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Engineering Nanoparticle-Coated Bacteria as Oral DNA Vaccines for Cancer Immunotherapy

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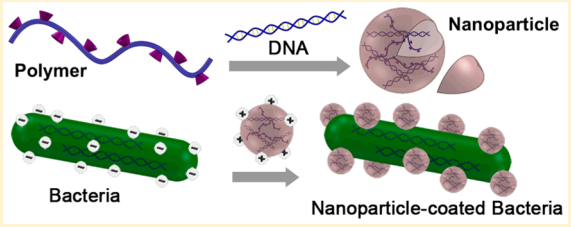
Supporting Information

ABSTRACT: Live attenuated bacteria are of increasing importance in biotechnology and medicine in the emerging field of cancer immunotherapy. Oral DNA vaccination mediated by live attenuated bacteria often suffers from low infection efficiency due to various biological barriers during the infection process. To this end, we herein report, for the first time, a new strategy to engineer cationic nanoparticle-coated bacterial vectors that can efficiently deliver oral DNA vaccine for efficacious cancer immunotherapy. By coating live attenuated bacteria with synthetic nanoparticles self-assembled from cationic polymers and plasmid DNA, the protective nanoparticle coating layer is able to facilitate bacteria to effectively escape phagosomes, significantly enhance the acid tolerance of bacteria in stomach and intestines, and greatly promote dissemination of bacteria into blood circulation after oral administration. Most importantly, oral delivery of DNA vaccines encoding autologous vascular endothelial growth factor receptor 2 (VEGFR2) by this hybrid vector showed remarkable T cell activation and cytokine production. Successful inhibition of tumor growth was also achieved by efficient oral delivery of VEGFR2 with nanoparticle-coated bacterial vectors due to angiogenesis suppression in the tumor vasculature and tumor necrosis. This proof-of-concept work demonstrates that coating live bacterial cells with synthetic nanoparticles represents a promising strategy to engineer efficient and versatile DNA vaccines for the era of immunotherapy.



KEYWORDS: *hybrid living material, Salmonella, phagosomal escape, acid tolerance, cationic polymer, vaccine delivery*

Immunotherapy has received considerable attention as an emerging cancer therapy modality, as this therapeutic strategy is of great potential in terms of its ability to break the immune tolerance and evoke an immune response to target cancer cells with much less side effects.[1−3] Although oral delivery of DNA vaccine represents one of the most promising approaches in cancer immunotherapy;[4,5] however, the low levels of DNA transfection is the main problem limiting efficacious immune response.[6−8] Live attenuated strains of a few bacteria have been developed as vaccines for a number of infectious diseases and several types of cancers and were also exploited as potential vaccine vectors to delivery different types of antigenic messages for activating antitumor immune responses.[9−11] For example, *Salmonellae* harboring cancer-specific antigen-expressing plasmid has been shown to be effective in DNA delivery and efficacious in the subsequent induction of immunity against antigens encoded by the plasmid.[5,12,13] Attenuated *Salmonella* vaccines have been successfully used as carriers for the oral delivery of vaccines.[4,14] As compared to bacterial vaccines delivered via intravenous injection, oral vaccination mediated by *attenuated Salmonella* is cost-effective and less toxic.[15−17] Orally administered *Salmonellae* are able to colonize the gut-associated lymphoid tissue through the microfold cells of Peyer's patches initially, where they could be engulfed and processed by dendritic cells or macrophages to stimulate a strong and durable immunological response.[18] Subsequently, the rest bacteria drain through the lymphatics to the thoracic duct into the blood and ultimately infect the liver and spleen, where they replicate inside phagocytic cells and also induce an innate immune response.[19] However, the oral infection efficiency of *Salmonellae* is often low, due to the digestion of live attenuated *Salmonellae* in the acidic stomach environment.[20] Additionally, *Salmonellae* lack the ability to escape phagosomes after they are captured by phagocytes, which has severely restricted its replication within macrophages following the invasion into intestinal mucosa.[18] As a result, the induction of MHC class-I-restricted immune response is largely limited, which is the key reason for the failure of DNA vaccination against cancer.[21,22] Collectively, it is of paramount importance to develop highly effective vectors to overcome current hurdles of oral DNA vaccine delivery.

