Effective Cancer Vaccine Platform Based on Attenuated Salmonella and a Type III Secretion System

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Abstract

Vaccines explored for cancer therapy have been based generally on injectable vector systems used to control foreign infectious pathogens, to which the immune system evolved to respond naturally. However, these vectors may not be effective at presenting tumor-associated antigens (TAA) to the immune system in a manner that is sufficient to engender antitumor responses. We addressed this issue with a novel orally administered Salmonella-based vector that exploits a type III secretion system to deliver selected TAA in the cytosol of professional antigen-presenting cells in situ. A systematic comparison of candidate genes from the Salmonella Pathogenicity Island 2 (SPI2) locus was conducted in the vaccine design, using model antigens and a codon-optimized form of the human TAA survivin (coSVN), an oncoprotein that is overexpressed in most human cancers. In a screen of 20 SPI2 promotoreffector combinations, a PspseA::sseJ combination exhibited maximal potency for antigen translocation into the APC cytosol, presentation to CD8 T cells, and murine immunogenicity. In the CT26 mouse model of colon carcinoma, therapeutic vaccination with a lead PspseA::sseJ-coSVN construct (p8032) produced CXCR3-dependent infiltration of tumors by CD8 T cells, reversed the CD8:Treg ratio at the tumor site, and triggered potent antitumor activity. Vaccine immunogenicity and antitumor potency were enhanced by coadministration of the natural killer T-cell ligand 7DW8-5, which heightened the production of IL12 and IFNγ. Furthermore, combined treatment with p8032 and 7DW8-5 resulted in complete tumor regression in A20 lymphoma-bearing mice, where protective memory was demonstrated. Taken together, our results demonstrate how antigen delivery using an oral Salmonella vector can provide an effective platform for the development of cancer vaccines.

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Introduction

Despite the identification of potentially immunogenic tumor-associated antigens (TAA) in many types of cancer, current therapeutic cancer vaccines remain largely ineffective (1). One of the major limitations of the modern vectors for cancer vaccines is that, unlike infectious pathogens to which the immune system has evolved to respond, they are unable to effectively deliver TAAs in an immunogenic form to intact professional antigen-presenting cells (APC). To address this challenge, attenuated viral or bacterial vectors, including Salmonella, have been used in experimental cancer vaccines to deliver TAAs to host APCs in situ (2). Because Salmonella naturally migrates from the intestine to the mesenteric lymph nodes and the spleen, Salmonella-based vectors induce a systemic immune response to the bacterially expressed antigens (3–5).

We recently reported a new strategy for an oral vaccine for cancer, in which the type III secretion system (T3SS) of Salmonella is exploited for translocation of TAAs into the cytosol of APCs that generate tumor-specific CTLs (6–9). Some T3SS effector proteins are encoded by the Salmonella Pathogenicity Island 2 (SPI2) locus and are activated only when Salmonella is inside macrophages or dendritic cells (DC; refs. 10–12). This enables the use of live attenuated Salmonella vectors for delivery of the heterologous antigens of interest into the class-I antigen presentation pathway of intact professional APCs in situ. We have reported the use of the SPI2 system to construct cancer vaccines in which human survivin (SVN) or its codon-optimized version (coSVN) was expressed under control of the SPI2 promoter PseA and fused to the gene for effector SseF for translocation (6, 7). As human and murine survivin are highly homologous (13), these vaccines induced CD8 T-cell-mediated antitumor activity in murine tumors that overexpress SVN (6, 7). However, without additional manipulation of the tumor microenvironment, the therapeutic activity was modest and transient.
CD1d-restricted natural killer T (NKT) cells play a critical role in bridging innate and adaptive immune responses and may be recruited for effective immunotherapy of cancer (14, 15). Indeed, we have shown that synthetic NKT ligands, GSL1 or αGalactosylceramide (αGalCer), enhanced the immunogenicity and antitumor efficacy of our first-generation Salmonella-based SVN vaccine (6). Since then, new NKT-cell ligands have been discovered, including an analogue of αGalCer, 7DW8-5, which has a stronger binding affinity to CD1d and NKT-cell TCR compared with αGalCer (16). During in vivo testing, 7DW8-5 exhibited a superior adjuvant effect compared with αGalCer for HIV and malaria vaccines in mice (16) and is currently being tested in primates, thus representing the primary candidate for entering into clinical testing as a vaccine adjuvant.

