monoclonal antibody (mAb), was developed by Bristol-Myers Squibb and approved by FDA in 2011 for the treatment of melanoma. A fusion protein consisting Fc region of IgG₁ and extracellular domain of CTLA-4 targeting B7 molecules on APCs, called Abatacept (Orencia), was also developed by Bristol-Myers Squibb and licensed in the US for the treatment of

rheumatoid arthritis [77]. Furthermore, another fusion protein called Belatacept, which differs from Abatacept with only two aminoacids, has also been approved by the FDA in 2011 to provide engraftment in transplantation [78]. The mechanism of these fusion proteins is to prevent T cell activation by binding to B7 molecules and blocking CD28 mediated co-stimulatory signaling to T cells.

Employment of the molecules targeting IgSF members has the common goal of targeting APCs, as a subsequent event they either induce or prevent T cell activation. Moreover, cytokines such as IL-2 and IFN- γ have also been utilized to target T cells by inducing their proliferation and responsiveness to the signals generated by APCs in response to antigen, but these treatments showed limited efficacy [79, 80]. Therefore, an alternative approach to directly target activated T cells is the use of TNFRSF members, due to their expression on only activated T cells and their important roles in the activation, acquisition of effector function, and establishment of long-term T cell memory.

TNF receptor superfamily:

Development of vaccines which are capable of inducing T cell responses, especially CTLs, is critical for infectious diseases and cancer. Regulation of Treg suppressive function is another important aim for immunotherapy. While in the case of autoimmune or inflammatory diseases, enhancing Treg numbers or activity is beneficial; in cancer or infectious diseases decreasing Treg numbers and suppressing their function is essential for the therapy [70]. In this context, TNFRSF play important roles in regulating both adaptive and regulatory immunity. Moreover, these receptors are inducibly expressed on activated T cells upon antigen stimulation, unlike CD28, which

gives another advantage to serve as adjuvant candidates for vaccines to generate antigen-specific immune responses [70]. Importantly, the expression of TNF ligands on the APCs are also induced after the receiving innate immune signals, such as TLRs, and adaptive signals, such as IFN- γ .

Targeting TNFR/TNFL interaction could be divided into two strategies: (1) Blocking these interactions to suppress the pathogenic immune responses in autoimmune and inflammatory diseases and (2) augmenting the signaling generated by TNFR ligation to stimulate the protective immune responses in cancer.

Different types of therapies, such as gene therapy, recombinant molecules, antibodies and Fc fusion proteins containing the TNFRSF ectodomains, targeting TNF ligands or TNFRSF members have been tested in the clinic and approved for human use. Infliximab (Remicade; Centocor Ortho Biotech), a chimeric TNF-specific antagonistic antibody, was the first drug approved in 1998 to treat RA, Crohn's disease, and ulcerative colitis [81]. Adalimumab was the first fully human TNF-specific antagonistic antibody approved in 2002 to treat RA, JIA, psoriatic arthritis, psoriasis, and Crohn's disease [81]. In the case of cancer, agonistic therapies and depleting antibodies have been evaluated in clinical trials. Tasonermin, a recombinant TNF molecule, and Brentuximab, depleting antibody specific for CD30 molecule have been approved for the treatment of sarcoma and Hodgkin's lymphoma, respectively [81]. Other agonistic antibodies targeting the members of TNFRSF, such as CD40, OX40, or 4-1BB, have been tested in clinical trials for cancer, but the main concern with the use of such antibodies is the potential of deleterious side-effects [82, 83].

Adjuvant systems:

The combination of immunostimulatory components in different adjuvant systems have been developed to promote appropriate protective and therapeutic immune responses generated by vaccination. When alum adjuvanted Gardasil vaccine and alum + MPL adjuvanted Cervarix vaccine were compared, it has been reported that Cervarix treatment resulted in significant increase in the neutralizing antibody titers for HPV-16 compared to Gardasil [84], which displays the contribution of MPL and importance of combining adjuvants with different action mechanisms.

MPL + alum is present in AS04, and MPL + QS-21 is present in AS01 (liposome based) and AS02 (emulsion based) adjuvant systems [85]. AS04 adjuvant system is a part of hepatitis B vaccine called Fendrix (GSK) and HPV vaccine: Cervarix (GSK). AS04 adjuvant system directly activates the innate immunity by activation of NF- κ B and induction of cytokine production, which in turn, induces the activation of antigen specific adaptive immune response [6]. Although synergistic effect of alum and MPL has not been noted, it has been suggested that alum prolongs the cytokine response of AS04 at the injection site [6].

AS01 and AS02 adjuvanted candidate malaria vaccines consist of two proteins, RTS (a sporozoite surface antigen of *P. falciparum*) and S (surface antigen of Hepatitis B virus). Although there are no AS01 and AS02 adjuvanted vaccines licensed yet, they have been studied in clinical trials. GSK's RTS, S vaccine candidate for malaria containing AS-01 adjuvant system has reduced the clinical malaria cases up to 46% in children and 27% in infants in a phase III clinical trial.

AS03 is another adjuvant system composed of squalene, α -tocopherol, and polysorbate 80 in an oil-in-water emulsion similar to MF59, has been licensed for H1N1

influenza vaccine, Pandemrix (GSK), in Europe for 2009 H1N1 pandemic flu [84]. Recently, AS03 adjuvanted H5N1 influenza vaccine Q-pan (GSK) has been approved by FDA. On the other hand, Pandemrix was discontinued in Europe due to an increased risk of narcolepsy found following vaccination. It has been shown that AS03 activates the innate immunity and enhances antigen uptake and presentation by APCs in draining lymph nodes, which in turn, activates adaptive immune responses, principally antibody response and immune memory [86].

Generation of potent T cell responses against tumor and infection is critical for the elimination of transformed and infected cells. In the case of infection where the generation of neutralizing antibodies is not potent enough to block the infection and cancer where TAAs induce host tolerance, cellular immunity comprising Th₁ and CTL responses is important. It is possible that the success of recombinant protein based vaccines, such as HBV and HPV, was because of the use of adjuvants (AS04 adjuvant system) with a distinct mechanism of action and targeting different immune cells for activation [10, 87]. Therefore for most of the infectious diseases and cancer vaccines, development of adjuvant systems which can induce both T cell, especially CD8⁺ T cells, and antibody responses is an important goal. In the case of enhancement of T cell responses, cytokines, TLR ligands and costimulatory molecules have been used as adjuvants in vaccine formulations.

Costimulatory SA-4-1BBL as an adjuvant platform for development of vaccines:

Although development of adjuvants targeting T cell stimulation is an important goal, in the case of therapeutic cancer vaccines, regulation of immune suppressive mechanisms is another important aim for immunotherapy since tumor cells employ these

mechanisms to evade immune attack. Not only tumor cells, but also viruses, such as hepatitis B virus, herpes, influenza, and retroviruses, exploit Tregs to evade immune responses. Therefore, strategies targeting the trafficking of Tregs to the site of immunization by small molecule antagonists have been explored [88, 89].

Among all the members in TNFSR family, targeting 4-1BB has been having growing interest due to its pleiotropic effects on cells of innate, adaptive, and regulatory immunity [90-94]. Its expression on the activated CD4⁺ T, CD8⁺ T, NK and NK T cells provides another advantage to target activated cells of innate and adaptive immunity, as such antigen specific CTL have been selected based on 4-1-BB expression [95]. 4-1BB signaling has been shown to be critical for T cell expansion, survival, acquisition of effector function and establishment of long-term memory [96, 97]. Additionally, we have previously reported that 4-1BB is constitutively expressed on a subset of DCs which may heighten antigen uptake and cross-presentation to T cells [98]. Ligation of 4-1BB has been shown to inhibit Treg suppressive activity in vivo and in vitro [93] through a direct effect by blocking Treg suppressive function or an indirect effect by rendering effector T cells resistant to Treg suppression [94].

In clinical studies, agonistic human anti-4-1BB antibodies have been evaluated either alone or in the combination with rituximab (anti-CD20 antibody) to treat melanoma (BMS-663513; Phase I/II) and non-Hodgkin's lymphoma (PF-05082566; Phase I), respectively. BMS-663513 mAb is also being used to treat a number of different solid tumors including renal cell carcinoma, ovarian carcinoma, and non-small cell lung carcinoma either as a single agent or in combination with chemotherapy, radiotherapy, or both [99]. However, liver toxicity was reported in the melanoma patients, which might be

because of nonspecific or excessive NK cell activity in liver, or accumulation of CTLs into the liver [81]. Toxicity issues may impede future studies and lead to termination of studies using agonistic antibodies.

To overcome toxicity problems alternative strategies should be developed to target 4-1BB. For this purpose, we have focused to generate a novel form of 4-1BB ligand which is functional in soluble form and without having toxicity. Natural 4-1BBL is a cell membrane-bound protein and has no activity in soluble form [100]. Therefore, we generated a novel form of this ligand composed of the extracellular domain of murine 4-1BBL fused to the C-terminus of a modified core-streptavidin (SA-4-1BBL) [101]. The SA domain allows the formation of tetramers and higher structures that have the ability to crosslink the 4-1BB receptor on immune cells for effective signaling [98, 102]. More importantly, this chimeric molecule has better costimulatory activity than an agonistic anti-4-1BB antibody without antibody associated severe toxicity [103]. The activity of this natural SA-4-1BBL has been established in our lab in various preclinical tumor models, and demonstrated that SA-4-1BBL adjuvanted recombinant protein or peptide-based vaccines generate robust Th₁ and CTL responses with therapeutic efficacy [98, 102, 104-107]. More importantly, we have reported that SA-4-1BBL inhibits the formation of inducible Tregs and confers CD4⁺ and CD8⁺ T effector cells refractory to Treg suppressive activity [106]. Based on our previous observations, we sought to investigate if SA-4-1BBL can be utilized as a novel adjuvant for plague vaccine in an infection model, a tolerant tumor model with a self TAA, and if the efficacy of SA-4-1BBL can be further improved by addition of other immune modulators to the vaccine formulation. Therefore, the first major goal of this Ph.D. dissertation was to test the SA-

4-1BBL efficacy as monotherapy and in combination with alum for bubonic plague infection and determine the mechanistic insights of the vaccine efficacy. The second goal was to determine the therapeutic efficacy of SA-4-1BBL and MPL adjuvanted Her-2/neu based protein vaccine in A2L2 breast cancer model and to evaluate the mechanisms behind the efficacy.

CHAPTER 2

SA-4-1BBL AS A NOVEL ADJUVANT FOR PLAGUE VACCINE

Introduction

Yersinia pestis is a Gram-negative facultative intracellular bacterium which causes bubonic and pneumonic plague and has a potential to be used as a bioweapon [108-110]. Currently there is no FDA approved vaccine available to protect the public from a potential epidemic or bioterrorism event. While *Y. pestis* infection can be treated with antibiotics, the effective treatment window for primary pneumonic infection is very short (less than 24 hours after exposure) [111]. Furthermore, naturally acquired resistance to antibiotics has been reported [112], and weaponized *Y. pestis* could likely be modified to be resistant to antibiotic treatment. Therefore, development of novel vaccines with protective efficacy against plague is imperative.

Historically, plague vaccines were made from live-attenuated or inactivated forms of the microbe. While these vaccines were effective against plague, there were concerns about stability during storage, occurrence of adverse effects and safety, especially in immunocompromised individuals [113]. Advancement in recombinant DNA technology allowed for the development of subunit vaccines containing well-characterized proteins serving as antigens. Such subunit vaccines overcome various issues related to the use of complete pathogens as vaccine. However, protein-based subunit vaccines are poorly immunogenic and need to be administered with immune-stimulant components known as

adjuvants [11, 12]. Although various *Y. pestis* antigens have been assessed for the development of subunit vaccines, the 17 kDa F1 extracellular capsule protein and the 37 kDa LcrV virulent factor are currently considered the most promising antigens [114]. Immunization with a mixture of F1 and LcrV antigens provided protection against plague [113, 115, 116]. The current lead candidate subunit vaccine for plague is based on a recombinant fusion protein between F1 and LcrV called rF1-V. This vaccine is adjuvanted with alum and its protective efficacy against plague has been shown in rodent, guinea-pig, and non-human primate (NHP) models [117, 118], although in cynomolgus macaques it was protective 80-100 %, studies in African green monkeys were not satisfactory enough [119], and it is currently under clinical evaluation by the US Department of Defense to protect military personnel to aerosolized *Y. pestis* [120]. However, the inconsistent efficacy in NHP raises a significant concern for the protective efficacy in humans and indicates the need for further improvement of this subunit vaccine.

The protection in rodents correlated with the generation of high titers of antibodies against rF1-V [121, 122]. Indeed, passive transfer of antibodies was effective in protecting mice against plague [121, 123, 124]. However, studies in nonhuman primate models have not been as clear [125][126]. For example, while cynomolgus macaques consistently generated high titers of antibodies against both the F1 and LcrV antigens when immunized with the rF1-V vaccine that correlated with protection, African green monkeys generated much lower titers against F1 (though consistent), demonstrated high variability in anti-LcrV antibody titers, and did not show a correlation between antibody

titers and protection [127]. Importantly, animals that failed protection had bacteria reservoir, suggesting antibodies failed to kill bacteria and disease progressed [128].

On the other hand, the Th₁ cytokines TNF- α and IFN- γ also appear to play a requisite role for protection against *Y. pestis* infection [129-132]. As such, exogenous administration of IFN- γ and TNF- α protects naïve mice against *Y. pestis* infection [132], and neutralization of either cytokines abrogates the protection in actively vaccinated mice [129]. Furthermore, IFN- γ and TNF- α also contribute to protection mediated by passive serum transfer or administration of F1 or LcrV specific mAbs which is demonstrated by abrogated efficacy in IFN- γ R- and TNF- α -deficient mice and neutralization of these cytokines [129, 130]. Contribution of cellular immunity to plague vaccine has further been demonstrated by adoptive transfer of *Y. pestis*-primed T cells to naive B-cell-deficient μ MT mice. This resulted in protection against lethal *Y. pestis* challenge. Moreover, treatment of μ MT mice with T-cell-depleting mAbs resulted in abrogation of protection conferred by vaccination with live *Y. pestis* [133]. Given the demonstrated role of Th₁ responses in protection against plague, one potential way to improve the efficacy of the rF1-V plague vaccine would be to increase the Th₁ response by altering the adjuvant system.

Although alum, as the adjuvant component of the lead rF1-V vaccine, generates robust antigen-specific Th₂ antibody responses, its efficacy in generating Th₁ responses is minimal [29]. Inasmuch Th₁ responses play a critical role in long term immune memory and clearance of intracellular pathogens, many subunit vaccines, including rF1-V, would likely benefit from a more balanced Th₁/Th₂ responses. Signaling through the costimulatory 4-1BB receptor of the TNFR superfamily is critical for the generation of

adaptive Th₁ and CTL responses and establishment of long-term immune memory [98, 102, 104]. Therefore, we have recently hypothesized that agonists of 4-1BB receptor may serve as an effective adjuvant with a Th₁ bias. Inasmuch as the natural ligand, 4-1BBL, functions as a cell-surface protein and has no activity in soluble form, we generated a chimeric form of this agonist (SA-4-1BBL) by fusing its functional extracellular domain to a modified form of core streptavidin [98, 102, 134, 135]. SA-4-1BBL activates DCs and T cells to generate a robust antigen-specific Th₁ response with demonstrated therapeutic efficacy in several preclinical cancer models [98, 102, 136].