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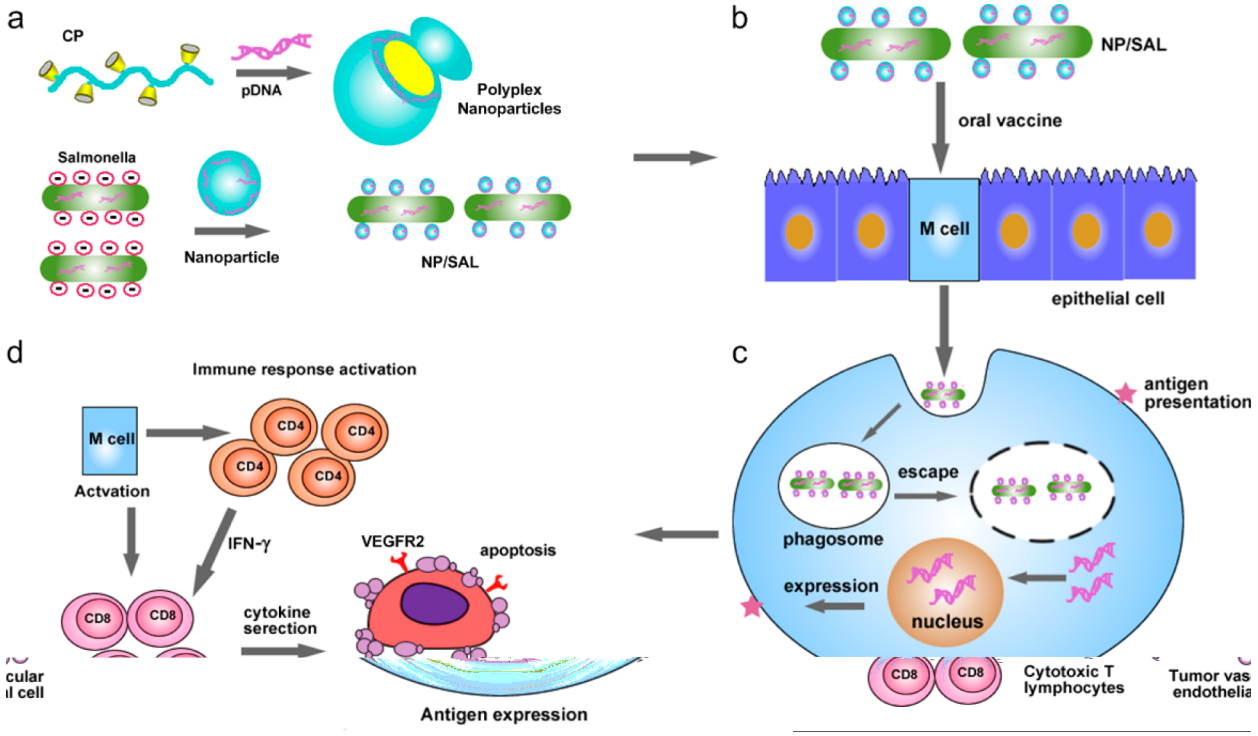
Scheme 1. Schematic Illustration of the Cationic Nanoparticle-Coated Attenuated *Salmonellae* for Improved Antigen Expression and Tumor Targeting Immune Responsive Activation[a]



[a](a) Engineering of polyplex nanoparticle-coated *Salmonellae*. (b) Oral DNA vaccine delivery mediated by nanoparticle-coated *Salmonellae*. (c) Intracellular trafficking of nanoparticle-coated *Salmonellae* and antigen expression. (d) Activation of antitumor immune response.