The goal of this study was to explore and exploit the full potential of SPI2-encoded T3SS of Salmonella typhimurium for construction of an effective cancer vaccine using coSVN as the TAA of choice. We show that vaccination with PsifB::sseJ-coSVN (p8032) not only increased the frequency of antigenic-specific CD8 T cells compared with the previous coSVN vaccine (P3342Max), but also resulted in a dense tumor infiltration with CD8 T cells and enhanced antitumor activity in the CT26 colon cancer model. The addition of 7DW8-5 as an adjuvant further increased vaccine immunogenicity and the persistence of circulating antigen-specific CD8 T cells compared with p8032 alone. Finally, we found that therapeutic vaccination with p8032/7DW8-5 resulted in complete tumor regression in the A20 lymphoma model.

Materials and Methods

Cell lines

Murine CT26 colon carcinoma and A20 B-cell lymphoma cell lines were obtained from Dr. Terabe (NIH/NCI, Bethesda, MD) and ATCC, respectively. The identities of both cell lines were authenticated using short tandem repeats profiling by DDC Medical, Inc. on June 23, 2014.

Strains of Salmonella typhimurium, plasmids, and constructs

Salmonella enterica serovar Typhimurium (S. typhimurium), purD/htrA double-deficient strain MvP728 was previously described (6). For the generation of recombinant plasmids, Escherichia coli DH5α was used as host. Low copy-number plasmid pWSK29 was used for the generation of expression cassettes consisting of SPI2 gene fusions with the indicated antigens as summarized in Table 1. Generation of plasmids and the sequence of codon-optimized human survivin are described in Supplementary Methods.

Table 1. Plasmids used in this study

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<th>Designation</th>
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In vitro antigen presentation assay

Mature bone marrow-derived dendritic cells (BM-DC) were generated and used in the antigen presentation assay as previously described in ref. 17 and Supplementary Methods.

Immunofluorescence microscopy

To detect and quantify the intracellular expression of recombinant proteins produced by MvP728 transformed with the experimental or control plasmids, BM-DCs were infected with MvP728 at a multiplicity of infection of 10. At 16 hours after infection, the cells were fixed and processed for immunostaining of Salmonella LPS (rabbit anti-Salmonella O4,5, Difco, BD), Armenian hamster anti-CD11c (BD), and mouse anti-HA epitope tag (Roche). Fluorescence images were acquired on a Zeiss LSM700 laser-scanning confocal microscope using ZEN software.

Tumor models

BALB/c female mice 6 to 8 weeks of age (Jackson Laboratory) were maintained at Baylor College of Medicine (Houston, TX) animal care facility and were treated according to the appropriate Institutional Biosafety Committee and Institutional Animal Care and Use Committee-approved protocols. The tumor models and vaccination protocols have been described (Supplementary Methods; ref. 6).

ELISpot assay

Splenocytes were isolated from vaccinated or control mice and restimulated with a peptide mix from human survivin library (JPT) and high amounts of translocated fusion proteins (17). We hypothesized that these parameters affect the efficacy of antigen presentation. Figure 1A demonstrates that the PsseJ::sseF combination (p2629) induced the highest level of antigen presentation compared with all other examined combinations. Compared with PsifB::sseF (p2629), which served as a basis for our first-generation SPI2-based vaccines (6), p3643 produced 2.5-fold higher antigen presentation activity (P < 0.001).

To test the efficacy of target protein translocation into the APC cytosol, we expressed the Listeria gene lics (encodes listerioliysin O protein, LLO) with P_{gsp}:ssesf (p8011) or P_{sifb}:ssesf (p2810) and infected BM-DCs with MvP728 carrying either vector control, p2810 or p8011. The immunofluorescence microscopy analysis of HA-tagged LLO demonstrates that p8011 translocated LLO into BM-DC cytosol much more effectively than p2810 (Fig. 1B). Next, we compared the in vivo immunogenicity of P_{gsp}:ssesf vaccine with that of P_{sifb}:ssesf. To that end, we examined generation of LLO-specific CD8 T cells after immunization of mice with p2810, p8011, or vector control. Figure 1C and D demonstrates that p8011 induced a frequency of LLO-specific CD8 T cells nearly five times higher than that of p2810 (P < 0.001). Therefore, we selected the P_{gsp}:ssesf promoter/effector combination for the expression and delivery of heterologous antigens in the second generation of the SPI2-based vaccination platform.