Given that both humoral and Th₁ cell mediated immune responses are important for the control/clearance of *Y. pestis*, we herein assessed the efficacy of SA-4-1BBL and alum as an adjuvant system to produce a balanced Th₁/Th₂ immune response in a short term prime-only setting and the efficacy of SA-4-1BBL alone to generate humoral response in a long term prime-boost setting. In the short term study, when we compared SA-4-1BBL and alum, SA-4-1BBL generated robust Th₁ cellular responses against rF1-V without a significant antibody response while in combination with alum, SA-4-1BBL generated a mixed Th₁ and Th₂ cellular response without a dramatic positive or negative impact on the titers of anti-rF1-V antibodies generated by alum alone. In this short term prime only study, a vaccine formulation containing the combined adjuvant system had better protection against plague as compared with those containing individual adjuvants. On the other hand, in the prime-boost long term study, vaccination with rF1-V antigen (different doses) and SA-4-1BBL generated significant titers of anti-rF1-V antibodies that lasted over a 190-day observation period and mice immunized with lowest dose of rF1-V antigen and SA-4-1BBL were completely protected against plague, displaying a dose-

response. Taken together, our data demonstrate that SA-4-1BBL improves the efficacy of the lead rF1-V vaccine adjuvanted with alum for the generation of Th₁ responses and SA-4-1BBL alone generates a long-lasting anti- rF1-V humoral response and protects from plague in a prime-boost setting. This study provides the scientific rationale for the use of SA-4-1BBL for the development of subunit vaccines against infections that may benefit from a balanced Th₁/Th₂ response.

Materials and Methods

Mice, vaccinations, and bacterial challenge

Female, 6 to 8-week-old C57BL/6 mice were purchased from The Jackson Laboratory or bred at the University of Louisville. Animals were cared for in accordance with NIH guidelines and all procedures were approved by the University of Louisville IACUC (Protocol No. 10-117 and 13-080). In the prime only short term study, vaccine formulations were prepared and incubated at 4°C O/N to allow for the adsorption of the antigen to alum, and mice were vaccinated once subcutaneously (s.c.) on day 0 with 0.2 ml of one of the following vaccine formulations containing 1 µg of rF1-V protein: 1) rF1-V, 2) rF1-V + SA-4-1BBL (12.5 µg), 3) rF1-V + alum (200 µg), or 4) rF1-V + SA-4-1BBL (12.5 µg) + alum (200 µg). Anti-rF1-V antibody titers were assessed using serum collected on days 14 and 28 post-vaccination by sub-mandibular bleed.

In the prime-boost long term study, mice were vaccinated twice subcutaneously (s.c.) on days 0 and 21 with 0.2 ml of one of the following vaccine formulations containing rF1-V protein: 1) 5 µg rF1-V, 2) 1 µg rF1-V + SA-4-1BBL (12.5 µg), 3) 5 µg rF1-V + SA-4-1BBL (12.5 µg), or 4) 10 µg rF1-V + SA-4-1BBL (12.5 µg). Anti-rF1-V

antibody titers were assessed using serum collected on days 14, 28, 35, 58, 98, 147, and 190 post-prime by sub-mandibular bleed.

For bubonic plague infection studies, immunized mice were transferred and maintained in the ABSL-3 vivarium at the University of Louisville's Center for Predictive Medicine Regional Biocontainment Laboratory. In the prime only setting 35 days post-vaccination, and in prime-boost setting 202 days post-prime mice were lightly anesthetized with isoflurane and inoculated intradermally (i.d.) at the base of the tail with 20 μ l of ~200 colony forming units of fully virulent *Y. pestis* CO92 Lux_{P_{cysZK}} [137]. Mice were monitored twice daily for survival and moribund animals were humanly euthanized. Bacterial dissemination was assessed by optical imaging and images were taken using the IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA). Regions of interest within the images were generated using the LivingImage 4.4 (PerkinElmer) to calculate the average radiance (photons/sec/cm²) at the site of infection [137, 138].

Reagents and antibodies

Y. pestis recombinant rF1-V fusion protein (NR-4526) was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH by BEI Resources. SA-4-1BBL protein was expressed and purified in the laboratory using standard techniques as previously published [101]. Alhydrogel was purchased from Brenntag Biosector, Denmark. Anti-CD8-APC-Cy7, anti-CD4-Alexa700, anti-CD44-APC, anti-TNF α -PE, anti-IFN γ -PE-Cy7, and isotype-matched antibodies with the same fluorochromes were purchased from BD Bioscience, eBioscience, or BioLegend, and used for flowcytometry.

Intracellular cytokine analysis

In the short term study, C57BL/6 mice were vaccinated twice s.c. on day 0 and 35 with vaccine formulations containing 1 µg of rF1-V protein: 1) rF1-V, 2) rF1-V + SA-4-1BBL (12.5 µg), 3) rF1-V + alum (200 µg), or 4) rF1-V + SA-4-1BBL (12.5 µg) + alum (200 µg). Lymphocytes were isolated from draining lymph nodes five days after booster vaccination (Day 40) and processed into single-cell suspension. For intracellular cytokine analysis, 0.5 ml of lymphocytes (8×10^6 cells/ml) were plated in a 48 well-plate and stimulated with 20 µg of rF1-V protein in the presence of 20 U IL-2 and 25 ng SA-4-1BBL for 24h. Cultures without rF1-V served as controls. Golgi Plug (1 µl/mL, BD PharMingen) was added to the cultures during the last 4h of incubation. Cells were surface stained first with anti-CD4-Alexa700, anti-CD8-APC-Cy7, and anti-CD44-APC and then fixed with 4% paraformaldehyde for 15 min. Following permeabilization, cells were stained with anti-IFN γ -PE-Cy7, anti-TNF α -PE, or isotype controls, acquired using multiparameter flow cytometer (BD FACS LSR-II), and analyzed by FACSDiva software.

Anti-rF1-V antibody analysis

The titers of anti-rF1-V antibodies in vaccinated C57BL/6 mice were determined using a standard ELISA. Briefly, 96-well titer plates were coated with 1 µg/ml of rF1-V overnight at 4°C, blocked with PBS containing 5% dry milk + 0.5% Tween 20 for 45 minutes at RT, and then washed with PBS + 0.5% Tween 20. Fifty µl of two-fold serial dilutions of serum was added to wells and incubated for 90 min at RT. Wells were then washed 3 times with PBS + 0.5% Tween 20 and incubated with anti-mouse IgG-, IgG₁- or IgG_{2c}-HRP (Sigma and Jackson ImmunoResearch) for 60 min. Wells were washed 3 times with PBS + 0.5% Tween 20, incubated with fifty µl TMB substrate (BD

biosciences) for 30 min, and reaction was stopped with 2N sulfuric acid. Absorbance was measured at 450 nm and anti-rF1-V antibodies were reported as Log₁₀ titers of the greatest serial dilution with a mean OD₄₅₀ > two-fold the OD₄₅₀ value of naïve serum with the same dilution. Samples with an antibody titer of Log₁₀ 1.4 or less were considered negative.

Statistical analysis

Analysis of variance (ANOVA) was used to compare antibody titers and CD4⁺ and CD8⁺ effector responses among experimental groups. The Student t-test (2-tail) was used to compare titers of IgG subclasses (IgG₁ and IgG_{2c}) and the ratios in alum and SA-4-1BBL+alum treatment groups. Kaplan-Meier log-rank test was used to generate the survival curves.

Results

SA-4-1BBL and alum as adjuvant platform generates a Th₁ response to the rF1-V antigen

The lead rF1-V vaccine includes alum as a strong adjuvant for the generation of a robust Th₂ regulated humoral response in mice [29, 139-141]. However, alum does not generate antigen-specific Th₁ responses as assessed by Th₁-regulated IgG_{2a} titers and IFN γ production [140, 142]. Because Th₁ cellular response plays an important role in protection against plague and SA-4-1BBL has robust efficacy in generating such a response in other vaccine settings [98, 102, 104, 105], we tested SA-4-1BBL as an adjuvant for the generation of a Th₁ response against rF1-V antigen. C57BL/6 were immunized with vaccine formulations containing 1 μ g rF1-V antigen alone or antigen

admixed with 12.5 µg of SA-4-1BBL, 200 µg alum, or a mixture of both adjuvants followed by a booster injection 35 days later. As expected, immunization with rF1-V alone or rF1-V + alum did not result in significant increases in the absolute numbers of CD4⁺ or CD8⁺ T cells expressing the Th₁ signature cytokine IFN γ and proinflammatory cytokine TNF α (Fig. 1). In contrast, we observed elevated numbers of both CD4⁺ (Fig. 1A-B) and CD8⁺ (Fig. 1C-D) T cells producing TNF α and IFN γ from mice immunized with rF1-V + SA-4-1BBL. Interestingly, in spite of the Th₂ bias of alum, the combined adjuvant platform was even more effective in generating TNF α and IFN γ responses, with the only exception being in the IFN γ response in CD8⁺ T cells, where SA-4-1BBL performed as well as the combined adjuvant system (Fig. 1D). In this model, generation of more robust Th₁ response with SA-4-1BBL + alum therapy is consistent with previous findings where alum adsorbed IL-12 promoted antibody as well as type 1 cytokine responses to HIV-1 gp120 [143]. Collectively, these data demonstrate that: i) alum alone does not generate a significant rF1-V-specific Th₁ cellular response, ii) as an adjuvant SA-4-1BBL induces an rF1-V-specific Th₁ cellular response, and iii) alum is unable to inhibit the ability of SA-4-1BBL to generate a Th₁ response and in the contrary significantly improves such a response.

SA-4-1BBL and alum as adjuvant platform generates high antibody titers against rF1-V in a prime-only setting

While SA-4-1BBL has been shown in multiple vaccines to generate robust antigen specific CD4⁺ and CD8⁺ cellular immune responses (Fig. 2 and [102, 104]), the ability of SA-4-1BBL to also generate humoral responses has not been examined. Furthermore, while alum did not appear to inhibit the SA-4-1BBL cellular response in the

combined adjuvant platform, the effect of SA-4-1BBL on alum-generated humoral responses has not been investigated. To address the impact of SA-4-1BBL on the generation of anti-rF1-V antibodies, serum samples were collected from vaccinated mice on days 14 and 28 post-prime and assessed for total IgG titers against rF1-V protein (Fig. 2). Vaccination with 1 μ g rF1-V alone did not generate detectable titers at day 14 or 28, with the exception of one mouse with low IgG titers at day 14 that declined by day 28, and a separate mouse that did not have titers at day 14 but detectable levels (albeit low) at day 28. SA-4-1BBL therapy alone was also ineffective in generating a sustained antibody response and all animals in this group were negative for anti-rF1-V titers by day 28. In marked contrast, vaccine formulations containing alum rapidly generated significant IgG titers by 14 days post-vaccination (Fig. 2, $p < 0.0001$) that were maintained for at least 28 days post-vaccination ($p < 0.0001$). The combined adjuvant platform also yielded a significant increase in antibody titers compared to antigen alone and SA-4-1BBL therapy on days 14 ($p < 0.0001$) and 28 ($p < 0.01$). However, we did observe slightly lower IgG titers in the combined adjuvant vaccinated mice compared to the alum alone mice at both time points (Alum mean: 14 d =3.28 Log, 28 d =3.48 Log; Combination: 14 d =3.1 Log, 28 d =3.21 Log; $p < 0.05$ for both days). Taken together, these data suggest that SA-4-1BBL in a prime only situation does not elicit a robust rF1-V antibody response. However, SA-4-1BBL addition did not appear to drastically inhibit the generation of antibodies in alum immunized mice.

SA-4-1BBL and alum as adjuvant platform shifts the immune response towards Th₁ direction in a prime-only setting

Analysis of T cell responses indicate that SA-4-1BBL generates Th₁ cellular responses in vaccinated mice (Fig. 1). Generation of Th₁ cytokines by T cells can also impact the humoral response by altering IgG class switching from Th₂ associated IgG₁ to IgG_{2a} (IgG_{2c} in C57BL/6 mice [144]). To determine if SA-4-1BBL influences the generation of IgG subclasses, IgG₁ and IgG_{2c} serum titers were determined for mice immunized with alum or SA-4-1BBL+alum 28 days post-vaccination. As expected, mice immunized with alum generated a robust Th₂ IgG₁ antibody response. We also observed a predominantly IgG₁ response in animals immunized with the combination adjuvant. However, there was a significant reduction in the IgG₁ titers compared to alum only immunized mice (alum mean: 4.34 Log; combination: 3.86 Log; $p = 0.0087$, Fig.3.A). Moreover, immunization with the combined adjuvant increased the IgG_{2c} response compared to alum. As a result, the combined adjuvant therapy significantly increased the ratio of IgG_{2c} to IgG₁ compared to the alum alone mice ($p=0.05$, Fig. 3.B), suggesting a shift in the immune response towards a Th₁ direction consistent with the cytokine response (Fig. 3).

Vaccination with combination adjuvant platform provides protection against bubonic plague in a prime-only setting

Addition of SA-4-1BBL to the rF1-V vaccine significantly enhanced the rF1-V-specific Th₁ immune response in animals. Next we tested whether a vaccine containing SA-4-1BBL could provide protection against pathogen challenge. Four groups of mice ($n=10$) were vaccinated once with one of four vaccine formulations: 1) 1 μ g rF1-V antigen alone, 2) 1 μ g rF1-V + 200 μ g alum, 3) 1 μ g rF1-V + 12.5 μ g SA-4-1BBL, or 4) 1 μ g rF1-V + both adjuvants. Serum samples were collected at days 14 and 28 to

determine anti-rF1-V titers (Fig. 4.A). As seen previously, 30-40% of mice vaccinated with antigen alone or SA-4-1BBL generated modest titers on days 14 and 28, but significantly higher titers were achieved when alum was present in the vaccine (D14 $p < 0.05$; D28 $p < 0.01$). Seven days after the last serum sample, mice were challenged i.d. with fully virulent *Y. pestis* *Lux_{cysZ}*. Bacterial replication and dissemination were monitored throughout the course of infection by whole animal optical imaging for bacterial bioluminescence. As early as 24 hrs post-infection, we observed a difference in bioluminescence signal at the inoculation site of mice that received alum or SA-4-1-BBL+alum compared to those that did not receive alum as part of the vaccine (Fig. 4.C). Furthermore, while the mean bioluminescence of the antigen alone and SA-4-1BBL groups increased at the inoculation site by 48 h post-infection, indicating bacterial replication, we did not observe an increase in the alum and SA-4-1BBL+alum groups (Fig. 4.D). Eventually, all of the antigen-alone vaccinated animals developed systemic disease (Fig. 4.B) and succumbed to infection by 7.5 days post-infection (Fig. 4.E). We also observed systemic disease in the SA-4-1BBL group. However, 20% of these mice did not develop systemic disease and eventually cleared the infection (Fig. 4B and E). Interestingly, the two surviving mice did not have detectable antibody titers on day 28, suggesting that cellular immunity generated by SA-4-1BBL alone was responsible for protection against plague. In contrast to these groups, animals vaccinated with alum demonstrated greater protection, supporting previous studies that anti-rF1-V antibodies generated by alum are important mediators of protection against plague [121, 123, 145]. However, 20% of the alum group still succumbed to infection (Fig. 4.E). The greatest efficacy was seen in the combination adjuvant platform, in which all mice were protected

from infection (Fig. 4.E). Taken together, these data demonstrate that: i) while anti-rF1-V antibodies are important mediators of protection, they may not be sufficient to clear infection, as the animals that succumbed to infection in the alum group had similar titers to protected animals, ii) in the absence of protective antibody titers, an anti-rF1-V cellular response can protect against *Y. pestis* infection, as observed in the SA-4-1BBL groups, and iii) generating a balanced Th₁/Th₂ immune response by using a combinatorial adjuvant platform improved the outcome of vaccination with either adjuvant individually.

