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Improving the Th_1 cellular efficacy of the lead *Yersinia pestis* rF1-V subunit vaccine using SA-4-1BBL as a novel adjuvant

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ABSTRACT

The lead candidate plague subunit vaccine is the recombinant fusion protein rF1-V adjuvanted with alum. While alum generates Th_2 regulated robust humoral responses, immune protection against *Yersinia pestis* has been shown to also involve Th_1 driven cellular responses. Therefore, the rF1-V-based subunit vaccine may benefit from an adjuvant system that generates a mixed Th_1 and humoral immune response. We herein assessed the efficacy of a novel SA-4-1BBL costimulatory molecule as a Th_1 adjuvant to improve cellular responses generated by the rF1-V vaccine. SA-4-1BBL as a single adjuvant had better efficacy than alum in generating $CD4^+$ and $CD8^+$ T cells producing TNFα and IFNγ, signature cytokines for Th_1 responses. The combination of SA-4-1BBL with alum further increased this Th_1 response as compared with the individual adjuvants. Analysis of the humoral response revealed that SA-4-1BBL as a single adjuvant did not generate a significant Ab response against rF1-V, and SA-4-1BBL in combination with alum did not improve Ab titers. However, the combined adjuvants significantly increased the ratio of Th_1 regulated IgG_{2c} in C57BL/6 mice to the Th_2 regulated IgG_1. Finally, a single vaccination with rF1-V adjuvanted with SA-4-1BBL + alum had better protective efficacy than vaccines containing individual adjuvants. Taken together, these results demonstrate that SA-4-1BBL improves the protective efficacy of the alum adjuvanted lead rF1-V subunit vaccine by generating a more balanced Th_1 cellular and humoral immune response. As such, this adjuvant platform may prove efficacious not only for the rF1-V vaccine but also against other infections that require both cellular and humoral immune responses for protection.

1. Introduction

Yersinia pestis causes bubonic and pneumonic plague and has a potential to be used as bioweapon [1–3]. Currently there is no FDA-approved vaccine available. While *Y. pestis* infection can be treated with antibiotics, the effective treatment window for primary pneumonic infection is less than 24 h [4]. Furthermore, naturally acquired resistance to antibiotics has been reported [5], and weaponized *Y. pestis* could likely be modified to be resistant to antibiotics. Therefore, development of a plague vaccine is imperative.

Historically, plague vaccines were made from live-attenuated or inactivated forms of the microbe, but there were concerns about vaccine stability, occurrence of adverse effects, and safety [6]. Although, subunit vaccines using recombinant proteins could help overcome these issues, they are poorly immunogenic and need adjuvants for efficacy [7,8]. The lead candidate subunit plague vaccine is a recombinant fusion protein between the *Y. pestis* F1 and LcrV proteins (rF1-V). This vaccine is adjuvanted with alum and its protective efficacy against plague has been shown in rodent and guinea-pig models [9,10]. However, the rF1-V vaccine did not show consistent protection against *Y. pestis* infection in nonhuman primates. While vaccine efficacy was between 80 and 100% in Cynomolgus macaques, efficacy in African green monkeys has been less consistent (ranging between 0 and 75%) [11–14]. It is unclear why there is such a difference between these two species, but it appears that it is independent of antibody responses. Regardless, the lack of protection in African green monkeys raises a concern about the protective efficacy of a F1-V-based vaccine in humans

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[12,13] and indicates the need for further improvement of this subunit vaccine.

Alum adjuvanted rF1-V vaccination generates high serum antibody (Ab) titers that can protect rodents from *Y. pestis* infection [15,16]. However, recent studies have shown that protection against plague also requires cellular immunity [17–20]. For example, exogenous administration of IFNγ and TNFα can protect naïve mice against *Y. pestis* infection [20], neutralization of either cytokine abrogates protection in actively or passively immunized mice [17,18], and transfer of serum to IFNγR- and TNFα-deficient mice does not provide protection [17]. Finally, adoptive transfer of *Y. pestis*-primed T cells to naive μMT mice can protect against lethal *Y. pestis* challenge [21]. Therefore, one potential way to improve the efficacy of the rF1-V plague vaccine would be to increase the Th_1 response by altering the adjuvant system. Signaling through the costimulatory 4-1BB receptor of the TNFR superfamily is critical for the generation of Th_1 responses [22–24]. Thus, we hypothesized that a chimeric form of its ligand developed in our lab, SA-4-1BBL, may serve as an effective adjuvant with a Th_1 bias [22,23,25,26]. This is supported by several preclinical cancer models demonstrating robust antigen-specific Th_1 response and therapeutic efficacy using SA-4-1BBL [22,23,27].