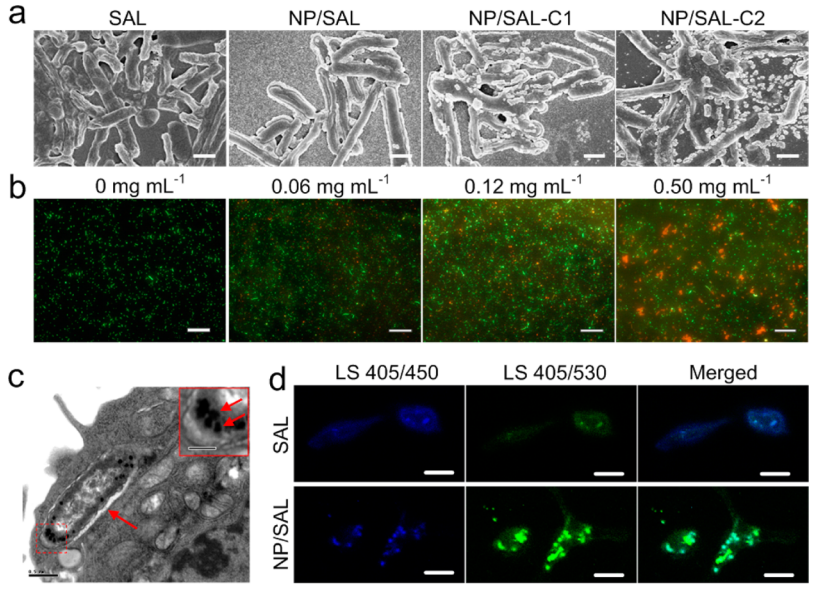


Figure 1. (a) Morphology of naked *Salmonellae* and coated *Salmonellae* with different concentrations of polyplex nanoparticles, as observed by scanning electron microscopy. The scale bars represent 1 μm. (b) Fluorescence microscopic images of the attenuated *Salmonella* incubated with different concentrations of polyplex nanoparticles. The *Salmonella* (2×10^6 CFU/mL) was coated with 0 mg/mL, 0.06 mg/mL (NP/SAL), 0.12 mg/mL (NP/SAL-C1), and 0.50 mg/mL (NP/SAL-C2) of polyplex nanoparticles. Naked bacteria with green fluorescence represent the intact cell membrane, whereas those in red represent damaged cell membrane. The live and dead *Salmonellae* were stained by LIVE/DEAD BacLight bacterial viability kits. The scale bars represent 50 μm. (c) TEM image of the intracellular location of NP/SAL in the RAW 264.7 cells. The scale bar represents 0.5 μm (image) and 0.2 μm (insert), respectively. The arrows denote the internalized coated bacteria (image) and nanoparticles adherent to bacteria (insert), respectively. (d) Infection of peritoneal macrophage mediated by NP/SAL and *Salmonellae*. The cells were stained by LysoSensor Yellow/Blue DND-160 after infection. The scale bars represent 20 μm.

"Living materials" that combine the advantages of living cells with nonliving nanomaterials are emerged as a new class of functional materials in the past few years. The intrinsic features of live bacterial cells with the merits of synthetic materials provides

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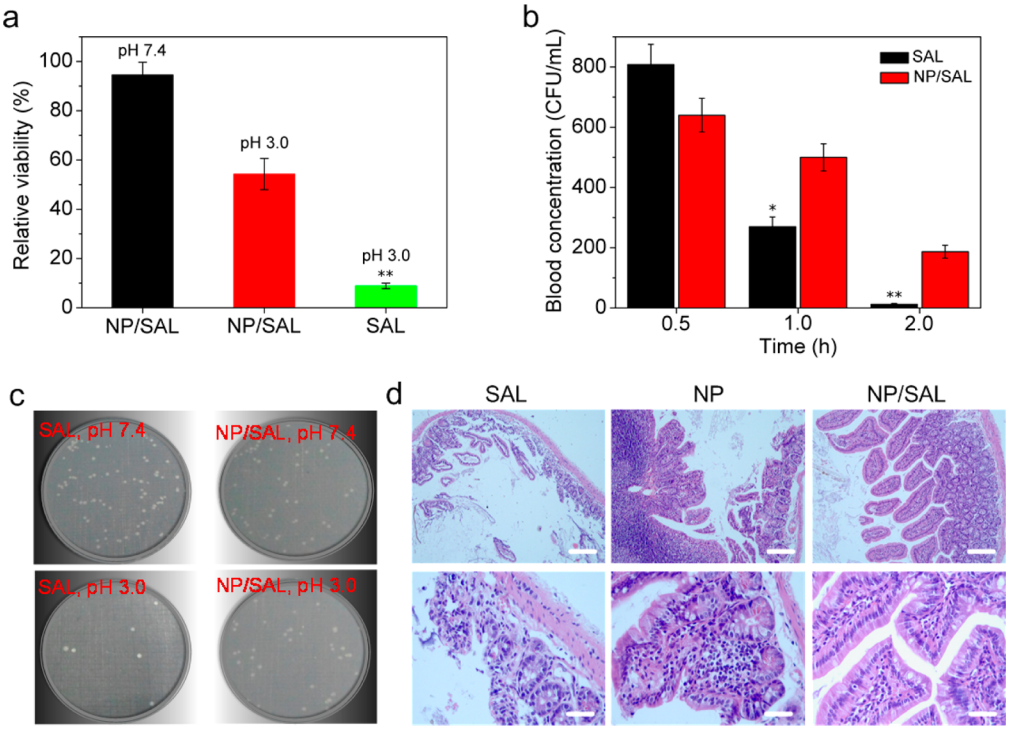