SA-4-1BBL generates a long lasting anti-rF1-V humoral response and protects mice from plague in a prime-boost setting

Since we observed that, SA-4-1BBL therapy does not elicit a humoral response for rF1-V in a prime-only setting, we wanted to explore the impact of SA-4-1BBL on the generation of anti-rF1-V titers in the prime-boost setting. To test this, mice were vaccinated twice with (1) 5 µg rF1-V alone, (2) 1 µg rF1-V + 12.5 µg SA-4-1BBL, (3) 5 µg rF1-V + 12.5 µg SA-4-1BBL, and (4) 10 µg rF1-V + 12.5 µg SA-4-1BBL with 21 days interval and serum samples were collected from vaccinated mice on days 14, 28, 35, 58, 98, 147, and 190 post-prime and assessed for total IgG titers against rF1-V protein (Fig. 5). Mice that received SA-4-1BBL as part of the vaccine generated significant titers of anti-rF1-V antibodies that lasted over a 190-day observation period. Interestingly, although 5 µg rF1-V antigen-alone generated faster and better titers than SA-4-1BBL adjuvanted vaccines early on, these titers were short lived and all mice scored negative by day 98 post-vaccination.

Since we observed that SA-4-1BBL induces the generation of long-lasting humoral response in a prime-boost setting (Fig. 5), and anti-rF1-V antibodies generated

by alum are important mediators of protection against plague [121, 123, 145], we wanted to examine the protective efficacy of SA-4-1BBL in a long term bubonic model. We challenged the mice i.d. 202 days after the prime with fully virulent *Y. pestis* *Lux_{cysZ}*, and monitored the survival and bacterial replication. Eventually, 5 µg antigen alone vaccinated mice developed systemic disease (Fig. 6.A) and succumbed to infection by 7 days post-infection (Fig. 6.B). We also observed systemic disease in the 5 µg rF1-V + SA-4-1BBL, and 10 µg rF1-V + SA-4-1BBL groups. However, one out of three mice in 5 µg rF1-V + SA-4-1BBL group did not develop systemic disease and eventually cleared the infection (Fig. 6.A-B), while in the 10 µg rF1-V + SA-4-1BBL group all mice developed systemic disease and succumbed to infection by 10.5 days (Fig. 6.A-B). Interestingly, mice vaccinated with 1 µg rF1-V + SA-4-1BBL did not develop systemic disease and showed greatest efficacy in which all mice were protected from infection cleared the infection (Fig. 6.A-B). Taken together, these data suggest that SA-4-1BBL in a prime-boost setting induce the generation of long-lasting anti-rF1-V antibody response as compared to antigen alone and provides protection in an antigenic dose-dependent manner.

Discussion

The lead rF1-V subunit vaccine against plague includes alum as a strong adjuvant for the generation of a robust Th₂ regulated humoral response in mice and protects mice against plague [29, 139-141]. However, the protective efficacy of the vaccine in NHP models has been variable, questioning the benefit of this vaccine for human use [120, 146][147]. This may be due to the inability of the vaccine to generate a Th₁ cellular

response. Indeed, accumulating evidence in the literature suggests that Th₁ cellular responses are important and complement humoral responses for protection against plague [129-131]. Alum, as the adjuvant component of the lead rF1-V subunit vaccine, does not generate antigen-specific Th₁ responses as assessed by Th₁-regulated antibody titers and IFN γ production [140, 142]. Therefore, we projected that the rF1-V vaccine may benefit from SA-4-1BBL as a strong Th₁ adjuvant. Consistent with this notion, a single immunization with SA-4-1BBL + alum adjuvanted rF1-V antigen generated a strong mixed humoral and Th₁ cellular immune responses against rF1-V antigen that translated into better protective efficacy against plague in a mouse model as compared with the alum only adjuvanted vaccine. Further, prime-boost immunization with SA-4-1BBL and 1 μ g rF1-V antigen provided 100% efficacy in bubonic plague model with generation of a long-lasting antibody response.

Recombinant protein-based subunit vaccines to infectious agents are attractive due to their ease of production and safety features. However, protein-based subunit vaccines heavily depend on immunostimulatory adjuvants for the generation of protective immunity against target infection [11, 12]. Alum is the most widely used adjuvant and is very effective in generating robust Th₂-regulated humoral responses against the antigen component of subunit vaccines [29]. However, cellular immune responses are also important, and in select settings are required for optimal immunity to infections, especially in the case of viruses and intracellular bacterial pathogens. It has been suggested that aluminum adjuvants may not drive complete protection against diseases that require a strong Th₁ response, such as malaria and cancer [148]. As such, to improve cellular responses of subunit vaccines, alum based vaccines have been adjuvanted with

immune modulators, such as IL-12 or synthetic ODNs containing CpG motifs, that promote Th₁ cellular responses [149]. We herein proposed that the cellular response and overall efficacy of the plague subunit rF1-V vaccine could be improved by using SA-4-1BBL costimulatory molecule as an adjuvant either alone or in combination with alum. The reasons behind the choice of SA-4-1BBL are several fold. First, signaling via 4-1BB is critical for T cell expansion, survival, acquisition of effector function, and generation of long-term immune memory [90, 96, 97]. Second, a subset of dendritic cells (DCs) constitutively express 4-1BB receptor and signaling via this receptor increases survival of DCs. This leads to increased antigen uptake and cross-presentation to T cells, and migration to T cell zone, culminating into enhanced T cell immune responses [98, 150, 151]. Third, 4-1BB/4-1BBL interaction selectively promotes the generation/expansion of T cells producing IFN γ and TNF α type 1 cytokines [152-154] that play an important role in protection against *Y. pestis* infection [129]. Fourth, 4-1BB expression is inducible and up-regulated on activated CD4⁺ and CD8⁺ T cells [70]. This provides an opportunity to specifically target antigen-specific, activated T cells for a more robust response, and as such avoid systemic/nonspecific activation of immune responses and associated adverse effects reported for other Th₁ immunomodulators, such as IL-2, IL-12 or CpG ODN [155-157]. Finally, and important in the context of this study, 4-1BB costimulation primarily and directly works on T cells, whereas alum targets APCs [29, 30] and as such these two adjuvants may work in synergy to enhance immunity against rF1-V antigen.

A prime-boost vaccination with rF1-V + SA-4-1BBL resulted in increased numbers of both CD4⁺ and CD8⁺ T cells producing TNF α and IFN γ . In particular, the most significant increase was observed for CD8⁺ T cells producing IFN γ . Given the

recently demonstrated role of CD8⁺ T cells in LcrV DNA-based vaccine protection against *Y. pestis* infection [158], our observations are significant. The administration of SA-4-1BBL with alum further improved the Th₁ cellular responses, except for IFN γ in CD8⁺ T cells where SA-4-1BBL therapy performed as well as the combination therapy. These findings are consistent with the previously published studies by us [102, 103] and others [159, 160] demonstrating the CD8⁺ T cell-centric function of 4-1BB signaling. Importantly, our findings are consistent with previously published studies demonstrating that alum in combination with Th₁ immune modulators, such as IL-12 or TLR4 agonists, promotes both Th₂-mediated humoral as well as Th₁ cytokine responses [6, 143].

We observed that SA-4-1BBL alone increased the number of CD4⁺ T cells producing TNF α and IFN γ as compared with alum, but such increases were not statistically significant. Alum when used in combination boosted the activity of SA-4-1BBL, resulting in a statistically significant increase in the number of CD4⁺ T cells producing TNF α and IFN γ . These observations are novel as they demonstrate synergy between alum and SA-4-1BBL in generating a strong CD4⁺ Th₁ cellular response. It has been shown that CD4⁺ T cells are required for the protective efficacy against *Y. pestis* conferred by CD8⁺ T cells in an adoptive transfer model [161]. Therefore, this synergy has important implications for the development of vaccines against intracellular infections where CD4⁺ Th₁-regulated CD8⁺ cytotoxic T cell responses play important roles in clearance. Further, treatment with Th₁ cytokines TNF α and IFN γ has been shown to diminish the survival of *Y. pestis* within the macrophages [162].

However, CD4⁺ T cells are not only critical for the generation of primary CD8⁺ T cell responses and the establishment of long-term memory, but also play important roles

in the generation and establishment of B cell responses that produce Ig isotypes, such as IgG_{2a} (IgG_{2c} in case of C57BL/6), involved in opsonization [163]. Although *Y. pestis* can evade from phagocytosis [164], it has been reported that neutrophils can control the bacteria growth within the first 2 days after infection [162]. Moreover, anti-LcrV antibodies enhances the *Y. pestis* phagocytosis indirectly by inhibiting Yop translocation [165] or production within the cells, and neutrophils are the major cell types in this protection against *Y. pestis* [166]. Here, we observed an increase in IgG_{2c} antibody response while there was a significant decrease in IgG₁ antibody response in mice vaccinated with the adjuvant system. As a result, the ratio of IgG_{2c} to IgG₁ was significantly increased, although the IgG₁ was the predominant isotype. Cytokines can regulate the generation of antibody isotypes, for example, IFN γ prevents IL-4 induced IgG₁ responses, while IL-4 blocks IFN γ induced IgG_{2a} response [167]. As a result, the observed increased ratio of IgG_{2c} to IgG₁ in the combined adjuvant therapy might be due to the elevated levels of IFN γ in these animals. Although all IgG subclasses contribute to clearance of bacteria, IgG_{2a} can fix the complement system greater than IgG₁ [163]. Therefore it can enhance the phagocytosis of *Y. pestis* by neutrophils [168].

Many studies have shown that anti-rF1-V antibodies contribute significantly to protection and that passive transfer of antibodies can confer protection in mice challenged with *Y. pestis* [121, 123, 145]. Our data do not contradict these assumptions, as in the prime-only study, the alum alone animals had high antibody responses to rF1-V and 80% of these animals were protected against *Y. pestis* challenge. However, 20% of these mice still succumbed to infection despite no significant differences in anti-rF1-V titers, suggesting that while important, antibodies alone may not be sufficient for protection.

This notion is consistent with studies in nonhuman primates demonstrating lack of correlation between antibody titers and protection against plague [169]. Cynomolgus macaques generated high and consistent titers of F1 and V antibodies and there was a correlation with protection, while African green monkeys generated more individual titers of V antibodies and they did not show a correlation [170]. Furthermore, in the prime-only setting, SA-4-1BBL alone provided 20% protection, in spite of no detectable antibody titers against rF1-V in the mice that cleared infection, and mice immunized with the combined adjuvant were 100% protected in spite of having lower IgG titers compared to alum alone therapy. These observations are consistent findings from Elvin and Williamson which demonstrated that protection conferred by a similar F1 and V subunit vaccine requires a Th₁ response. They reported that mice lacking the signal transducer and activator of transcription protein family 4 (Stat4), which regulates the Th₁ response, were not protected against plague following vaccination. [171]. In marked contrast, mice deficient in Stat6, which regulates the Th₂ response, had vaccine-induced protection. Importantly the antibody titers and IgG isotypes in both mice were similar, providing direct evidence for an important role of the Th₁ cellular response in protection. This notion is further consistent with the studies reporting important roles of T cells and Th₁ cytokines IFN γ and TNF α in protection against *Y. pestis* [129, 131].

However, in the case of prime-boost study, SA-4-1BBL adjuvanted vaccine with different rF1-V doses generated significant titers of anti-rF1-V antibodies that lasted over a 190-day observation period. On the other hand, vaccination with rF1-V alone was failed to do so. Although it has been demonstrated that agonistic 4-1BB antibody suppresses the T cell dependent humoral immunity through the induction of CD4⁺ T helper cell anergy

[172], and treatment of several autoimmune diseases have taken advantage from this [173, 174], it has also been reported that, the form of 4-1BB agonistic adjuvant can also influence the outcome of the cellular and humoral immune responses [175]. As such, treatment with agonistic anti-4-1BB antibody, and secreted form of the 4-1BBL suppress the humoral response to HIV-1 Gag DNA vaccine, while DNA plasmid expressing membrane bound 4-1BBL enhances the Gag-specific antibody titers [175]. Therefore, generation of long-lasting anti-rF1-V titers with the natural soluble form of SA-4-1BBL adjuvanted vaccine may not be surprising, and may need a booster dose as seen in prime-boost setting for the generation of potent humoral response since in the prime-only setting we did not observe an increase in the antibody response against rF1-V antigen. Interestingly, here we observed an antigen-dose dependent efficacy of SA-4-1BBL therapy where administration of SA-4-1BBL with 1 μ g rF1-V resulted in complete protection, while increasing the antigen dose abrogated the protection to 33% and 0% with 5 μ g and 10 μ g rF1-V, respectively. This could be due to the over-activation of CD8⁺ T cells with higher antigen doses, and subsequent generation of CD8⁺ T suppressor cells which inhibit or suppress CD4⁺ T cell responses via IFN γ -dependent release of TGF β [176]. On the other hand, vaccination with 1 μ g rF1-V and SA-4-1BBL induced the generation of a robust long-lasting antibody response with a balanced effector cellular response which resulted in 100% efficacy in bubonic plague model in the prime-boost study.

Collectively, our data demonstrate that in the short term setting, addition of the Th₁ adjuvant SA-4-1BBL improves the efficacy of the lead alum adjuvanted rF1-V subunit vaccine by generating a strong Th₁ cellular immune response without significant

impact on the generation of Th₂ regulated antibody responses. The combined adjuvant system has better efficacy over individual adjuvants in protection against plague. Importantly, since we did not observe a correlation between antibody titers and survival in mice immunized with SA-4-1BBL (either alone or in combination with alum), we provide the first evidence that rF1-V-specific cellular immune responses can protect against plague and may provide an important contribution to the future success of the rF1-V subunit plague vaccine. This hypothesis is supported by previous reports that show that adaptive cellular modulators, such as CpG ODN or cytokines, can enhance the immune response in alum vaccinated animals [143, 177]. However, giving one more shot of SA-4-1BBL adjuvanted rF1-V vaccine induces the generation of long-lasting antibody response against the antigen, and confers 100% protection, highlighting the importance of humoral immune response for *Y. pestis*. Therefore, generation of a balanced Th₁ and Th₂ immune response is critical for the elimination of bacteria. Finally, while these studies focused on the rF1-V subunit vaccine, we believe that our findings are not specific to rF1-V or plague, and that addition of SA-4-1BBL to other subunit vaccines will have similar effects and could dramatically improve efficacy of vaccines, especially against intracellular pathogens.

