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Development of an Effective Cancer Vaccine Using Attenuated *Salmonella* and Type III Secretion System to Deliver Recombinant Tumor-Associated Antigens

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Abstract

One of the major limitations of modern cancer vaccine vectors is that, unlike infectious pathogens, to which the immune system has evolved to respond, they are not sufficiently effective in delivering tumor-associated antigens (TAAs) in an immunogenic form to intact professional antigen-presenting cells (APCs) at their anatomic location. To overcome this challenge, we exploited *Salmonella* Pathogenicity Island 2 (SPI2) and its type III secretion system (T3SS) to deliver a TAA of choice into the cytosol of APCs *in situ*. We have systematically compared candidate genes from the SPI2 locus of *Salmonella typhimurium* in the vaccine design, using model antigens and a codon-optimized human TAA, survivin (coSVN). In a screen of 20 SPI2 promoter/effector combinations, the *P_{sifB}::sseJ* pair demonstrated the maximal potency for antigen translocation in the APC cytosol, presentation to CD8 T cells, and immunogenicity in mice. Therapeutic vaccination with the *P_{sifB}::sseJ*-coSVN construct (p8032) resulted in CXCR3-dependent tumor infiltration with CD8 T cells, reversal of the CD8:Treg ratio at the tumor site, and potent anti-tumor activity in a CT26 colon carcinoma model. The vaccine's immunogenicity and anti-tumor potency were further enhanced by co-administration of an NKT-cell ligand, 7WD8-5, which strongly enhanced production of IL-12 and IFN γ in vaccinated mice. Furthermore, therapeutic vaccination with p8032/7WD8-5 resulted in complete tumor regression in an A20 lymphoma model, with the generation of protective memory. Thus, oral antigen delivery via SPI2-encoded T3SS of *Salmonella* may be the foundation for an effective cancer vaccine platform.

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Keywords

cancer vaccines; *Salmonella*; type III secretion system; CXCR3; NKT cells; survivin

Introduction

Despite the identification of potentially immunogenic tumor-associated antigens (TAAs) 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 (APCs). 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 SPI2 locus and are activated only when *Salmonella* is inside macrophages or dendritic cells (DCs) (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 *P_{sseA}* 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 anti-tumor 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 (NKTs) 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 α Galactosylceramide (α GalCer), enhanced the immunogenicity and anti-tumor efficacy of our first-generation *Salmonella*-based SVN vaccine (6). Since then, new NKT-cell ligands have been discovered, including an analogue of α GalCer, 7DW8-5, which has a stronger binding affinity to CD1d and NKT-cell TCR compared with α GalCer (16). During *in vivo* testing, 7DW8-5 exhibited a superior adjuvant effect compared with α GalCer 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 *S. typhimurium* for construction of an effective cancer vaccine using coSVN as the TAA of choice. We show that vaccination with *P_{sifB::sseJ}*-coSVN (p8032) not only increased the frequency of antigenic-specific CD8 T cells compared with the previous coSVN vaccine

(P3342Max), but resulted in a dense tumor infiltration with CD8 T cells and enhanced anti-tumor activity in the CT26 colon cancer model. The addition of 7WD8-5 as an adjuvant further increased vaccine immunogenicity and the persistence of circulating antigen-specific CD8 T cells compared with p8032 alone. Lastly, we found that therapeutic vaccination with p8032/7WD8-5 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) and ATCC, respectively. The identities of both cell lines were authenticated using Short Tandem Repeats (STR) profiling by DDC Medical, inc. on 06/23/2014.

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, *E. coli* DH5 α 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 “Supplemental Methods”.

In vitro antigen-presentation assay

Murine bone marrow-derived dendritic cells (BM-DC) were generated and used in the antigen-presentation assay as previously described ((17) and “Supplemental Methods”).

Immunofluorescence Microscopy

In order 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 MOI of 10. At 16 h after infection, the cells were fixed and processed for immuno-staining 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 6–8 weeks of age (Jackson Laboratory) were maintained at Baylor College of Medicine animal care facility and were treated according to the appropriate IBC and IACUC approved protocols. The tumor models and vaccination protocols have been described ((6) and “Supplemental Methods”).

ELISpot assay

Splenocytes were isolated from vaccinated or control mice and restimulated with a peptide mix from human survivin library (JPT) *in vitro* followed by 7 days culture in the presence of

50 unit/ml IL-2. The frequency of survivin-specific IFN- γ secreting cells was determined using an ELISpot assay kit (R&D Systems) according to the manufacturer's instructions.

ELISA

The concentrations of IL-12 p70 and IFN γ were quantified in mouse serum using respective ELISA kits (R&D Systems) according to the manufacturer's instructions.