Statistical analyses

The comparisons between groups were based on the two-sided unpaired Student t test or one-way ANOVA with the Tukey–Kramer posttest comparison of group means. The Kaplan–Meier method was used to compute survival probabilities and produce survival curves. The analysis of the difference in survival between groups was based on the log-rank test. Statistical computations were performed with GraphPad Prism 5.02 software (GraphPad). P values < 0.05 were considered significant. All experiments were repeated at least twice, and data are presented as Mean ± SD. **, P < 0.01; and ***, P < 0.001.

Results

Selection of optimal SPI2 promoter and effector protein for antigen delivery to APC cytosol via Salmonella T3SS

Our original SVN vaccine used the sseA promoter and effector sseF for antigen expression and intracellular translocation, respectively (6). To comprehensively evaluate the potential of genes of the SsrAB virulon of S. typhimurium, we compared their promoter and effector activities to express and translocate heterologous proteins for antigen presentation. The results from these studies are summarized in two recent publications from our laboratories (8, 17). On the basis of these results, we selected the four strongest in vitro-induced promoters (P_{gsp}, P_{sifb}, P_{sifA}, and P_{sifC}; ref. 8) and tested combinations thereof with five genes sseJ, sifA, steC, sseL, and sseF encoding translocated effector proteins (17) using OVA as a model antigen in an in vitro antigen presentation assay. We selected the effector proteins SseJ, SifA, SteC, and SseL, for comparison with the previously used effector protein SseF because these proteins show a characteristic association with endosomal membranes of the host cell after translocation, long half-life, and high amounts of translocated fusion proteins (17). We hypothesized that these parameters affect the efficacy of antigen presentation.
the translocation of coSVN into the cell cytosol, murine BM-DCs were infected with MvP728 carrying p8032, p3342Max, or vector control followed by immunofluorescence analysis. Figure 2B and C demonstrates that p8032 delivered significantly larger amounts of the recombinant protein into the cell cytosol compared with p3342Max (P < 0.001). Moreover, ELI-Spot assays showed that vaccination of mice with p8032 induced a higher frequency of SVN-specific IFN-γ-producing T cells in the spleen compared with p3342Max (Fig. 2D). Finally, vaccination with p8032 more effectively inhibited tumor growth in the CT26 colon carcinoma model than p3342Max (P < 0.01; Fig. 2E). Therefore, compared with the first-generation SPI2-based vaccines, PsifB::sseJ-based vaccines possess a higher rate of antigen translocation into APC cytosol, increased immunogenicity, and enhanced antitumor activity in mice.

Vaccination with p8032 results in tumor infiltration with CD8 T cells

To determine whether the enhanced antitumor efficacy of p8032 is associated with enhanced CD8 T-cell localization to the tumor site, we performed immunofluorescence analysis of CT26 tumor tissues from mice that received vector control,
p3342Max, or p8032. Figure 3A demonstrates that, in contrast with the limited CD8 T-cell infiltration in the tumors of control or p3342Max-treated mice, the tumors of mice vaccinated with p8032 had dense CD8 T-cell infiltrates. We also performed FACS on cell preparations homogenized from the same tumor tissues and examined the frequencies of CD8 and CD4 T cells, including Tregs (CD4⁺CD25⁺FOXP3⁺). Figure 3B demonstrates that vaccination with p3342Max alone had no impact on the composition of tumor-infiltrating T cells. In contrast, vaccination with p8032 reversed the CD8:CD4 ratio and increased the CD8 T-cell frequency 2-fold, compared with the vector control (P < 0.001). Moreover, the p8032 induced >3-fold increase of the CD8:Treg ratio and CD8:tumor cell ratio in the tumor parenchyma compared with the vector control or p3342Max (P < 0.01, Fig. 3C and D).