Figure 1

SA-4-1BBL+Alum increases the cellular response of the plague vaccine. C57BL/6 mice were immunized with rF1-V with or without the indicated adjuvants or PBS only (n=5) on days 0 and 35. 5 days after the boost (Day 40), antigen specific cytokine response was determined in the dLN. Each bar represents the mean and S.D. of absolute numbers of CD4⁺CD44^{hi} and CD8⁺CD44^{hi} T cells producing TNF α (*left*) and IFN γ (*right*) cytokines in each group. Data are representative of two independent experiments. *Upper panel* shows the absolute numbers of CD4⁺ T cells, *lower panel* shows the absolute numbers of CD8⁺ T cells. **** = p< 0.0001; *** = p<0.001; ** = p<0.01; ns= not statistically significant.

Figure 2

SA-4-1BBL + Alum induces antibody response for rF1-V in prime-only setting. C57BL/6 mice were immunized with rF1-V with or without the indicated adjuvants (n=5). 14 and 28 days post-immunization serum samples were harvested and IgG titers were determined. Dotted lines indicate limit of detection. Graph shows the individual values in each group. **** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.05$.

Figure 3

SA-4-1BBL + Alum shifts the cellular response towards Th₁ in prime-only setting. C57BL/6 mice were immunized with rF1-V and indicated adjuvants (n=5). 28 days post-immunization serum samples were harvested and titers of IgG₁ and IgG_{2c} subtypes were determined. A) The bar graph represents average log titers of IgG₁ and IgG_{2c} antibodies with SD, and dotted lines indicate limit of detection. Alum (Black); Alum + SA-4-1BBL (White). B) The bar graph represents average x value of IgG_{2c}/IgG₁ ratio in each group.

Figure 4

One shot immunization with SA-4-1BBL + Alum protects mice from bubonic plague. C57BL/6 mice were immunized with rF1-V with or without the indicated adjuvants (n=10 per group). A) 14 d and 28 d post-immunization serum samples were harvested and IgG titers determined. Dotted lines indicate limit of detection. 35 days post-immunization mice were challenged with *Y. pestis* *Lux_{P_{cysZK}}* by i.d. injection. B) Representative optical image of animals from each group at 24 h post-infection or at the time of euthanasia. C) 24 h and D) 48 h post-infection bacterial numbers (as a function of bacterial bioluminescence) were determined at the inoculation site by optical imaging (mean radiance (photons/sec/cm²) $\pm$ st. dev.). E) Survival comparison of vaccinated groups. * = $p < 0.05$; ** = $p < 0.01$; ns = not statistically significant.

Figure 5

SA-4-1BBL generates a long-lasting anti-rF1-V humoral response in a prime-boost setting. C57BL/6 mice were immunized with vaccine formulations containing (1) 5 µg rF1-V alone (n=3), (2) 1 µg rF1-V + 12.5 µg SA-4-1BBL (n=3), (3) 5 µg rF1-V + 12.5 µg SA-4-1BBL (n=3), and (4) 10 µg rF1-V + 12.5 µg SA-4-1BBL (n=3) on days 0 and 21. The role of SA-4-1BBL on humoral immune response was evaluated by the generation of anti-F1-V titers detected in the serum collected on days 14, 28, 35, 58, 98, 147, and 190 post-prime. The bar graph represents average log value of IgG titers in each group. Dotted lines indicate limit of detection.

Figure 6

SA-4-1BBL provides protection against bubonic plague with an rF1-V dose dependent manner. C57BL/6 mice (n=3 per group) were immunized with indicated vaccine formulations on days 0 and 21, and inoculated i.d. at the base of the tail with ~200 CFU of fully virulent *Y. pestis* CO92 Lux_{P_{cy}SZK}. A) Representative optical image of animals from each group at 2.5, 6.5, 13.5 d post-infection. B) Survival comparison of vaccinated groups.

CHAPTER 3

SA-4-1BBL AND MPL COMBINATION AS AN ADJUVANT SYSTEM FOR THE TREATMENT OF BREAST CANCER

Introduction

Breast cancer is the most common cause of cancer related death among women worldwide. Nearly 30% of human breast cancers over-express the human epidermal growth factor receptor 2 (Her-2/neu) [178]. Although Her-2/neu positive breast cancer could be treated with trastuzumab, a humanized anti-Her-2/neu mAb, these therapies require frequent administrations and more importantly tumor cells acquire resistance against antibody therapies [179, 180]. As a result, tumors are being relapsed. Moreover, response rate to trastuzumab therapy is limited in patients with metastatic breast tumor [181]. Therefore, active immunization against Her-2/neu is an alternative approach to mAb therapy primarily because of the activation of cytotoxic T cell responses, generation of humoral response and long-term immunological memory to control recurrences [182].

Her-2/neu is an attractive TAA for therapeutic vaccines against Her-2/neu positive breast cancers since it is over-expressed in cancer cells. Active immunization strategies against Her-2/neu include peptide and protein based, DNA based, dendritic cell based, and whole tumor cell based vaccines. In the clinical trials, the mostly tested vaccine strategy is peptide based, since they can induce antigen specific T cell (CD8⁺ and

CD4⁺ T cell epitopes) and antibody responses (B cell epitopes). However, this vaccine approach has some limitations due to the restricted immune response to one or two epitopes, or specific HLA haplotypes [182], and without generation of CD4⁺ Th cells, the induced CTL response cannot be long lived [183]. However, employing protein (entire or truncated) based vaccines may overcome these restrictions since they include both HLA class I and II epitopes, therefore activates both CTL and Th cellular, and humoral responses. As such, in several clinical trials it has been reported that use of Her-2/neu protein as a vaccine antigen, induces antigen specific T cell and antibody responses [184][185].

Even though Her-2/neu is an immunogenic molecule since it generates specific antibody and CTL responses in some patients [16, 17], soluble Her-2/neu protein as a recombinant vaccine is not immunogenic [186]. Therefore, to generate potent immune responses to Her-2/neu protein, adjuvants should be formulated in Her-2/neu recombinant vaccines [184]. In cancer vaccines, induction of CD8⁺ T cell response is critical to generate potent anti-tumor immune response since antigen specific CTL response is required for the elimination of tumor cells and establishment of long term memory. For antigen specific T cell activation, the first target among the immune cells is the APCs since in turn they prime the adaptive arm of the immunity. As such, the importance of innate immunity for activation of cellular responses has put the emphasis on the development of adjuvants, which are capable of activating innate immune cells, and the only adjuvants approved by FDA for cancer vaccines are primarily targeting the innate arm of the immune system through APCs. For example, live-attenuated tuberculosis vaccine Bacille Calmette-Guérin (BCG) is the first adjuvant approved by FDA in 1990

for the treatment of bladder cancer, and MPL is the second one as a component of the adjuvant system (AS04) in Cervarix HPV vaccine [187, 188]. Consistent with the importance of activation of APCs and enhancement of antigen cross-presentation by APCs, vaccination with live attenuated *Listeria monocytogenes* vector expressing rat or human Her-2/neu gene segments induces tumor regression and prevent metastasis in murine models [189, 190]. The anti-tumor efficacy of the immunizations against Her-2/neu has also been enhanced by targeted delivery of Her-2/neu to the APCs by fusion proteins consisting soluble CTLA-4 [191], or single-chain fragment variable specific for CD11c [192], or Fc domain of IgG [193] in preclinical studies.

Nevertheless, activation of the CD8⁺ and CD4⁺ T cells requires the first signal delivered upon MHC/peptide complex and TCR interaction together with a second signal from costimulatory receptors [8]. Failure to receive second signal can lead to T cell anergy or apoptosis. Moreover, regulation of Treg suppressive function is another important aim for cancer immunotherapy since tumor-derived soluble factors may induce the generation of regulatory cells as an immune escape mechanism. Among the T cell costimulatory receptor families, TNFRF signaling is crucial for T cell survival, cell cycle progression, effector function, and memory responses [69, 70], and plays important role regulating both adaptive and regulatory immunity. Moreover, these receptors are inducibly expressed on activated T cells upon antigen stimulation which provides another advantage to serve as adjuvant candidates for vaccines to generate antigen specific immune responses [70].

Since the importance of CD8⁺ T cells in breast cancer has been determined in preclinical and clinical studies [194-197], we, herein, particularly focused on 4-1BBL

among TNFRF members due to its critical role in the generation and maintenance of CD8⁺ T cell responses [92, 96]. Further, 4-1BBL co-stimulation confers CD8⁺ T cells resistant to suppression by Tregs [70], which may help to improve anti-tumor immune responses. However, 4-1BBL functions as a cell membrane protein and has minimal to no function in soluble form. Therefore, we generated a chimeric molecule (SA-4-1BBL) that exists as functional oligomers [102] and showed that SA-4-1BBL induces T cell activation, acquisition of effector functions, and generation of long-term memory [98, 102, 103, 107, 198]. Most importantly, we reported that SA-4-1BBL modulates regulatory immunity by rendering Teff cells resistant to Treg suppressive activity [98], and inhibiting the conversion of Teff cells into Tregs through the production of IFN- γ [106]. As a result, SA-4-1BBL provides significant therapeutic efficacy in various preclinical models [98, 102-104, 136].

Here, we tested the efficacy of a combined adjuvant therapy; SA-4-1BBL, for the activation of adaptive immunity, and MPL, for priming innate immunity, in a preclinical Her-2/neu positive breast cancer model. Since MPL primarily targets APCs, such as DCs and macrophages, for the initiation of adaptive immunity [6] and 4-1BBL targets CD8⁺ T cells for activation, acquisition of effector function, survival, and long-term memory [90, 199, 200], here we hypothesized that these immunomodulators may work in synergy to improve the anti-tumor efficacy of rat Her-2/neu subunit breast cancer vaccine. We demonstrated that a prime-boost vaccination with combined adjuvant system (SA-4-1BBL + MPL) generates 30% therapeutic efficacy in Her-2/neu positive A2L2 breast cancer model in BALB/c mice. On the other hand, SA-4-1BBL monotherapy provides 10% survival, while MPL monotherapy cannot confer therapy. Furthermore, vaccination

with combined adjuvant system eradicates 20% of transplanted A2L2 tumors, while depletion of Treg cells further increases its therapeutic efficacy to 40% in tolerant Her-2/neu transgenic BALB/neuT mice. Consistent with the therapeutic efficacy, combined adjuvant therapy induced better tumor specific killing responses than individual agents. Further, combined adjuvant therapy induced significant increase in pro-inflammatory cytokine IFN- γ production. Taken together, these data demonstrate the utility of SA-4-1BBL + MPL as a novel adjuvant system for the development of therapeutic rat Her-2/neu-based breast cancer vaccine with significant therapeutic efficacy.

Materials and Methods

Cloning, expression, and purification of recombinant rat Her-2/neu ECD

Extracellular domain (ECD) of the rat Her-2/neu cDNA (from Genscript) was subcloned into both 6X-His-pTWIN-1 and pTWIN-1-6X-His bacterial expression vectors (New England Biolabs) using Nde I and BamH I restriction sites. After transformation into C2566H competent *E. coli* cells (New England Biolabs), cultures were grown at 37°C for 2 h and induced using 1 μ l/ml of IPTG. After 3 h induction, cells were harvested, centrifuged, and resuspended in 100 ml lysis buffer containing 20mM Tris, pH 7.0, 500 mM NaCl, 10 μ M ZnCl₂, 5mM imidazole, and 5mM β -ME. Cells were lysed using ultra-sonication, inclusion bodies were pelleted at 10,000xg for 10 min. Inclusion bodies were washed three times by lysis buffer + 1% Triton X-100 with rotation at RT for 30 min, and centrifuged at 35,000xg for 30 min. After three cycles, the final pellet was resuspended in 100 ml lysis buffer + 6M Guanidine-HCl and rotated O/N at RT to solubilize inclusion bodies. Next day, supernatant was collected after centrifugation at

35,000xg for 30 min at 4°C, and used for IMAC purification using Talon resin with the advantage of His-Tag according to manufacturer's protocol (Clontech). The only exception was adding 0.1 % Triton X-114 to help to remove endotoxin. In all purification steps, 10µM ZnCl₂ and 5mM β-ME were used to enhance proper folding. Protein was dialyzed against PBS, concentrated using an Amnicon Ultra MWCO 50,000, and sterile filtered using a 0.22 µm filter. Protein concentration was measured using BCA method (Pierce).

ECD of the rat Her-2/neu cDNA (from Genscript) was subcloned into the Flag-pMT/BiP vector and expressed in Drosophila Schneider (S2) cells (Drosophila expression system inducible/secreted kit from invitrogen life technologies). Briefly, 9x10⁵ cells in 2 ml serum free media (SFM, Hyclone SFX-insect) containing P/S and L-glu were plated. Cells were led to attach for 1 hour. Meanwhile, transfection solution was prepared by adding solution A consisting 2 µg rat Her-2/neu construct and 100 ng of pCoHygro in 100 µl SFM to solution B consisting 5 µl of Cellfectin in 95 µl of SFM and incubated for 45 minutes. After incubation, 0.8 ml SFM was added to Mix A+B to make transfection solution. Growth medium was removed from the cell culture and washed with SFM. 1 ml transfection solution was added to cells and incubated at 27°C for 5h. After 5 hours, transfection solution was removed and replaced with 2 ml SFM containing hygromycin and incubated at 27°C for 48h. Expression of rat Her-2/neu was induced with 1 mM CuSO₄ in HyClone (w/o FBS) with the advantage of MT promoter. pMT/BiP vector carrying the Drosophila BiP signal sequence induced rat Her-2/neu protein secretion to the culture supernatant. Culture supernatant was collected and rat Her-2/neu protein was purified and eluted using anti-FLAG M2 affinity gel and 3xFLAG peptide, respectively.

Protein was dialyzed against PBS, concentrated using an Amnicon Ultra MWCO 50,000, and sterile filtered using a 0.22 mm filter. Protein concentration was measured using BCA method (Pierce). Construction, expression, purification, and characterization of SA-4-1BBL have been previously described [98].

Mice and cell lines

Female BALB/c and BALBneuT mice were bred in University of Louisville animal facility. All animals were cared for in accordance with institutional and NIH guidelines. Female BALB-neuT mice 6–8 weeks old were used before autochthonous tumor appearance. Heterozygous females expressing rNEU verified by PCR were used throughout this work. Mouse mammary breast cancer cell line A2L2 transfected with rat Her-2/neu (H-2^d) and its parental 66.3 cell line were kindly provided from Dr. Janet Price from UT M.D. Anderson Cancer Center, and maintained in MEM media supplemented with 5% FBS, 2X MEM vitamins, 0.1 mM non-essential aminoacids, 1 mM sodium pyruvate, 20 mM HEPES, 2 mM L-glutamine, 1% P/S and 400 µg/ml gentamicin. All the reagents were purchased from Gibco except for FBS from Atlanta biological.