Dendritic Cell Vaccine

BM-DCs were pulsed with human Survivin PepMixTM peptide library consisting of a pool of 33 peptides (15 mers with 11 aa overlap, JPT) for 24 h. Cells were then harvested, washed, and intravenously administrated (5×10^5 cells per mouse) at the same schedule as the *Salmonella*-based vaccine.

Flow cytometry

The reagents for flow cytometry are described in "Supplemental Methods". The analysis was performed on a LSR-II flow cytometer (BD Biosciences) using BD FACDiva software v. 6.0.

Statistical analyses

The comparisons between groups were based on the two-sided unpaired Student's t-test or one-way ANOVA with the Tukey-Kramer post-test 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 PrismTM 5.02 software (GraphPad). P values < 0.05 were considered significant. All experiments were repeated at least twice, and data are presented as Mean $\pm$ SD. * p <0.05, ** 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 labs (8;17). Based on these results, we selected the four strongest *in vivo*-induced promoters (P_{sifB} , P_{sseJ} , P_{sifA} and P_{sseA}) (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 to the previously used effector protein SseF since 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. Fig. 1A demonstrates that the

P_{sifB}::sseJ combination (p3643) induced the highest level of antigen presentation compared with all other examined combinations. Compared with *P_{sseA}::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 *lisA* (encodes listeriolysin O protein, LLO) with *P_{sifB}::sseJ* (p8011) or *P_{sseA}::sseF* (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_{sifB}::sseJ* vaccine with that of *P_{sseA}::sseF*. To that end, we examined generation of LLO-specific CD8 T cells after immunization of mice with p2810, p8011, or vector control. Figs. 1C,D demonstrate that p8011 induced a frequency of LLO-specific CD8 T cells nearly 5-times higher than that of p2810 ($P < 0.001$). Therefore, we selected the *P_{sifB}::sseJ* promoter/effector combination for the expression and delivery of heterologous antigens in the second generation of the SPI2-based vaccination platform.

Enhanced TAA translocation, immunogenicity, and anti-tumor activity of new survivin vaccine

Our previously reported *P_{sseA}::sseF*-driven cancer vaccines expressed human survivin (SVN, p3342 (6)) or codon-optimized survivin (coSVN, p3342Max (7)) as a TAA. Here we expressed coSVN under the control of *P_{sifB}::sseJ* (p8032) and compared it with p3342Max (Fig. 2A). To visualize and quantify 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. Figs. 2B,C demonstrate that p8032 delivered significantly larger amounts of the recombinant protein into the cell cytosol compared to p3342Max ($P < 0.001$). Moreover, ELISpot assays showed that vaccination of mice with p8032 induced a higher frequency of SVN-specific IFN γ -producing T cells in the spleen compared to 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, *P_{sifB}::sseJ*-based vaccines possess a higher rate of antigen translocation into APC cytosol, increased immunogenicity and enhanced anti-tumor activity in mice.

Vaccination with p8032 results in tumor infiltration with CD8 T cells

To determine whether the enhanced anti-tumor 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. Fig. 3A demonstrates that, in contrast to 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+). Fig. 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 two-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 ($P < 0.01$, Figs. 3C,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 and naïve mice using a Luminex assay and found that only CXCL10 and CCL2 were consistently increased in the serum of tumor-bearing mice ($P < 0.01$, 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 (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 to the control vector, vaccination with p8032 (but not p3342Max) significantly increased the frequency of CXCR3-positive CD8 T cells in the spleen (Fig. 3E,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 Fig. S2) and anti-tumor 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 up-regulated by p8032 vaccine.

Adjuvant activity of NKT ligand, 7DW8-5

To examine whether immunogenicity of *P_{sifB}::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. Figs. 4A,B demonstrate 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 to 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 IFN γ -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 up-regulation of co-stimulatory molecules in spleen DCs (Fig. S3). Compared to the vaccine alone, the administration of 7DW8-5 either alone or with the vaccine resulted in dramatic increases of IL-12 and IFN γ serum concentrations within 6 h and 24 h, respectively (Fig. 4D). Therefore, 7DW8-5 has a potent adjuvant activity for the *P_{sifB}::sseJ*-driven vaccines that is associated with the up-regulation of IL-12 and IFN γ , which support Th-1 immune response and CTL generation.