Given that both Ab and Th_1 cellular immune responses are important for the control/clearance of *Y. pestis*, we here in hypothesized that SA-4-1BBL will improve the efficacy of alum adjuvanted rF1-V subunit vaccine to generate a balanced Th_1 and humoral response. Vaccination with SA-4-1BBL + alum adjuvanted rF1-V antigen generated a robust Th_1 cellular response without a significant impact on Ab titers. Single vaccination with combined adjuvant therapy generated better protection against plague than individual adjuvants. Taken together, our data demonstrate that SA-4-1BBL improves the Th_1 cellular efficacy of the lead rF1-V vaccine and provide the scientific rationale for the use of this adjuvant system as a platform for the development of subunit vaccines against infections that may benefit from a balanced Th_1 and humoral immune responses.

2. Materials and methods

2.1. Mice, vaccination, 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). Vaccine formulations were prepared and incubated at 4 °C O/N to allow for the adsorption of the antigen to alum. 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).

For bubonic plague infection studies, immunized mice ($n = 10$ per group) were transferred to the University of Louisville's Center for Predictive Medicine Regional Biocontainment Laboratory. On day 35 post-vaccination, mice were anesthetized with isoflorane and inoculated intradermally (i.d.) at the base of the tail with 20 μl of ~200 colony forming units (~20 LD_{50}s) of fully virulent *Y. pestis* CO92 Lux_{PcysZK} [28]. Mice were monitored twice daily for survival and moribund animals were humanly euthanized. Bacterial dissemination was assessed by optical imaging using the IVIS Spectrum in-vivo Imaging System (PerkinElmer, Waltham, MA). Regions of interest were generated using LivingImage 4.4 (PerkinElmer) to calculate the average radiance (photons/s/cm^2) at the site of infection [28,29].

2.2. Reagents and Abs

Y. pestis rF1-V 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 as previously published [30]. The basic description of the construct used in this study is as follows: 6xHis-(Gly)linker-corestreptavidin-(Ala)linker-extracellular domain (aa 104–309) of mouse 4-1BBL cloned in the PMT/BiP/V5-HisA expression vector for expression and purification using 6xHis Tag [31]. The theoretical pI and molecular weight of the protein is 6.52 and 39,160.8 Da, respectively. However, the molecule runs as a ~41 kDa on denatured SDS-PAGE gels, possibly due to glycosylation. Alhydrogel was purchased from Brenntag Biosector, Denmark. Antibodies for flow cytometery were purchased from BD Bioscience, eBioscience, or BioLegend.

2.3. Intracellular cytokine analysis

Mice ($n = 5$ per group) were vaccinated s.c. on days 0 and 35 and lymphocytes were isolated from draining lymph nodes on day 40. For intracellular cytokine analysis, 4×10^6 lymphocytes were plated in 0.5 ml 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 24 h. Cultures without proteins served as controls. Golgi Plug (1 μl/ml, BD Pharmingen) was added during the last 4 h of incubation. Cells were surface stained with anti-CD4-Alexa700, anti-CD8-APC-Cy7, and anti-CD44-APC Abs and then fixed with 4% paraformaldehyde for 15 min. Following permeabilization, cells were stained with anti-IFNγ-PE-Cy7, anti-TNFα-PE, or isotype controls, and acquired and analyzed using flow cytometry (BD FACS LSR-II and FACSDiva software).

2.4. Anti-rF1-V Ab analysis

Anti-rF1-V Ab titers were determined by ELISA from serum samples collected 14 and 28 days post-vaccination ($n = 5$ per group). Briefly, 96-well titer plates were coated with 1 μg/ml of rF1-V overnight at 4 °C, blocked with PBS + 5% dry milk + 0.5% Tween-20, and then washed with PBS + 0.5% Tween20. Two-fold serial dilutions of serum were added and incubated for 90 min. Wells were washed, incubated with anti-mouse IgG-, IgG_1- or IgG_{2c}-HRP Abs (Sigma and Jackson ImmunoResearch) for 60 min, and washed. TMB substrate (BD biosciences) was added, and the reaction was stopped with 2 N sulfuric acid. Absorbance was measured at 450 nm and anti-rF1-V Abs were reported as $\log_{10}$ titers of the greatest serial dilution with a mean OD_{450} value >two-fold the OD_{450} value of naïve serum with the same dilution. Samples with an Ab titer of $\log_{10}$ 1.4 or less were considered negative.

2.5. Statistical analysis

Analysis of variance (ANOVA) was used to compare total IgG Ab titers and $CD4^+$ and $CD8^+$ effector responses. The Student *t*-test (2-tail) was used to compare titers of IgG subclasses and IgG_{2c}/IgG_1 ratios. Kaplan–Meier log-rank test was used to generate survival curves.

3. Results

3.1. SA-4-1BBL + alum adjuvant platform generates a Th_1 response to the rF1-V antigen

The alum adjuvanted rF1-V subunit vaccine generates a strong Ab response [32]. Because the Th_1 cellular response plays an