Antibodies and other reagents

For intracellular cytokine analysis, fluorochrome-conjugated anti-CD3-V500, anti-CD8-APC-Cy7, anti-CD4-Alexa700, anti-CD62L-PerCp-Cy5.5, anti-CD44-APC, anti-CD69-PE, anti-CD25-PE-Cy7, anti-Foxp3-Alexa-488, anti-TNF α -PE, anti-IFN γ -PE-Cy7, anti-IL-2-PerCp-Cy5.5, and isotype matched antibodies were purchased from BD Bioscience, eBioscience, and BioLegend.

For toxicity studies, anti-CD3-FITC, anti- CD8-APC-Cy7, anti- CD4-Alex700, anti- CD11b-PerCp-Cy5.5, anti- CD19-PE-Cy7, anti- NK1.1-PE, anti- F4/80-Alexa647,

anti- CD11c-PE-Texas Red, and isotype matched antibodies with same fluorochrome were purchased from BD Bioscience, Invitrogen, eBioscience, and Caltag Lab. Fc Block was purchased from BD Bioscience.

MPL was purchased from Sigma Aldrich. Rat Her-2/neu p66 (TYVPANASL) peptide (Her-2₆₆₋₇₄) corresponding to H-2K^d-restricted, dominant CTL epitope was used for *in vitro* stimulation of draining lymphocytes in intracellular cytokine assay was purchased from Peptide 2.0 Company.

Tumor models and vaccination

Tolerant BALB-neuT and BALB/c mice were challenged subcutaneously (s.c.) with 1×10^5 live A2L2 cells in the right flank. For therapy, mice were vaccinated s.c. on days 5 and 10 post-tumor challenge with various vaccine formulations containing rat Her-2/neu protein (50 μ g) with SA-4-1BBL (25 μ g), or MPL (25 μ g), or the combination of both agents (25 μ g/agent). The doses of SA-4-1BBL, and MPL used in this study were based on our previously published and preliminary studies [98]. Tumor size was measured with a caliper twice weekly. Mice were sacrificed when tumor size reached 12 mm in diameter, or showed external ulceration, or signs of poor conditions (weight loss, dehydration, inactivity, or moribund). Anti-Her-2/neu antibody titers were assessed using serum collected on termination days when tumor size 12 mm diameter from tumor bearing mice and on Day 59 from tumor-free (TF) mice. Treg cells were depleted using anti-CD25 Ab (clone PC-61) at 300 μ g/mice via intra peritoneal (i.p.) injection two days before A2L2 tumor inoculation. In the case of established tumor study, to determine T cell numbers and cytokine responses, mice with 3-5 mm established tumors were

vaccinated with indicated formulations and a booster dose was given 5 days later. 3 days after the boost mice were terminated to collect the dLNs.

For toxicity studies, C57BL/6 mice were injected with vaccine formulations containing E7 protein (50 µg) with SA-4-1BBL (25 µg), or MPL (25 µg), or the combination of both agents (25 µg/agent). 18 h after immunization, mice were terminated.

Cytotoxicity assay

BALB/c mice were vaccinated twice on days 0 and 5 s.c. with different vaccine formulations containing rat Her-2/neu protein (50 µg) with SA-4-1BBL (25 µg), or MPL (25 µg), or the combination of both agents (25 µg/agent). 5 days after booster vaccination, animals were sacrificed, spleens were harvested and single cell suspensions were prepared. Splenocytes (3×10^6 /ml) from vaccinated animals were re-stimulated *in vitro* for 5 days with 10 µg of Her-2/neu protein/ml in complete MLR medium supplemented with 50 IU/mL of IL-2 and 0.05 µg/mL of SA-4-1BBL. Viable lymphocytes were harvested 5 days later using a Ficoll gradient and cultured with A2L2, or 66.3 neo, or TC-1 target cells at various ratios for 5 hours at 37°C. The percentage of killing was determined by the formula: percent specific lysis = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

Flow cytometry and cytokine bead array analysis

5 days after booster vaccination, animals were sacrificed, dLNs were harvested, and single cell suspensions were prepared. Lymphocytes (8×10^6 cells/ml) were plated to the wells of 48 well plate and stimulated with 10 µg/ml Her-2 p66 peptide for a total of 6 hours and GolgiPlug (1 µl/ml, BD PharMingen) was added at the last 4 hours. Cells were

surface stained with anti-CD3-V500, anti-CD44-APC, anti-CD8-APC-Cy7 and anti-CD4-Alex700, fixed with 4% paraformaldehyde, and after permeabilization stained intracellular with anti-IFN- γ -PE-Cy7, anti-IL-2-Percp-Cy5.5, anti-TNF- α -PE, or isotype controls, acquired using multiparameter flow cytometer (BD FACS LSR-II), and analyzed by FACSDiva software.

For toxicity studies, spleens, mLNs and dLNs were collected 18h post-immunization to determine the number of different lymphocyte subsets in each treatment group. Cells were incubated with Fc Block, and stained with anti-CD3-FITC, anti-CD8-APC-Cy7 and anti-CD4-Alex700, anti-CD11b-PerCp-Cy5.5, anti-CD19-PE-Cy7, anti-NK1.1-PE, anti-F4/80-Alexa647, anti-CD11c-PE-Texas Red, or isotype controls followed by acquisition and analysis using multiparameter flow cytometer (BD FACS LSR-II), and FACSDiva software respectively.

For CBA analysis, mice were terminated 3 days after the boost and dLNs were harvested. Cells (2×10^6 cells/mL) were plated triplicate to the wells of 96 well U-bottom plate, and stimulated *in vitro* with 10 μ g/ml of both Her-2/neu ECD protein and p66 peptide for 48h at 37 °C. After 48h incubation, culture supernatants were harvested and IL-2, IL-4, IL-10, TNF α , and IFN γ cytokines were determined using Mouse Th₁/Th₂/Th₁₇ Cytokine Cytometric Bead Array Kit and protocols from BD Biosciences. For each assay tube, 10 μ l aliquot of each capture bead were added and mixed in the same tube. 50 μ l of mixed cytokine captured beads were mixed with 50 μ l of sample or standart, and 50 μ l of PE Detection reagent was added into each assay tube and incubated in the dark at RT for 2h. After incubation, beads were washed with and resuspended in 300 μ l wash buffer.

Beads were acquired using multiparameter flow cytometer (BD FACS LSR-II), and analyzed by BD CBA software.

Analysis of Her-2/neu antibodies

ELISA was performed to assess the presence of anti-Her-2/neu antibodies in vaccinated mice. Briefly, ninety six titer plates coated with 1 µg/well of rat Her-2/neu protein for 4 hours at 37 °C and blocked with PBS containing 5% BSA + 0.5% Tween 20 for overnight at 4°C. Serum dilutions were added to wells and incubated at 37°C for 2 hours. Wells were washed 3 times, incubated with anti-mouse IgG-HRP (Jackson ImmunoResearch) diluted in blocking buffer for 1 hour at 37°C, washed, substrate was added and incubated at RT for 30 minutes. Absorbance was measured at 450 nm.

Liver and Kidney functional status:

C57BL/6 mice were vaccinated with E7 protein (50µg) + either MPL (25 µg), or SA-4-1BBL (25 µg), or the combination of both agents MPL + SA-4-1BBL (25 µg/agent). Mice were sacrificed 18 hours after vaccination. Serum was collected and status of liver and kidney function was evaluated by serum alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and Creatinin (CREA) levels respectively.

Hematoxylin-eosin staining of liver tissues:

Mice treated with vaccine formulations as indicated above were terminated 18 h post immunization, and livers were collected from each treatment groups. PBS vaccinated mice used as negative control. Livers were fixed in 3.7% formaldehyde,

embedded in paraffin wax, sliced and stained with Hematoxylin and Eosin for pathological analysis.

Statistical analysis:

Analysis of variance (ANOVA) was used to compare antibody titers, CD4⁺ and CD8⁺ effector T cell responses, and CBA analysis among experimental groups. Two-tail student t-test was used to compare the number of each lymphocyte population in the spleen, mLN, and dLNs among the experimental groups for toxicity studies. Kaplan-Meier log-rank test was used to generate the survival curves.

Results

Expression and purification of recombinant rat Her-2/neu ECD

To generate a recombinant Her-2/neu protein as a TAA for the therapeutic vaccine, we first generated plasmid containing the 6X-His-pTWIN-1 expression vector, carrying the rat Her-2/neu ECD cDNA (Fig. 7.A). The recombinant Her-2/neu was expressed in bacteria, and its expression was induced with IPTG (Fig. 7.B). However, as shown in Fig. 7.B, the protein was degraded. Then, we expressed the rat Her-2/neu ECD cDNA in pTWIN-1-6X-His vector (Fig. 8.A), transfected the bacteria with this construct, and induced the protein expression with IPTG (Fig. 8.B). Although, we were able to express the ECD of rat Her-2/neu protein with the right size (approximately 85 kDa, Fig. 8.C), after elution the protein was precipitated out and found to have high endotoxin levels.

To overcome the endotoxin issue, we generated plasmid containing the pMT-BiP expression vector, carrying the rat Her-2/neu ECD cDNA and Flag residues at C-

terminus (Fig. 9.A). The recombinant Her-2/neu was expressed in Drosophila S2 cells, its expression was induced with CuSO₄ (Fig. 9.B), secreted into the culture supernatant, and purified and eluted using anti-FLAG M2 affinity gel and 3xFLAG peptide, respectively. The purified protein runs approximately as an 85 kDa band on SDS-PAGE (Fig. 9.C), and reacts with anti-ErbB2 antibody on a western blot gel (Fig. 9.D).

Next, we tested if the A2L2 breast cancer cell line that we are planning to use for the tumor studies expresses rat Her-2/neu protein. We evaluated the Her-2/neu expression in the tumor cell lysate. As shown in Fig. 9.E, the whole Her-2/neu (185 kDa) was detected in the A2L2 cell lysate.

SA-4-1BBL adjuvanted rat Her-2/neu TAA-based vaccine induces elimination of established A2L2 tumors in BALB/c mice

We examined the therapeutic efficacy of SA-4-1BBL in different preclinical tumor models, such as E7 expressing TC-1 tumors [102], and survivin expressing 3LL tumors [98, 107]. We sought to investigate whether SA-4-1BBL with demonstrated adjuvant activity can generate an anti-tumor efficacy in a more tolerant self-TAA using rat Her-2/neu expressing A2L2 mouse breast cancer model in BALB/c mice. Immunization with rat Her-2/neu as a TAA and SA-4-1BBL as an adjuvant resulted in eradication of A2L2 tumors in 10% of BALB/c mice in a prime-boost setting. Moreover, mice that expired from tumor in this treatment group had significantly slow tumor progression and prolonged survival as compared to PBS control group ($p < 0.05$; Fig. 10.A-B). We hypothesized that SA-4-1BBL vaccine efficacy could be improved with the recruitment of MPL as a second adjuvant with primary effect on the innate immunity [6, 201]. A prime-boost immunization with rat Her-2/neu protein mixed with SA-4-1BBL +

MPL resulted in the eradication of A2L2 tumors in 30% of BALB/c mice, which remained TF over an observation period of 90 days. Further, this combined adjuvant treatment resulted in slower tumor progression and prolonged survival significantly as compared to control group ($p < 0.05$; Fig. 10.A-B). In contrast, rat Her-2/neu + MPL monotherapy did not provide protection and all mice expired from the tumor burden within 45 days (Fig. 10.A-B). Taken together, these data demonstrate that SA-4-1BBL + MPL as an adjuvant system is effective in eradicating the established A2L2 tumors with better therapeutic efficacy than the individual agents, and SA-4-1BBL monotherapy has better efficacy than MPL monotherapy.

MPL induced antibody response against Her-2/neu does not correlate with survival

Since antibody therapies against Her2/neu have been utilized and trastuzumab therapy has demonstrated clinical efficacy [183], generation of humoral response with long lasting antibodies against Her-2/neu ECD may increase the anti-tumor efficacy. Therefore, we collected serum samples from mice with different treatments as indicated above. Serum samples were collected from tumor bearing mice on termination days when tumor size reached 12 mm in diameter average and from TF mice on day 59, and assessed for anti-Her-2/neu titers. Tumor bearing PBS treated mice did not develop anti-Her-2/neu antibody response, while mice vaccinated with Her-2/neu + SA-4-1BBL, and combined adjuvants generated moderate Ab response, which did not result in statistical significance compared to PBS alone (Fig. 11). Two TF mice in the combination treatment group were negative for anti-Her-2/neu titers while one mouse showed moderate titer against Her-2/neu protein on day 59, and one TF mouse in the SA-4-1BBL monotherapy did not generate antibody response for the target antigen. On the other hand, Her-2/neu +

MPL vaccination induced significant anti-Her-2/neu titers compared to other treatment groups. However, vaccination with Her-2/neu + MPL did not provide protection against A2L2 tumor challenge.

Vaccination with combined adjuvants and rat Her-2/neu induces better anti-tumor killing response

CD8⁺ T cell effector and memory responses is critical for the elimination of primary tumor and control recurrences in various tumor settings [98, 102, 198], and their importance in breast cancer has also been determined in preclinical and clinical studies [194-197]. As such, the number of tumor infiltrating CD8⁺ T cells is associated with the clinical responsiveness in breast cancer patients and they are critical for the elimination of primary tumor and control of metastasis [194-197]. Therefore, we assessed the anti-tumor killing responses elicited by vaccine formulations containing rat Her-2/neu and MPL, or SA-4-1BBL, or MPL + SA-4-1BBL. Mice (n=3 per group) were vaccinated twice with 5 days interval s.c. with the same formulations, and euthanized 5 days later to test the cytotoxic activity of CD8⁺ T cells against rat Her-2/neu expressing A2L2 and Her-2/neu negative parental cell line 66.3neo breast cancer cells, (Fig. 12.A). Consistent with the therapeutic efficacy, vaccination with combined adjuvants generated a better A2L2 killing response than single adjuvants, and SA-4-1BBL as monotherapy generated better killing responses than MPL monotherapy (Fig. 12.A). The high cytotoxic killing response against Her-2/neu negative parental cell line 66.3neo in combined adjuvant treatment was not expected (Fig. 12.A). Therefore, we sought to determine the anti-tumor killing response in the combined adjuvant therapy against E7 expressing TC-1 cervical tumor cells to eliminate the possibility of low Her-2/neu expression on the 66.3 cell line

and killing response against other antigens common for both A2L2 and 66.3 cell lines. We observed that treatment with combined adjuvants did not result in killing of unrelated control TC-1 cervical cancer cells (Fig. 12.B), suggesting that the killing response generated by combined adjuvant therapy is not only Her-2/neu antigen specific, but also specific for other tumor antigens common for both A2L2 and its parental cell line. This might be due to ‘epitope spreading’; as vaccine kills tumor cells, they release more tumor associated antigens resulting in generation of immune responses to new epitopes [202].