Potent anti-tumor 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. Figs. 5A,B demonstrate that p8032 vaccine significantly delayed tumor growth and prolonged survival compared with p3342Max ($P < 0.001$). The addition of 7DW8-5 further enhanced the anti-tumor 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 ($P < 0.001$, Figs. 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 anti-tumor 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. Fig. 5D shows that while 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 cells, 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 anti-tumor activity with or without the adjuvant. Therefore, p8032/7DW8-5 vaccine has a potent anti-tumor 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. Figs. 6A,B demonstrate 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. While 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 anti-tumor 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 *P_{sifB}* with effector gene *sseJ* provides maximal anti-tumor 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 anti-tumor efficacy were further enhanced by an NKT-cell ligand, 7WD8-5, 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 are 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_{sifB}::sseJ*, which enables the maximal level of antigen presentation in APCs to CD8 T cells. Importantly, *P_{sifB}::sseJ*-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 anti-tumor 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 anti-tumor activity in B16F10 melanoma model was 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- γ 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, while 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 induced production of large amounts of IL-12 and IFN γ 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 IL-12 by DCs and IFN γ by both NKT and NK cells (32;33). In the current study, the anti-tumor activity of p8032/7DW8-5 was unequivocally dependent on CD8 T cells and CD4 cells, which include about half of NKT cells (34) and conventional T-helper cells. While 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Evaluation of SPI2 promoter/effector combinations

(A) BM-DC were infected with MvP728 (MOI: 25), carrying an empty plasmid (negative control) or the indicated promoter/effector combinations with ovalbumin (OVA), or were pulsed with SIINFEKL peptide (positive control). B3Z reporter cell line was used to evaluate the strength of antigen presentation by measuring β -galactosidase activity in a colorimetric assay. Results are Mean $\pm$ SD from three experiments in triplicates. (B) BM-DCs were fixed 16 h after infection with MvP728 with the indicated LLO-HA-expressing plasmids and stained for intracellular *Salmonella* (green) and translocated fusion protein (red). Shown are representative confocal microscopy images from three experiments. (C) Mice received MvP728 with empty vector or LLO under control of the indicated promoter/effector combinations followed by boost vaccinations on days 14 and 28. LLO-specific CD8

T cells were quantified by FACS using H-2K^d/LLO91-99 pentamer. Shown are representative density plots on day 35. **(D)** Means $\pm$ SD of LLO-specific CD8 T cells, a representative of three experiments.

Figure 2. Enhanced antigen translocation, immunogenicity, and anti-tumor activity of *P_{sifB::sseJ}*-driven coSVN vaccine

(A) A schematic presentation of P3342Max and P8032 constructs. (B) BM-DCs were fixed 16 h after infection with MvP728 with the indicated plasmids and stained for intracellular *Salmonella* (green), CD11c (blue) and translocated SseJ-Survivin-HA fusion protein (red). Shown are representative confocal microscopy images from three experiments. (C) Mean $\pm$ SD of green fluorescence intensity per cell (50 infected cells per condition). (D) Mice were vaccinated with MvP728-vector or the indicated MvP728-coSVN constructs with boosts on days 14 and 28. On day 35, splenocytes were examined for reactivity to survivin peptides using IFN- γ ELISpot assay. Bars are means and SD of the frequency of IFN- γ -producing cells from 6 mice per group in one of three experiments. (E) Mice were treated with MvP728, carrying vector control or the indicated coSVN-expressing plasmids as depicted in

the immunization schedule. Shown are means $\pm$ SD of tumor weight on day 28, one of three experiments.

Figure 3. Vaccination with p8032 results in tumor infiltration with CD8 T cells

(A) Mice with 5-day established CT26 tumors were vaccinated with the MvP728 carrying the indicated plasmids followed by boost on days 12 and 19. On day 28, tumor sections were analyzed by immunofluorescence microscopy for CD8⁺ T cells (red). Shown are representative images from 5 mice per group, one of three experiments. (B) CD8 and CD4 T cells were quantified by FACS from the same tumors as in “A” after gating on CD3-positive cells. (C) Tregs were identified within CD4⁺ T cells as a subset of cells positive for FOXP3 and CD25 (not shown). Shown are means and SD of CD8:Treg ratios from one of three experiments. (D) Using images in “A”, the absolute numbers of CD8⁺ cells and tumor cells were counted in 10 100-μm fields per mouse, 5 mice per group. Shown are Mean ± SD of

CD8:tumor cell ratios. **(E)** CXCR3 expression by FACS in spleen and tumor-infiltrating CD8 T cells in mice vaccinated with the indicated vectors. **(F)** Mean $\pm$ SD of CXCR3-positive cells within CD8 T cells in spleen on day 28. **(G)** Mice with 5-day established CT26 tumors received anti-CXCR3 blocking or control mAb followed by P8032 vaccine. CD8:tumor cell ratio was analyzed as in “D”. **(H)** The same conditions as in “G”. Shown are Mean $\pm$ SD of tumor weight on day 28; one of two experiments.