To examine the mechanism responsible for CD8 T-cell trafficking to the tumor site, we first compared serum concentrations of 12 chemokines/cytokines in tumor-bearing mice using a Luminex assay and found that only CXCL10 and CCL2 were consistently increased in the serum of tumor-bearing mice (P < 0.01, Supplementary Fig. S1A). The CXCL10 concentration was about 10 times higher than that of CCL2. Moreover, CXCL10 could also be detected at high concentrations in tumor lysates, in which its concentration, normalized by tumor weight, was similar in mice that received therapeutic vaccines or vector control (Supplementary Fig. S1B). Next, we analyzed expression of the corresponding chemokine receptor, CXCR3 on tumor-infiltrating and spleen CD8 T cells in mice vaccinated with different vectors. Compared with the control vector, vaccination with p8032 (but not p3342Max) significantly increased the frequency of CXCR3-positive CD8 T cells in mice vaccinated with different vectors. Compared with the control vector, vaccination with p8032 (but not p3342Max) significantly increased the frequency of CXCR3-positive CD8 T cells in the spleen (Fig. 3E and F, P < 0.001). More than 90% of tumor-infiltrating CD8 T cells were CXCR3 positive regardless of the vaccination (Fig. 3E), suggesting that CXCL10-CXCR3 axis directs localization of CD8 T cells to the tumor site in this model. Furthermore, treatment of mice with an anti-CXCR3 blocking mAb abrogated the p8032-induced CD8 T-cell infiltration to the tumor site (Fig. 3G and Supplementary Fig. S2).
and antitumor activity of the vaccine (Fig. 3H). Therefore, the increased therapeutic activity of p8032 vaccine is associated with an increased tumor infiltration by CD8 T cells and the reversal of the CD8:Treg ratio. Tumor infiltration depends on CXCR3 expression on CD8 T cells, which is selectively upregulated by p8032 vaccine.

**Adjuvant activity of NKT ligand, 7DW8-5**

To examine whether immunogenicity of PsifB::sseJ-driven vaccines can be enhanced by NKT-cell ligand, 7DW8-5, we vaccinated mice with LLO construct (p8011) alone or in combination with 7DW8-5. All mice received two boosts with the vaccine alone. Figure 4A and B demonstrates that the frequency of LLO-reactive CD8 T cells in peripheral blood was significantly higher in mice that received LLO vaccine with 7DW8-5 compared with the vaccine alone at all examined intervals (P < 0.001). The control groups that received empty vector with 7DW8-5 had no detectable LLO-specific CTLs. Next, we tested 7DW8-5 in combination with the new coSVN vaccine p8032 and found that the ligand enhanced generation of SVN-specific IFNγ-producing T cells compared with the vaccine alone as measured by ELISpot assay (Fig. 4C, P < 0.01). Consistent with the reported properties of 7DW8-5 (16), its administration caused rapid activation of NKT and trans-activation of NK cells as well as upregulation of costimulatory molecules in spleen DCs (Supplementary Fig. S3). Compared with the vaccine alone, the administration of 7DW8-5 either alone or with the vaccine resulted in dramatic increases of IL12 and IFNγ serum concentrations within 6 and 24 hours, respectively (Fig. 4D). Therefore, 7DW8-5 has a potent adjuvant activity for the Ppsif::sseJ-driven vaccine that is associated with the upregulation of IL12 and IFNγ, which support Th-1 immune response and CTL generation.
Potent antitumor activity of p8032 vaccine with 7DW8-5 as an adjuvant

To test the therapeutic activity of the vaccine, mice with 5-day subcutaneous transplants of CT26 colon carcinoma cells were vaccinated with p3342Max, p8032, or p8032 with 7DW8-5. Control mice received an empty vector alone or with 7DW8-5. To compare the efficacy of the p8032 vaccine with a commonly used vaccination modality (18), we used ex-vivo generated DCs pulsed with a human survivin peptide library and 7DW8-5 as an adjuvant. Figure 5A and B demonstrates that p8032 vaccine significantly delayed tumor growth and prolonged survival compared with p3342Max (P < 0.001). The addition of 7DW8-5 further enhanced the antitumor activity of p8032 vaccine, so that median survival of the animals was nearly doubled compared with the control group (P < 0.001). Moreover, p8032/7DW8-5 was more effective than DC/7DW8-5 (P < 0.001, Fig. 5A–C). However, the addition of 7DW8-5 to p8032 vaccine did not further increase the number of tumor-infiltrating CD8 T cells or the CD8:Treg ratio compared with the vaccine alone (data not shown). Staining with CD1d/PBS57 tetramers found no detectable NKT cells within tumor-infiltrating leukocytes regardless of 7DW8-5 treatment (data not shown), suggesting that 7DW8-5-activated NKT cells function only at the initiation of the immune response in this model.