Vaccination with rat HER-2/neu + SA-4-1BBL induces robust antigen specific cytokine responses in CD4⁺ and CD8⁺ T cells

Although the importance of CD8⁺ T cells has been shown in breast cancer [194-196] for the generation of primary CD8⁺ T cell responses and the establishment of long-term memory, CD4⁺ T cells are also critical to provide help. We, therefore, assessed the Th₁, and CD8⁺ T cell effector responses elicited by each vaccine formulations. Mice (n=5 per group) were primed and boosted s.c. with rat Her-2/neu and either individual agents (MPL or SA-4-1BBL) or combination of both adjuvants with 5 days interval and euthanized 5 days the boost to test the intracellular cytokine response of CD4⁺ and CD8⁺ T cells to the dominant Her-2₆₆₋₇₄ CTL epitope in the vaccine dLNs.

While vaccination with rat Her-2/neu + MPL did increase antigen-specific IFN- γ , IL-2 and TNF- α cytokine production (Fig. 13) in both CD4⁺ (*upper panel*) and CD8⁺ (*lower panel*) T cells, rat Her-2/neu + SA-4-1BBL therapy evoked much stronger production in these cytokines in both T cell types (most differences are reaching statistically significance; $p < 0.05$, except for IL-2 responses in both CD4⁺ and CD8⁺ T cells and IFN- γ response in CD8⁺ T cells, Fig. 13). Interestingly, although vaccination

with combined adjuvants was more effective than MPL monotherapy in the generation of antigen-specific cytokine responses, it was either equal to (IL-2, and IFN- γ in CD8⁺ T cells) or not as effective as the SA-4-1BBL monotherapy (Fig. 13). Collectively, these data demonstrate that: i) vaccination with MPL does induce antigen-specific CD4⁺ and CD8⁺ cytokine responses, ii) SA-4-1BBL monotherapy induces higher cytokine production in both CD4⁺ and CD8⁺ T cells than MPL monotherapy, and iii) combined adjuvant therapy does not further improve the SA-4-1BBL effect on cytokine responses. Indeed, in the generation of some cytokines, it is less effective than SA-4-1BBL monotherapy.

Vaccination with rat HER-2/neu + SA-4-1BBL increases the number of effector T cell populations in the dLNs of tumor bearing mice

Since we observed the synergistic effect of MPL on the SA-4-1BBL therapy in therapeutic setting (Fig. 10), we sought to determine the anti-tumor mechanism of the vaccine formulations when tumor was in place. For this purpose, mice (n=3 per group) were inoculated with A2L2 tumors, and when the tumors were around 3-5 mm, mice were primed with the previously indicated vaccine formulations and received the booster dose 5 days later. Three days after the boost, absolute numbers of different effector CD4⁺ and CD8⁺ T cell populations were determined in the dLNs. Vaccination with Her-2/neu + SA-4-1BBL generated higher numbers of effector memory (CD44^{hi}CD62L^{lo}), resting memory (CD44^{hi}CD62L^{hi}), and activated (CD69⁺) CD4⁺ T cells compared to the PBS vaccinated group (control *vs.* SA-4-1BBL: *** = $p < 0.001$ in CD44^{hi}CD62L^{lo}; * = $p < 0.05$ in CD44^{hi}CD62L^{lo}; ** = $p < 0.01$ in CD69⁺ cells; Fig. 14), but in doing so it also increased the numbers of these subsets in CD8⁺ T cells (control *vs.* SA-4-1BBL: *** = $p < 0.001$ in

CD44^{hi}CD62L^{lo}; * = p<0.05 in CD44^{hi}CD62L^{lo} cells; Fig. 14). On the other hand, MPL monotherapy did not induce the numbers of effector subsets in CD4⁺ and CD8⁺ T cells as well as SA-4-1BBL monotherapy, although it was significantly higher than PBS vaccinated control group (Fig. 14). Therapy with rat Her-2/neu + both adjuvants did not further increase the number of effector CD4⁺ and CD8⁺ T cell populations generated by SA-4-1BBL monotherapy in the dLNs. Indeed, it was less effective than SA-4-1BBL monotherapy in CD8⁺ effector T cell populations (Fig. 14).

However, the combined adjuvant therapy induced the highest number of Tregs (CD4⁺CD25⁺Foxp3⁺) in the dLNs, which was followed by SA-4-1BBL, and then MPL therapy (Fig. 14). Taken together, SA-4-1BBL monotherapy generates higher numbers of effector CD4⁺ and CD8⁺ T cells than MPL monotherapy, and combined adjuvant therapy does not improve the efficacy of SA-4-1BBL in such responses. On the other hand, addition of MPL to SA-4-1BBL adjuvanted vaccine enhances the number of Tregs.

The therapeutic efficacy of SA-4-1BBL + MPL adjuvanted Her-2/neu vaccine is associated with the increased pro-inflammatory and decreased anti-inflammatory cytokine responses

In addition to examining the numbers of effector T cell subsets along with Tregs, we next determined the functional status of lymphocytes in the setting above mentioned. Vaccination induced changes in pro-inflammatory (IL-2, IFN- γ , and TNF- α) and anti-inflammatory (IL-4, and IL-10) cytokine production by lymphocytes from the dLNs were determined in the supernatant of 48 hrs cultured cells (Fig 15). SA-4-1BBL + MPL treatment resulted in highest IFN- γ production compared to other therapies (* = p<0.05 Naive vs. SA-4-1BBL + MPL Fig. 15). However, combined adjuvant therapy did not

further improve the SA-4-1BBL efficacy in IL-2 and TNF- α production, and production of TNF- α was higher in SA-4-1BBL adjuvanted groups than control or MPL monotherapy. On the other hand, SA-4-1BBL monotherapy resulted in the increased production of IL-4 and IL-10 cytokines leading to a mixed pro and anti-inflammatory response, while addition of MPL decreased the amount of IL-10 (** = $p < 0.01$ SA-4-1BBL vs SA-4-1BBL + MPL), and IL-4 anti-inflammatory cytokines.

Combined adjuvant therapy with SA-4-1BBL and MPL generates a significant anti-tumor effect in tolerogenic BALB/neuT mice

It has been demonstrated that treatment with agonistic antibodies against 4-1BB enhances the immune responses in BALB-neuT mice [203-206]. Therefore, we assessed the therapeutic effect of combined adjuvants in a more clinically relevant BALB-neuT model. First of all, we verified the expression of rat Her-2/neu gene (233 bp) in heterozygous female BALB/neuT mice with PCR (Fig. 16) [207], and used the transgenic mice in this study. Eight week old tolerant BALB/neuT mice ($n=5$) were challenged with A2L2 cells on day 0 and vaccinated with Her-2/neu + SA-4-1BBL + MPL on days 5 and 10, or left untreated (PBS). As shown in Fig. 17, combination of SA-4-1BBL and MPL resulted in eradication of A2L2 tumors in 20% of BALB/neuT mice, and mice that expired from tumor in this treatment group had significantly slow tumor progression (* = $p < 0.05$, Fig. 17.A-B). Since elevated Treg numbers is associated with a poor prognosis of cancer patients [208, 209] and depletion of Treg cells results in better immune efficacy of therapeutic vaccines [210, 211], we hypothesized that depletion of Treg cells in BALB/neuT mice may further increase the efficacy of combined adjuvant therapy. Therefore, mice ($n=5$) were depleted with Treg cells using PC61 mAb two days before

tumor challenge, and vaccinated with SA-4-1BBL + MPL adjuvanted Her-2/neu vaccine on days 5 and 10 after tumor inoculation. As expected, depletion of Tregs further increased the anti-tumor efficacy of combined adjuvant system, and increased the survival up to 40% (**= $p < 0.02$, Fig. 17.A-B). Taken together, these data demonstrate that SA-4-1BBL + MPL as an adjuvant system is effective in eradicating the transplanted A2L2 tumors and prolongation of survival of tumor bearing mice, and depletion of Tregs further increase its anti-tumor efficacy.

Therapeutic efficacy of the SA-4-1BBL + MPL adjuvant system is achieved in the absence of detectable toxicity

Toxicity and autoimmunity generated by effective self-TAA-based therapeutic vaccine formulations using potent adjuvants may lead to termination of the studies, and impede future trials [212]. Since, we used combination of two potent adjuvants, SA-4-1BBL and MPL, for the treatment of breast cancer, we wanted to evaluate the toxicity profile of each vaccine formulation in a more relevant exogenous E7 (TAA) expressing TC-1 model in Th₁ biased C57BL/6 mice, which are more conducive to toxicity studies [213]. C57BL/6 mice (n=4 per group) were immunized with each vaccine formulations (E7+ MPL, E7 + SA-4-1BBL, or E7 + both adjuvants) and terminated 18 h post-immunization. Absolute numbers of T cells, B cells, DCs and Macrophages, NK cells and NK T cells in the spleen, mLN, and vaccine draining LNs were determined (Table1). We also evaluated liver and kidney functional status by measuring serum levels of ALT and AST as an indicator of liver function, and BUN and CREA for renal function (n=3 per group). Although, liver toxicity was reported in the melanoma patients with anti-4-1BB Ab treatment [81], as shown in Fig. 18, there was no significant difference in these

enzymes levels in SA-4-1BBL treatment group compared to naïve mice. Moreover, MPL monotherapy or combined adjuvant therapy also did not increase these enzyme levels compared to naïve mice. We further analyzed the liver tissue histology to evaluate the pathology of inflammation in the vaccine treated mice, which appeared normal with no observable differences compared to the liver tissues of naïve mice (Fig. 19). Taken together, these results indicate that combined adjuvant therapy did not generate detectable signs of toxicity, demonstrating the safety profile of this adjuvant system.

Discussion

One out of 5 women with Her-2/neu expressing mammary carcinoma relapses in spite of trastuzumab treatment [214]. To overcome this problem, many active vaccine strategies targeting Her-2/neu have been evaluated in clinical studies [182]. These strategies include peptide and protein based, DNA based, dendritic cell based, and whole tumor cell based vaccines [182]. However, recombinant peptide or protein based vaccines require an adjuvant to generate potent anti-tumor immune responses to target Her-2/neu protein because of their weak antigenic nature [11, 12]. In the current study, we tested if the costimulatory ligand SA-4-1BBL and TLR4 agonist MPL with distinct mechanisms of action can serve as a novel adjuvant system for the development of recombinant TAA based breast cancer vaccine. Here we demonstrated that MPL synergized with SA-4-1BBL as the adjuvant component of rat Her-2/neu TAA-based vaccine to generate better anti-tumor killing response, enhanced pro-inflammatory, and reduced anti-inflammatory cytokine responses that translated into improved therapeutic efficacy in mouse A2L2

breast cancer model. More importantly, the combined adjuvant therapy induced the eradication of established A2L2 tumors in both BALB/c and more tolerant BALB/neuT mice, and its efficacy was further improved with the depletion of Tregs in BALB/neuT mice.

In the case of poorly immunogenic tumors, targeting two arms of immunity using TLR ligands and agonists for TNF receptors has been evaluated for the generation of potent anti-tumor immune response. As an example, combined stimulation of TLR-9 and 4-1BB enhances antigen specific CTL responses and induces the rejection of melanoma tumors [215]. Here we particularly focused on TLR4 ligand MPL, as an FDA approved adjuvant, to activate the innate arm of the immune system, and SA-4-1BBL, as a novel adjuvant for the activation of adaptive immunity. MPL interacts with TLR4 on the APCs, and induce their activation. This activation leads to the production of proinflammatory cytokines and upregulation of costimulatory molecules on the cell surface, which in turn, primes adaptive immune responses [6]. However, SA-4-1BBL, binds to the 4-1BB receptor on the activated CD4⁺ and CD8⁺ T cells, leading to their survival, expansion, acquisition of effector function, and long-term immune memory [90, 199, 200]. More importantly, 4-1BB costimulation has a predominant effect on CD8⁺ T cells that are critical for the elimination of tumors and generation of long term memory as demonstrated in various cancer settings including breast cancer [98, 194-198]. Therefore, here we hypothesized that MPL can co-operate with SA-4-1BBL for the activation of CD8⁺ T cells through APC activation and antigen presentation by APCs [216].

Anti-Her-2/neu antibodies have been detected in breast cancer patients in the early stages, enhanced antibody response was observed when Her-2/neu is overexpressed

in the primary tumor [183], and antibody treatment against Her-2/neu has shown to be efficacious. Although Trastuzumab has been evaluated and shown to be efficacious, its mechanism of action is not well known. Some possible action mechanisms are: (1) Her-2/neu downregulation; (2) reduced signaling of Her-2/neu; (3) inhibition of the shedding of Her-2/neu ECD; (4) blocking proliferation, and induction of apoptosis; (5) angiogenesis inhibition; and finally induction of Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) [217]. Therefore, in the recombinant protein-based vaccines, induction of humoral immunity to generate long-term anti-Her-2/neu titers along with cellular response might improve the protective efficacy of such vaccines. In this study, we reported that MPL monotherapy induced significant increase in the anti-Her-2/neu titers (Fig. 11), but it failed to provide protection. Even though, all these predicted mechanisms for Trastuzumab can inhibit the cancer cell growth, not all the antibodies are capable of doing so, instead they may stimulate cancer cell growth [218]. It is also possible that these different effects are due to epitope specificity of the antibodies and changes in signaling pathways [218]. Consistent with this, Ma et al. reported that immunization with four different domains of Her-2/neu ECD fused with CD19 scFv induced different IgG Ab levels, and while serum from mice immunized with scFv-D3 induced growth inhibition of SKBR-3 cells despite low titers of IgG Ab, scFv-D1 immunization did not generate an inhibitory effect despite high anti-Her-2/neu titers [219]. Therefore, anti-Her-2/neu antibodies generated by MPL treatment may not be helpful for tumor eradication. On the other hand, two TF mice in the combination treatment group were negative for anti-Her-2/neu titers while one mouse showed moderate titer against Her-2/neu protein on day 59, and one TF mouse in the SA-4-1BBL

monotherapy did not generate antibody response for the target antigen. Therefore, further studies such as evaluating the neutralization efficacy of the antibodies must be performed to understand the relation of antibody response with survival.

We did not observe the synergistic effect of MPL on the SA-4-1BBL therapy in terms of cytokine production in CD4⁺ and CD8⁺ T cell subsets in the vaccine dLNs 5 days after the boost. Instead, SA-4-1BBL monotherapy induced better cytokine responses than MPL and combined adjuvant therapy (Fig. 13). However, combined adjuvant therapy induced higher numbers of CD4⁺ and CD8⁺ T cell producing IFN- γ , TNF- α , and IL-2 cytokines than other treatment groups in the spleen (data is not shown). It is possible that cytokine producing effector T cells in the combined adjuvant therapy have already been migrated into the spleen, and determining such responses 5 days after the boost in the dLNs might be a late time-point. Next, we should investigate the cytokine responses in the dLNs at earlier time points such as 1 or 3 days after the booster immunization to determine the synergistic effect of SA-4-1BBL and MPL combined adjuvant therapy.