Figure 4. NKT-cell ligand 7DW8-5 enhances vaccine immunogenicity

(A) Mice received vector control, P8011 alone or with 7DW8-5 followed by two boosts without 7DW8-5 with weekly intervals. The frequency of LLO-specific CD8 T cells in peripheral blood was examined by FACS. Shown are representative plots on day 21. (B) Kinetic analysis of LLO-specific CTL response, a representative of three experiments. (C) Mice were treated as in “A” using survivin instead of LLO. The frequency of survivin-specific CD8 T cells was measured by IFN- γ Elispot assay. Bars are means and SD from one of three experiments. (D) Serum concentrations of IFN- γ and IL-12 were measured by ELISA. Shown are Mean $\pm$ SD from two experiments.

Figure 5. Potent anti-tumor activity of P8032 vaccine with 7DW8-5 in CT26 model

(A) Mice were s.c. injected with CT26 cells and 5 days later vaccinated with vector control, P3342Max, P8032, P8032 with 7DW8-5, or survivin peptide (PepMixTM) pulsed DCs with 7DW8-5 followed by two boost vaccinations (without 7DW8-5) with weekly intervals. Tumor size was measured every 2 days. (B) Kaplan–Meier survival analysis. (C) Resected tumors were photographed on day 28. (D) Mice with CT26 tumors were treated with P8032 or P8032/7DW8-5 and received anti-CD8, anti-CD4, or anti-asialo-GM1 depleting antibodies one day before vaccination and every three

Shown are Means $\pm$ SD of the tumor weight on day 28, a representative of three experiments.

Figure 6. Tumor regression and formation of protective memory in A20 lymphoma model (A) Mice were s.c. injected with 10^5 A20 lymphoma cells and 5 days later vaccinated with vector control, P3342Max, P8032 or P8032 with 7DW8-5 followed by two boost vaccinations (without 7DW8-5) with weekly intervals. Tumor volume was monitored every two days. (B) Kaplan–Meier survival analysis; a 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 two months. Data are from a representative of three experiments, 8 mice per group.

Table 1

Plasmids used in this study

Designation	Relevant characteristics	Source/reference
pWSK29	Low copy number vector, Amp ^R	Lab stock
p2629	pWSK29 <i>P_{sseA} sscB sseF::OVA::M45</i>	Lab stock
p2810	pWSK29 <i>P_{sseA} sscB sseF::lisA₅₁₋₃₆₃::HA</i>	Lab stock
p3342Max	pWSK29 <i>P_{sseA} sscB sseF::COhSurvivin</i>	Ref. 6
p3528	pWSK29 <i>P_{sifB} sscB sseF::OVA::HA</i>	Lab stock
p3530	pWSK29 <i>P_{sifA} sscB sseF::OVA::HA</i>	Lab stock
p3531	pWSK29 <i>P_{sseJ} sscB sseF::OVA::HA</i>	Lab stock
p3554	pWSK29 <i>P_{sseJ} sseF::OVA::HA</i>	This study
p3556	pWSK29 <i>P_{sifA} sifA::OVA::HA</i>	This study
p3631	pWSK29 <i>P_{sseA} sseF::OVA::HA</i>	Lab stock
p3632	pWSK29 <i>P_{sseA} sifA::OVA::HA</i>	Lab stock
p3633	pWSK29 <i>P_{sseA} sseL::OVA::HA</i>	Lab stock
p3634	pWSK29 <i>P_{sseA} steC::OVA::HA</i>	Lab stock
p3643	pWSK29 <i>P_{sifB} sseJ::OVA::HA</i>	This study
p3644	pWSK29 <i>P_{sifB} sifA::OVA::HA</i>	This study
p3645	pWSK29 <i>P_{sifB} sseL::OVA::HA</i>	This study
p3646	pWSK29 <i>P_{sifB} steC::OVA::HA</i>	This study
p3654	pWSK29 <i>P_{sseJ} sifA::OVA::HA</i>	This study
p3655	pWSK29 <i>P_{sseJ} sseL::OVA::HA</i>	This study
p3556	pWSK29 <i>P_{sseJ} steC::OVA::HA</i>	This study
p3663	pWSK29 <i>P_{sifA} sseJ::OVA::HA</i>	This study
p3664	pWSK29 <i>P_{sifA} sseL::OVA::HA</i>	This study
p3665	pWSK29 <i>P_{sifA} steC::OVA::HA</i>	This study
p8011	pWSK29 <i>P_{sifB} sseJ::lisA₅₁₋₃₆₃::HA</i>	This study
p8032	pWSK29 <i>P_{sifB} sseJ::COhSurvivin</i>	This study