To determine the requirement of the major lymphocyte subsets for the observed antitumor activity of p8032 vaccine with 7DW8-5 as an adjuvant, we repeated the treatment of CT26 tumor-bearing mice using anti-CD8 (CD8 T cells), anti-CD4 (a subset of NKT cells and T-helper cells), or anti-asialo-GM1 (NK cells) depleting antibodies before vaccine treatment. Figure 5D shows that although the therapeutic activity of the vaccine alone depends only on CD8 T cells, the p8032/7DW8-5 combination depends on both CD8 T cells and CD4, which include subsets of NKT and T cells. Despite the observed activation of NK cells early after 7DW8-5 injection, NK cells were not required for the vaccine antitumor activity with or without the adjuvant. Therefore, p8032/7DW8-5 vaccine has a potent antitumor activity, which depends on CD8 T cells and help from CD4-positive NKT-cell subset or T-helper cells.
Curative activity of p8032/7DW8-5 vaccine in A20 lymphoma model

To determine whether the therapeutic potential of the new SVN vaccine extends beyond the CT26 tumor model, we tested efficacy in the A20 B-cell lymphoma model. Figure 6A and B demonstrates that p8032 was again superior to p3342Max and the vaccine’s therapeutic efficacy was further enhanced by 7DW8-5. Although the trend was the same as in the CT26 model, the differences between groups in the A20 model were more striking. Although p3342Max only delayed tumor growth, it did not cure mice of tumors. In contrast, p8032 without or with 7DW8-5 cured half or all mice, respectively. Moreover, the cured animals developed protective immune memory, as none of them developed tumors after injection of A20 cells in the contralateral flank at day 60, whereas all naïve mice grew progressive tumors from the same A20 preparations (Fig. 6D). Therefore, p8032 vaccine has a potent antitumor activity in the A20 lymphoma model, which is further enhanced by 7DW8-5 as an adjuvant.

Discussion

In this work, we describe the systematic evaluation of Salmonella SPI2-T3SS effector proteins and their promoters for use in cancer vaccines. We found that the combination of SPI2 promoter popp with effector gene sseJ provides maximal antitumor immunogenicity. The resulting coSVN-expressing vaccine (p8032) induced CXCR3-dependent tumor infiltration by CD8 T cells and had potent therapeutic activity in two separate tumor models. The vaccine immunogenicity and antitumor efficacy were further enhanced by an NKT-cell ligand, 7DW8-5, used as an adjuvant.

Among multiple factors that contribute to the efficacy of SPI2-based vaccines, the choice of a promoter for target...
antigen expression and an effector protein as a fusion partner for antigen translocation is of particular importance. Previous studies from our groups have evaluated the promoter activities of genes of the SsrAB virulon in *S. typhimurium* (17) and various SPI2-T3SS effector proteins for translocation of heterologous antigens (8). In the present study, we have found a novel promoter/effector combination, \( P_{sisB::ssJ} \), which enables the maximal level of antigen presentation in APCs to CD8 T cells. Importantly, \( P_{sisB::ssJ} \)-based vaccines with either LLO or SVN as an antigen demonstrated a dramatically stronger immunogenicity in mice compared with our first-generation SPI2 vaccines with the same antigens.

Compared with the previous version of coSVN-expressing vaccine p3342max, p8032 demonstrated a much enhanced antitumor activity in two murine cancer models. Of particular importance, only vaccination with p8032 resulted in tumor infiltration with CD8 T cells and the reversal of the CD8:Treg ratio. This observation is consistent with accumulating evidence from both mouse models and clinical trials that shifting the balance toward CD8 T cells versus Tregs at the tumor site is critical to the success of cancer immunotherapy (19–21). To achieve such an effect, previously reported Salmonella-based vaccines were used in combination with other therapies that target the tumor microenvironment. For example, an attenuated *Salmonella* strain VNP20009 (22), modified with a DNA plasmid expressing the shRNA specific for indoleamine 2,3-dioxygenase, extended survival in murine models of melanoma and pancreatic cancer (23, 24). In the case of p3342Max, CD8 T-cell infiltration and potent antitumor activity in B16F10 melanoma model were achieved when the vaccine was combined with a tumor-targeted Stat3 shRNA (7).