Interestingly, *in-vitro* stimulation with rat Her-2/neu p66 peptide corresponding to H-2K^d-restricted, dominant CTL epitope increased the expression of proinflammatory cytokines in CD4⁺ T cells when compared to negative control (without p66 peptide stimulation in the *in-vitro* culture) (Fig. 13). This could be due to the memory response generated by a prime-boost vaccination, since *in vivo* generated CD8⁺ T cells were ready for a robust antigen specific secondary response *in vitro*. Therefore, cytokines, such as IFN- γ produced by CD8⁺ T cells *in vitro*, might have induced the production of Th₁ cytokines by CD4⁺ T cells. Further, since NK cells also express 4-1BB, IFN- γ produced

by *in vivo* activated NK cells may also enhance CD4⁺ T cell differentiation into Th₁ subtype, and production of type I cytokines [220-222].

Although it has been demonstrated that 4-1-BB signaling preferentially activates CD8⁺ T cells over CD4⁺ T cells by us [102, 103] and others [159, 160], in this study SA-4-1BBL monotherapy induced the production of inflammatory cytokines in both CD4⁺ and CD8⁺ T cells (Fig. 13). This could differ between models because Ganguly et al. demonstrated that agonistic anti-4-1BB antibody enhances Gag specific CD4⁺ but not CD8⁺ T cell responses, while membrane-bound 4-1BBL plasmid DNA induces antigen-specific CD4⁺ and CD8⁺ T cell responses in an HIV infection model [175]. Further, these results may provide clinical benefit since CD4⁺ T helper cells significantly contribute to generation of antitumor responses [223-225] by providing essential signals to CTLs directly [226] or through APCs [227, 228].

When tumor was present, MPL worked in synergy with SA-4-1BBL to induce pro-inflammatory cytokine IFN- γ production in the lymphocyte cultures from the dLNs (Fig. 15). These results are consistent with the clinical outcome (Fig. 10.A-B), and anti-tumor killing response (Fig. 12) since IFN- γ is required for the induction of MHC class I expression which is critical for the antigen specific CTL killing of the cancer cells [229]. On the other hand, immunization with Her-2/neu + SA-4-1BBL also induced anti-inflammatory cytokine IL-10, and IL-4 production, suggesting a mixed response generated by SA-4-1BBL. Addition of MPL to SA-4-1BBL adjuvanted vaccine decreased the IL-10, and IL-4 production, and shifted the immune response towards inflammatory response. However, IL-10 effect on the immune system has been controversial since it can suppress the inflammatory cytokine production, while, in doing

so, it can induce CTL and NK cytotoxic activity [230-232]. Furthermore, its impact on tumor growth has also been controversial since it has been shown that IL-10 suppresses tumor growth and inhibits metastasis in several models, including breast cancer [233-236], while it promotes tumor growth through Stat3 activation [237]. Nevertheless, both pro- and anti-inflammatory activity of IL-10 has been shown to be important in rejection of melanoma tumors [238]. As a result, the enhanced anti-tumor efficacy by combined adjuvant therapy might be due to the generation of a more balanced anti- and pro-inflammatory response.

Tregs are the critical players in the escape mechanism of cancer cells from the immune system attack [239], and they have a critical effect in tumor progression in different tumor models, including breast cancer [208, 240, 241]. Moreover, the number of intratumoral Tregs is a prognostic predictor in breast cancer patients [242, 243]. Therefore, in cancer immunotherapy, controlling the number and/or function of Tregs is another critical aim for better vaccine efficacy. As such, depletion of Tregs using antibodies or cyclophosphamide has been shown to improve the anti-tumor efficacy in preclinical models [244-247]. Consistent with preclinical studies, breast cancer patients with lower numbers of Tregs infiltrated into the tumor after neoadjuvant chemotherapy was associated with higher pathological response rates [243]. Our data further supports the role of Tregs in the vaccine efficacy since their depletion resulted in increased survival in BALB/neuT mice treated with combined adjuvants (Fig. 17.A-B). However, the difference was not statistically significant. This could be due to the timing, schedule of administration or dose of PC-61 antibody since we only used one dose of PC-61 at one time point. On the other hand, the augmented numbers of Tregs in the dLNs of the

treatment groups with better therapeutic efficacy was not expected. Combined adjuvant therapy induced the highest numbers of Tregs, which was followed by SA-4-1BBL, and MPL therapy (Fig. 14). However, our data do not contradict with previously published studies by us using SA-4-1BBL and others using agonistic anti-4-1BB antibody where 4-1BB costimulation enhances the proliferation of Tregs [101, 248]. It is likely that SA-4-1BBL renders T effector cells resistant to Treg suppression within draining lymph nodes and tumor [98, 101], or the high number of infiltrated Tregs in tumor draining lymph nodes may be a trafficking issue [72]. Therefore, the increased numbers of Tregs in the vaccine dLNs may not be a limiting factor for the efficacy of the SA-4-1BBL adjuvanted vaccines. On the other hand, it has been shown that TLR-4 is also expressed on CD4⁺ T effector and Tregs [249, 250], and stimulation with LPS induces Treg survival, expansion, and enhances their regulatory function *in vivo* [250], which might be the reason for increased number of Tregs in the dLNs of MPL treated group. Further studies must be performed to look at the Treg numbers within the tumor, to evaluate whether SA-4-1BBL adjuvanted vaccines may suppress the trafficking of Tregs into the tumor.

Costimulatory molecules have a critical role in generating protective cellular immune responses [70, 200]. Agonistic antibodies to costimulatory members of CD28 and TNFR families are effective in generating immune responses against cancer and infectious agents [251-253]. However, agonistic antibodies can cause severe toxicity due to nonspecific lymphocyte activation systemically [103, 254], and toxicity generated by vaccination is a critical limiting factor. Towards this goal, we have developed the costimulatory molecule 4-1BBL in a novel soluble form as a novel adjuvant. As we reported previously SA-4-1BBL generates more potent and qualitatively different

immunostimulatory activity without detectable toxicity as assessed by systemic cytokine response, non-specific lymphoproliferation, lymphomegaly and splenomegaly, and hepatitis compared to the agonistic 4-1BB mAb [103]. In this study, we further investigated the possible induction of toxicity by two potent immunomodulators. Importantly, the therapeutic efficacy of SA-4-1BBL + MPL adjuvant system was achieved in the absence of detectable toxicity as assessed by various indicators, such as total number of various lymphocyte populations in dLNs, mLNs and spleen (Table 1), liver and kidney enzyme levels to determine functional status of these organs (Fig. 18), and histological analysis of liver to measure inflammation (Fig. 19). The lack of toxicity in SA-4-1BBL monotherapy is consistent with our previously published study where treatment of mice with 4-fold higher SA-4-1BBL over the therapeutic dose used in this study did not induce toxicity [103]. MPL has also been shown to be safe in preclinical and clinical studies [6, 201, 255], and our results further supports the safety of MPL adjuvanted vaccine. Therefore, the lack of detectable toxicity in the combined and individual adjuvant therapies provides another advantage along with therapeutic efficacy for the human use.

In conclusion, here we demonstrate the efficacy of the SA-4-1BBL + MPL adjuvant system in inducing potent anti-tumor killing responses and antigen specific production of IFN- γ , a signature cytokine for CTL killing activity, and suppressing the anti-inflammatory IL-10 production by generating a more balanced pro- and anti-inflammatory environment compared to SA-4-1BBL monotherapy which translate into potent therapeutic efficacy in Her-2/neu expressing A2L2 breast cancer model. Moreover, depletion of Tregs further increases the therapeutic efficacy of the adjuvant

system in tolerant BALB/neuT mice. Importantly, the therapeutic efficacy of the vaccines was observed in the absence of detectable toxicity which was tested in E7 expressing TC-1 model. Enhancing the efficacy of individual adjuvants with the combined adjuvant therapy emphasizes the importance of combining the adjuvants with different action mechanisms for a better therapeutic efficacy. Finally, this combined adjuvant therapy can be utilized for other cancers, and infectious diseases for a better efficacy.

A **Figure 7**

Production of recombinant rat Her-2/neu ECD in bacteria. A) Schematic representation of 6X-His-pTWIN-1 expression vector with Her-2/neu. B) Coomassie blue staining of uninduced and induced samples with IPTG.

A **Figure 8**

Production of recombinant rat Her-2/neu ECD in bacteria. A) Schematic representation of pTWIN-1- 6X-His expression vector with Her-2/neu. B) Coomassie blue staining of uninduced and induced samples with IPTG. C) Western blot picture of recombinant rat Her-2/neu protein as detected by anti-ErbB2 antibody.

Figure 9

Production of recombinant rat Her-2/neu protein ECD in *Drosophila* S2 cells, and Her-2/neu expression in A2L2 cells. A) Schematic representation of pMT/BiP expression vector with Her-2/neu. B) Coomassie blue staining of CuSO_4 induced samples. C) Purified rat Her-2/neu pattern (FT: flow through, WB: wash buffer). D) Western blot picture of recombinant rat Her-2/neu protein. E) Whole Her-2/neu protein expression in A2L2 cell lysate as evaluated by anti-ErbB2 antibody on western blot.

Figure 10

A prime-boost vaccination with SA-4-1BBL and MPL adjuvant system results in eradication of established A2L2 tumors. BALB/c mice were challenged s.c. with 1×10^5 live rat Her-2/neu expressing A2L2 tumor cells and left untreated (PBS) or vaccinated twice s.c. on days 5 and 10 post-tumor challenge with rat Her-2/neu (50 μ g) mixed with MPL (25 μ g), or SA-4-1BBL (25 μ g), or combination of both agents (25 μ g/agent). A) Individual tumor growths in each treatment group. B) Survival comparison of treatment groups. *= $p < 0.05$.

Figure 11

A prime-boost vaccination with Her-2/neu + MPL induces significant antibody response. Serum was collected from each treatment groups as indicated above on termination day of mice when tumor was around 12 mm diameter or on day 59 when tumor was eradicated. Anti-Her-2/neu titers were determined by ELISA. Bar graph shows the average log titers of anti-Her-2/neu in each treatment group. Dotted line represents the limit of detection. *= $p < 0.02$; ** = $p < 0.01$.

Figure 12

SA-4-1BBL + MPL adjuvant system induces a strong tumor-specific killing response. BALB/c mice were vaccinated twice with 5 days interval with indicated formulations. 5 days after booster dose injection, lysis of target cells were determined. A) Tumor specific lysis of A2L2 and 66.3 neo target cells. B) Tumor specific (A2L2) and non-specific (TC-1) target cell lysis were determined in mice treated with combined adjuvants.

Figure 13

SA-4-1BBL based Her-2/neu vaccine induces a strong cytokine response. BALB/c mice were vaccinated twice with 5 days interval with Her-2/neu and indicated adjuvants. 5 days after the boost, peptide specific cytokine responses were determined in the dLNs. Each graph represents the individual numbers of CD4⁺CD44^{hi} (*Upper panel*) and CD8⁺CD44^{hi} (*lower panel*) T cells producing TNF α , IL-2, and IFN γ cytokines. Horizontal line represents the mean value of each group. *** = p<0.001; ** = p<0.01; * = p<0.05.

Figure 14

Vaccination with Her-2/neu + SA-4-1BBL induces high numbers of effector T cell subsets in the dLNs of tumor bearing mice. Tumor bearing (3-5 mm) BALB/c mice were vaccinated twice with 5 days interval. 3 days after the boost, absolute numbers of different CD4⁺ and CD8⁺ T cell populations were determined. Each bar represents the mean and S.D. of absolute numbers of T cell subsets in each treatment group. **** = $p < 0.0001$; *** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$.

Figure 15

Addition of MPL to the SA-4-1BBL adjuvanted vaccine increases pro-inflammatory and decreases anti-inflammatory cytokine production in the dLNs of tumor bearing mice. Tumor bearing (3-5 mm) BALB/c mice were vaccinated twice with 5 days interval. 3 days after the boost, lymphocytes from the dLNs were cultured in the presence of Her-2/neu protein and p66 peptide for 48h. Culture supernatants were collected and cytokine responses were determined by CBA analysis. Each bar represents the mean and S.D. of cytokine amount in each treatment group. **** = $p < 0.0001$; ** = $p < 0.01$; * = $p < 0.05$.

Figure 16

Verification of the expression of rat Her-2/neu gene in BALB/neuT mice with PCR.

DNA gel picture of Her-2/neu gene (233 bp) in female BALB/neuT mice.

Figure 17

While treatment with SA-4-1BBL and MPL adjuvanted vaccine results in eradication of established A2L2 tumors in 20% of BALB/neuT mice, depletion of Tregs further improves the vaccine efficacy. BALB/neuT mice were challenged s.c. with 1×10^5 live rat Her-2/neu expressing A2L2 tumor cells and left untreated (PBS) or vaccinated twice s.c. on days 5 and 10 post-tumor challenge with rat Her-2/neu (50 μ g) mixed with MPL (25 μ g) and SA-4-1BBL (25 μ g), or first treated with PC-61 depleting mAb (300 μ g) two days before tumor inoculation and then treated with the combined adjuvant therapy. A) Individual tumor growths of mice in each treatment group. B) Survival comparison of treatment groups. * = $p < 0.05$; ** = $p < 0.02$.

A. Total numbers of lymphocytes in the spleen

	CD4 ⁺ T cells	CD8 ⁺ T cells	B cells	NK cells	NK T cells	Macrophages	DCs
Naive	22±2 (x10 ⁶)	12±2 (x10 ⁶)	44±4 (x10 ⁶)	2±0.3 (x10 ⁶)	0.5±0.2 (x10 ⁶)	0.4±0.1 (x10 ⁶)	6±1 (x10 ⁶)
E7+MPL	20±3 (x10 ⁶)	11±2 (x10 ⁶)	44±12 (x10 ⁶)	0.9±0.2 (x10 ⁶) (**)	0.5±0.2 (x10 ⁶)	0.3±0.2 (x10 ⁶)	8±2 (x10 ⁶)
E7+SA-4-1BBL	22±2 (x10 ⁶)	12±2 (x10 ⁶)	47±3 (x10 ⁶)	1±0.1 (x10 ⁶)	0.5±0.1 (x10 ⁶)	0.2±0.06 (x10 ⁶) (*)	8±1 (x10 ⁶)
E7+MPL+S A-4-1BBL	21±4 (x10 ⁶)	12±2 (x10 ⁶)	52±10 (x10 ⁶)	2±0.5 (x10 ⁶)	0.7±0.2 (x10 ⁶)	0.4±0.1 (x10 ⁶)	8±1 (x10 ⁶)

B. Total numbers of lymphocytes in the dLNs

	CD4 ⁺ T cells	CD8 ⁺ T cells	B cells	NK cells	NK T cells	Macrophages	DCs
Naive	34±20 (x10 ⁵)	25±17 (x10 ⁵)	15±9 (x10 ⁵)	0.38±0.26 (x10 ⁵)	0.17±0.15 (x10 ⁵)	0.02±0.01 (x10 ⁵)	1.7±1.4 (x10 ⁵)
E7+MPL	21±5 (x10 ⁵)	17±5 (x10 ⁵)	14±5 (x10 ⁵)	0.19±0.03 (x10 ⁵) (*)	0.15±0.12 (x10 ⁵)	0.02±0.01 (x10 ⁵) (*)	2±1.2 (x10 ⁵)
E7+SA-4-1BBL	22±6 (x10 ⁵)	18±3 (x10 ⁵)	14±6 (x10 ⁵)	0.29±0.13 (x10 ⁵)	0.11±0.05 (x10 ⁵)	0.03±0.02 (x10 ⁵)	1.5±0.7 (x10 ⁵)
E7+MPL+S A-4-1BBL	24±8 (x10 ⁵)	20±7 (x10 ⁵)	17±7 (x10 ⁵)	0.28±0.06 (x10 ⁵)	0.13±0.04 (x10 ⁵)	0.05±0.01 (x10 ⁵)	1.7±0.6 (x10 ⁵)

C. Total numbers of lymphocytes in the mLN

	CD4 ⁺ T cells	CD8 ⁺ T cells	B cells	NK cells	NK T cells	Macrophages	DCs
Naïve	69±24 (x10 ⁵)	45±14 (x10 ⁵)	50±19 (x10 ⁵)	0.7±0.4 (x10 ⁵)	0.4±0.2 (x10 ⁵)	0.1±0.07 (x10 ⁵)	6±2 (x10 ⁵)
E7+MPL	43±11 (x10 ⁵)	32±8.3 (x10 ⁵)	29±13 (x10 ⁵)	0.3±0.1 (x10 ⁵) (*)	0.3±0.07 (x10 ⁵)	0.05±0.01 (x10 ⁵)	4±2 (x10 ⁵)
E7+SA-4-1BBL	55±29 (x10 ⁵)	34±22 (x10 ⁵)	49±16 (x10 ⁵)	0.6±0.2 (x10 ⁵)	0.3±0.1 (x10 ⁵)	0.07±0.02 (x10 ⁵)	5±2 (x10 ⁵)
E7+MPL+S A-4-1BBL	56±18 (x10 ⁵)	38±15 (x10 ⁵)	42±12 (x10 ⁵)	0.5±0.1 (x10 ⁵)	0.3±0.06 (x10 ⁵)	0.07±0.02 (x10 ⁵)	5±1 (x10 ⁵)

Table 1

SA-4-1BBL + MPL adjuvant system does not induce acute lymphocyte recruitment into the lymphatic tissues. C57BL/6 mice (n=3 per group) were vaccinated with indicated vaccine formulations or left untreated (PBS). 18 h after immunization, mice were terminated; spleen, dLNs, and mLN were harvested to determine the absolute numbers of indicated lymphocyte subsets. A) Absolute numbers of indicated lymphocytes in the spleen. ** $p \leq 0.02$ in E7+MPL vs E7+MPL+SA-4-1BBL; E7+MPL vs E7+SA-4-1BBL; E7+MPL vs Naïve in NK cells. * $p \leq 0.05$ in E7+SA-4-1BBL vs Naïve in macrophages. B) Absolute numbers of indicated lymphocytes in the dLNs. * $p \leq 0.05$ in E7+MPL vs E7+SA-4-1BBL+MPL in NK cells. * $p \leq 0.05$ in E7+MPL vs E7+SA-4-1BBL+MPL in macrophages. C) Absolute numbers of indicated lymphocytes in the mLN. * $p \leq 0.05$ in E7+MPL vs E7+SA-4-1BBL in NK cells.

Figure 18

Treatment with SA-4-1BBL + MPL adjuvant system does not induce liver and kidney damage. C57BL/6 mice (n=3 per group) were vaccinated with indicated vaccine formulations or left untreated (PBS). 18 h after immunization, serum was collected to evaluate the kidney and liver enzyme levels. Rectangle area indicates the normal range for each enzyme level. ALT range: 28-132 U/L, AST range: 59-247 U/L, BUN range: 18-29 mg/dL, CREA range: 0.2-0.8 mg/dL.

Figure 19

Treatment with SA-4-1BBL + MPL adjuvant system does not induce acute lymphocyte infiltration into the liver. C57BL/6 mice (n=3 per group) were vaccinated with indicated vaccine formulations or left untreated (PBS). 18h after immunization, livers were collected to determine the histo-pathology.

CHAPTER 4

CONCLUSIONS AND FUTURE PROSPECTIVES

Prophylactic vaccines against infections have had a tremendous effect on individual health since it has significantly reduced the rate of deadly infectious diseases and human mortality. However, historically, vaccines were made from live-attenuated or inactivated forms of the microbe and there were concerns about stability, side effects and safety. As a result, with the development of molecular biology, different vaccine strategies have been investigated to generate new human vaccines. Vaccines based on recombinant proteins can overcome the limitations relating to the safety, and present an attractive approach because of their safety, ease of transportation, and storage. However, recombinant protein-based subunit vaccines are poorly immunogenic and need to be administered with adjuvants to generate an efficient appropriate immune response with long lasting immune memory against target antigens.

Historically, the importance of the humoral immune response for vaccine effectiveness has put the emphasis on the development of adjuvants, which are capable of enhancing antibody responses. As a result, many adjuvants are effective in increasing antibody titers, but do not elicit significant Th₁ or CTL responses. However, for some viral and intracellular bacterial infections, generation of only antibody response is not

enough for the complete protection. In this regard, combination of adjuvants with distinct action mechanisms should provide a great potential against such infections. For example, combination of TLR ligands that target primarily innate immunity and stimulate Th₁ cytokine responses increases the efficacy of vaccines which primarily target the generation of antibody response (AS04 adjuvant system).

Plague is one of those infections that require both antibody and Th₁ cellular responses. Therefore, in this study, we utilized SA-4-1BBL that targets primarily adaptive immunity as an adjuvant component of prophylactic bubonic plague vaccine either alone or in combination with alum that targets the innate immunity to induce the generation of cellular immune responses, and enhance the protective efficacy of the vaccine. Here, it has been shown that combination of SA-4-1BBL + alum induced a robust antigen specific Th₁ cytokine response in CD4⁺ and CD8⁺ T cells, along with high anti-rF1-V titers which lead to 100% efficacy against bubonic plague in a prime-only setting, while SA-4-1BBL monotherapy resulted in 20% efficacy without generation of antibody response. However, administration of SA-4-1BBL monotherapy twice in a prime-boost setting induced robust and long-lasting antibody response and enhanced cellular response which translated into 100% efficacy. These results further support the requirement of a balanced cellular and humoral response in the case of plague infection, and future vaccines should be designed to target both humoral and cellular immunity with the utilization of combination adjuvants.

Cancer, on the other hand, is a complex disease to overcome since tumor microenvironment can highly affect the disease progression and includes multiple cell types which interact with each other. Therefore, it affects millions of people and its incidence and human mortality is increasing all over the world. Although cancer immunotherapy has been chosen as breakthrough of the year for 2013 by *Science's* editors, as two strategies have been shown to be effective including anti-CTLA-4 antibody therapy, but the cost of these antibody therapies is too high, \$120,000, for a course therapy. On the other hand, the complexity of the preparation of individualized vaccines limits the use of whole tumor cell and dendritic cell-based vaccines. Therefore, development of recombinant sub-unit based vaccines might present an attractive approach because of their potential low cost, and ease of production.

Due to the low immunogenic nature of TAAs, adjuvants should be formulated in the subunit vaccines. However, not all the adjuvants are effective to generate anti-tumor immune responses with a therapeutic efficacy since the tumor-induced immunosuppressive mechanisms in the tumor microenvironment (TME) which are the biggest obstacles for therapeutic cancer vaccine development. The immunosuppressive mechanisms constitute expansion and/or recruitment of regulatory cells such as Tregs, MDSCs, and TAMs within the TME, and production and release of immune suppressive soluble factors such as TGF- β , IL-10, IDO via tumor cells. Therefore, immune modulators such as antibodies against CTLA-4, and PD-1 have been used to enhance the anti-tumor effector responses. However, such strategies are established to block or remove negative regulatory mechanisms, and may not be sufficient to induce potent anti-tumor efficacy due to the lack of the stimulation of antigen specific immune responses,

and tumors are also capable of interfering innate and adaptive immune responses to evade from immune system attack. In this context, vaccines utilizing adjuvants which can modulate innate, adaptive and regulatory immunity will have better efficacy. Therefore, here we hypothesize that utilization of SA-4-1BBL as an adjuvant component of recombinant Her-2/neu subunit vaccine might improve the therapeutic efficacy of Her-2/neu positive breast cancer vaccine due to its pleiotropic effects on the cells of innate, adaptive, and regulatory immunity either alone or in combination with MPL. Furthermore, its expression on the activated CD4⁺ T, CD8⁺ T, NK and NK T cells provides another advantage to target antigen specific cells of innate and adaptive immunity. We show that combination of SA-4-1BBL + MPL generated robust tumor specific killing response, and inflammatory cytokine IFN- γ production. The sum of these responses translated into better efficacy than the individual agents without detectable toxicity. As such, combined adjuvant therapy resulted in 30% eradication of established Her-2/neu breast cancer in BALB/c mice, while SA-4-1BBL monotherapy provided 10% protection and MPL monotherapy failed to do so. Furthermore, combined adjuvant therapy eradicated the tumors in 20% of BALB/neuT mice, while depletion of Tregs further improved its efficacy to 40%. These results further support the requirement of targeting both innate and adaptive immunity to generate potent immune response to eliminate the cancer cells and overcoming immune suppressive mechanisms employed by tumors.

On the other hand, although critical, augmented CTL responses may not translate into better anti-tumor efficacy since another obstacle is the trafficking of tumor-reactive CTLs into the tumor from the periphery and for the immune surveillance activated

effector T cells must accumulate and function within the tumors. In this regard, SA-4-1BBL seems to be a potential adjuvant candidate since our lab has previously shown that SA-4-1BBL adjuvanted HPV E7-based therapeutic cervical cancer vaccine enhances the number of infiltrating CD8⁺ T cells to the tumor, and addition of MPL to the SA-4-1BBL therapy further increases the number of intratumoral CD8⁺ T cells infiltration. Moreover, a significant reduction in the number of intratumoral Tregs in mice vaccinated with either SA-4-1BBL as a single adjuvant or in combination with MPL has also been observed in that study. In this regard, chemokines such as CCL5, CXCL9, CXCL10, and Leukotriene B4 receptor BLT1 have been shown to regulate the selective CTL recruitment. Although it is not known whether SA-4-1BBL is up-regulating the expression of such chemokines, it is likely that it enhances the production of leukocyte chemoattractants due to the high CD8⁺ T cell numbers in the tumor.

There are three distinct steps that must be achieved to generate effective immunity against tumor or infection. First of all, DCs must be activated and present antigens derived from tumor, or pathogens. Activation signals could be generated with the recruitment of TLR ligands or agonistic antibodies against activating receptors such as CD40 in the vaccines. Next, antigen specific T cell activation must be induced in the lymphoid organs. In this context, the importance of co-stimulatory molecules for the activation of effector T lymphocytes has put the emphasis on the use of the agonists of co-stimulatory receptors as adjuvants in vaccines. Now, it has been realized that generation of the first signal through antigen presentation is not enough for T cell activation; rather this signal should be followed by signals from costimulatory molecules. Further, the quality of effector CD4⁺ and CD8⁺ T cells is more important than their high

quantity. Finally, antigen-specific T cells must be recruited into the site of infection or tumor to perform their effector function where immune suppression takes place. Any vaccine approaches have to overcome such barriers to be successful. As a result, the failure of immunotherapy is very likely, and combining immunotherapies has shown to be efficacious and encourage scientists to investigate other combination approaches.

Although animal models show promising results for immunotherapy, human clinical trials have been somewhat disappointing because of the complexity and undefined differences between animals and humans. Despite the challenges to translate efficacy from animals to humans, vaccines against some infectious diseases and cancers have been developed and shown to be efficacious in humans with therapeutic efficacy, and some vaccines are already being tested in clinical trials and the ones which are effective and safe will be used in near future as well. However, there is still a dire need for developing vaccines against various acute and chronic infections and cancer.

It still remains challenging to develop effective vaccines against infectious diseases and cancer with recombinant proteins because of their poor immunogenic nature. Development of adjuvants to improve immune response to such vaccines has been increased dramatically in the last several years, and some adjuvants are currently employed in human vaccines. Targeting 4-1BB receptor presents an attractive approach due to its 1) pleiotropic effect on the innate, adaptive, and regulatory immunity, 2) expression on the activated but not resting T cells, and 3) CD8⁺ T cell centric function which is critical for the elimination of pathogen infected and tumor cells, and generation of immune memory. Moreover, significant progress has been made for the development of antibodies specific for 4-1-BB receptor for human use.

The promising results from this overall Ph.D. study may provide a new scientific rationale for the utility of SA-4-1BBL costimulatory molecule as an adjuvant platform for the development of subunit vaccines either alone or in combination with other immune stimulators targeting primarily innate immunity against viral or bacterial infections requiring both humoral and cellular immune responses, and cancer. Use of immune stimulators targeting innate immunity provides enhanced APC activation and function which primes T cells, and use of SA-4-1BBL further increases T cell effector functions, survival, and memory responses.

Although treatment with SA-4-1BBL + MPL therapy resulted in eradication of transplanted tumors in 30% of BALB/c mice and 20% of more tolerogenic BALB/neuT mice, vaccine schedule and dosage should be further evaluated for a better therapeutic efficacy. After finding the most efficacious schedule and administration dosage, this potent combined adjuvant therapy should be tested for a spontaneous breast cancer model in BALB/neuT mice. Moreover, despite the 100 % protection generated by SA-4-1BBL + alum in prime-only setting, and SA-4-1BBL adjuvanted vaccine in a prime-boost setting in the bubonic plague model, this potent adjuvant system should be tested in a more clinically relevant pneumonic plague model. To mimic the aerosol exposure to *Y. pestis*, and induce the immune response at mucosal surfaces, the vaccine could be administered intranasal. If it demonstrates efficacy in murine model, it should be tested in NHP models such as African green monkeys that have been difficult to confer protection. Although here we demonstrated effectiveness of the combined adjuvant systems in the clearance of intracellular bacteria and cancer, the mechanisms behind these therapies, and the synergistic effect should be further investigated for the clinical translation. SA-4-

1BBL either alone or in combination with other adjuvants targeting different pathways should be tested in future pre-clinical models and clinical trials against plague and breast cancer. Eventually, testing these adjuvant systems in clinical trials will assess the therapeutic potential of these combined adjuvant platforms, and if efficacy is demonstrated, they can be utilized against other infection and cancer types.

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2. Dinc G, *et al.* Improving the cellular efficacy of the lead *Yersinia pestis* rF1V subunit vaccine using SA-4-1BBL as a novel adjuvant. Under review in *Vaccine*.
3. Dinc G, *et al.* Vaccination with rat Her-2/neu and combined adjuvant system of SA-4-1BBL and MPL eradicates established breast tumors in BALB/c and tolerogenic BALB/neuT mice. Manuscript ready for submission.
4. Srivastava AK, Dinc G, *et al.* 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. Manuscript ready for submission.

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