The increased tumor infiltration with CD8 T cells and the therapeutic activity induced by the p8032 vaccine were dependent on the induction of CXCR3 expression on CD8 T cells. CXCL10, the ligand for CXCR3, was abundantly produced by CT26 tumor grafts. Although normal epithelial cells produce CXCL10 in response to IFN\( \gamma \) at the initiation of immune response (25), CXCL10 expression in colon cancer cells has been reported to be under control of oncogenic Ras signaling (26), and human colon cancer cells expressing both CXCL10 and CXCR3 have been shown to have enhanced growth and invasion properties (27). Moreover, a subset of Tregs expresses CXCR3 and preferentially accumulates in CXCL10-producing solid tumors (28). Thus, although tumor cells produce CXCL10 to support growth, metastasis, and immune evasion, effective vaccines may exploit this phenomenon for tumor eradication via induction of CXCR3 expression on tumor-specific CD8 T cells.

We found that new synthetic NKT-cell ligand 7DW8-5 is a potent adjuvant for the p8032 vaccine. The addition of 7DW8-5 was monitored every 2 days. B, Kaplan–Meier survival analysis; representative of three experiments. C, mice with regressed A20 tumors were challenged with \( 10^5 \) A20 cells in the contralateral flank on day 60 after the initial tumor cell injection. Naïve mice were used as a control and received the same dose of A20 cells. Mice were monitored for the evidence of tumor growth for 2 months. Data are from a representative of three experiments, 8 mice per group.
7DW8-5 induced production of large amounts of IL12 and IFNγ in mice and increased both the magnitude and duration of the vaccine-induced CD8 T-cell response that may explain the increased therapeutic efficacy of the combined treatment. The effect of 7DW8-5 is similar to that of other NKT ligands used as vaccine adjuvants (6, 16, 29). The mechanism of the adjuvant activity of NKT ligands involves bidirectional interactions between NKT cells and DCs that have been extensively reviewed elsewhere (15, 30, 31). However, no cure has been achieved in the CT26 model. A recent study demonstrated that PD1- and CTLA-4-mediated inhibition contributes to the immune suppressive microenvironment in CT26 tumor (21). That study also found that GVAX vaccination in combination with the blockade of both PD-1 and CTLA-4 produced tumor regression. Therefore, a systematic evaluation of Salmonella-based vaccines in combination with the checkpoint blockade therapies would be a logical avenue for a future investigation.

The therapeutic activity of p8032/7DW8-5 was curative in an A20 lymphoma model. Moreover, cured animals developed a long-lasting protective memory response. These observations are consistent with the properties of NKT ligands to potentiate development of memory CD8 T-cell responses via enhanced production of IL12 by DCs and IFNγ by both NKT and NK cells (32, 33). In the current study, the antitumor activity of p8032/7DW8-5 was unequivocally dependent on CD8 T cells and CD4 T cells, which include about half of NKT cells (34) and conventional T-helper cells. Although NKT cells were specifically activated by 7DW8-5, the contribution of conventional CD4 T cells cannot be excluded as antigen-specific CD4 T cells can help CD8 T cells during the priming and maintenance of memory response (35, 36). In conclusion, the vaccination approach we described, using the molecular machinery of SPI2-encoded T3SS system of Salmonella, may provide a foundation for clinical trials of new oral vaccines for many types of cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** X. Xu, W. Hegazy, M. Hensel, L.S. Metelitsa

**Development of methodology:** X. Xu, W. Hegazy, L. Guo, X. Gao, S. Kurbanov, M. Hensel, L.S. Metelitsa

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** X. Xu, L. Guo, A.N. Courtney, D. Liu, G. Tian, E.R. Manuel, D.J. Diamond

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** X. Xu, A.N. Courtney, S. Kurbanov, D. Liu, L.S. Metelitsa

**Writing, review, and/or revision of the manuscript:** X. Xu, E.R. Manuel, D.J. Diamond, M. Hensel, L.S. Metelitsa

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** X. Xu, L. Guo, S. Kurbanov

**Study supervision:** M. Hensel, L.S. Metelitsa

**Other (construction of used plasmids and in vitro testing of attenuated strains):** W. Hegazy

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