Figure 2. (a) Relative viability of NP/SAL and uncoated *Salmonellae* at physiological pH (pH 7.4) and simulated stomach pH (pH 3.0). The absolute number of viable *Salmonellae* was defined as 100%. Data represent mean ± SD ($n = 3$, Student's t test, $^{**}P < 0.01$, SAL (pH3.0) vs NP/SAL (pH3.0)). (b) Blood concentration of *Salmonellae* and NP/SAL at different time points after oral administration. Data represent mean ± SD, and comparison of group means was made between SAL group and NP/SAL group at the same time point after oral administration. ($n = 3$, Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$) (c) Growth of uncoated and coated *Salmonellae* in Petri plates after exposure to different pH buffers. (d) H&E stained small intestine section of mice treated with *Salmonella*, NP or NP/SAL by oral administration successively for 3 days. The scale bar represents 200 μm (upper panel ×100; lower panel ×400).

the potential for several applications such as electricity conducting and light emitting, cell imaging and barcoding, as well as pathogen detection.[23−25] Synthetic polymer-coated live bacteria were also designed for damaging cancer cells with multimodal features and modulating tumor-targeting efficiency.[26,27] Furthermore, bacteria-mediated nanoparticle and cargo delivery approach (termed as microbotics) also exhibited excellent potential for the delivery of nucleic acids by taking advantages of the invasive nature of bacteria.[28] Inspired by these works, we attempt to coat *Salmonella* with nanoparticles self-assembled from cationic polymers and DNA in overcoming multiple barriers in the oral delivery of DNA vaccines. Cationic polymers represent a promising type of nonviral gene delivery vectors and are able to spontaneously complex with DNA (or other nucleic acids) to form nanoscaled polymer/DNA complexes (polyplexes) through electrostatic interactions.[29] The strong buffering capacity of cationic polymers could effectively help themselves escape from endo/lysosome as a result of "proton sponge" effect.[30,31] For example, 25 kDa polyethylenimine (PEI) is well known for its excellent transfection activity in vitro largely due to its strong buffering capacity. The PEI/DNA complexes escape the endosomal through a "proton-sponge" mechanism.[32] Similarly, cross-linked β-cyclodextrin-PEI600 (CP) with degradable PEI network was demonstrated as an efficient vector for nucleic acid delivery in vitro and in vivo in our previous study.[33−36] On the basis of the merits of cationic polymers, we herein disclose that a living hybrid system composed of synthetic CP nanoparticles and live attenuated *Salmonellae* acts as an efficient vector to deliver oral DNA vaccines and achieves potent antitumor immune response in vivo. The nanoparticle-coated *Salmonella* we developed is expect to fullfill the two requirements in oral DNA vaccine delivery, efficient phagosomal escape and enhanced acid tolerance, thereby effectively modulating antitumor immune response (Scheme 1). To our knowledge, this is the first example of nanoparticle-modified bacterial delivery system for oral DNA vaccination in cancer immunotherapy in vivo. It may constitute a promising approach for engineering DNA vaccine delivery systems for a wide spectrum of immunotherapies in the future.

The morphology of the nanoparticle-coated *Salmonella* was first observed by scanning electron microscopy (SEM) to obtain direct insight into the interaction between *Salmonellae* and nanoparticles. As shown in Figure 1a, the uncoated naked *Salmonella* exhibited a typical rod shape with a smooth membrane surface, whereas polyplex nanoparticles were clearly observed to stick on the *Salmonella* surface in the case of nanoparticle-coated *Salmonellae*. Due to the electronegative nature of bacterial cell walls, the positively charged nanoparticles can be self-assembled onto *Salmonella* surface via electrostatic interaction, forming a dense coating layer over the rod-shaped *Salmonella*. The self-assembly approach of nanoparticle-coated bacteria in the current study is much simpler and more straightforward as compared to that of microbots where the surface premodification of both bacteria and nanoparticles is required before assembly.[28] The layer thickness is also found to increase with the nanoparticle coating concentration. LIVE/DEAD bacteria viability assay suggests the concentration of nanoparticle has a dose-dependent effect on the *Salmonella* viability because the strong electrostatic and hydrophobic interactions between polyplex nanoparticles and bacteria may

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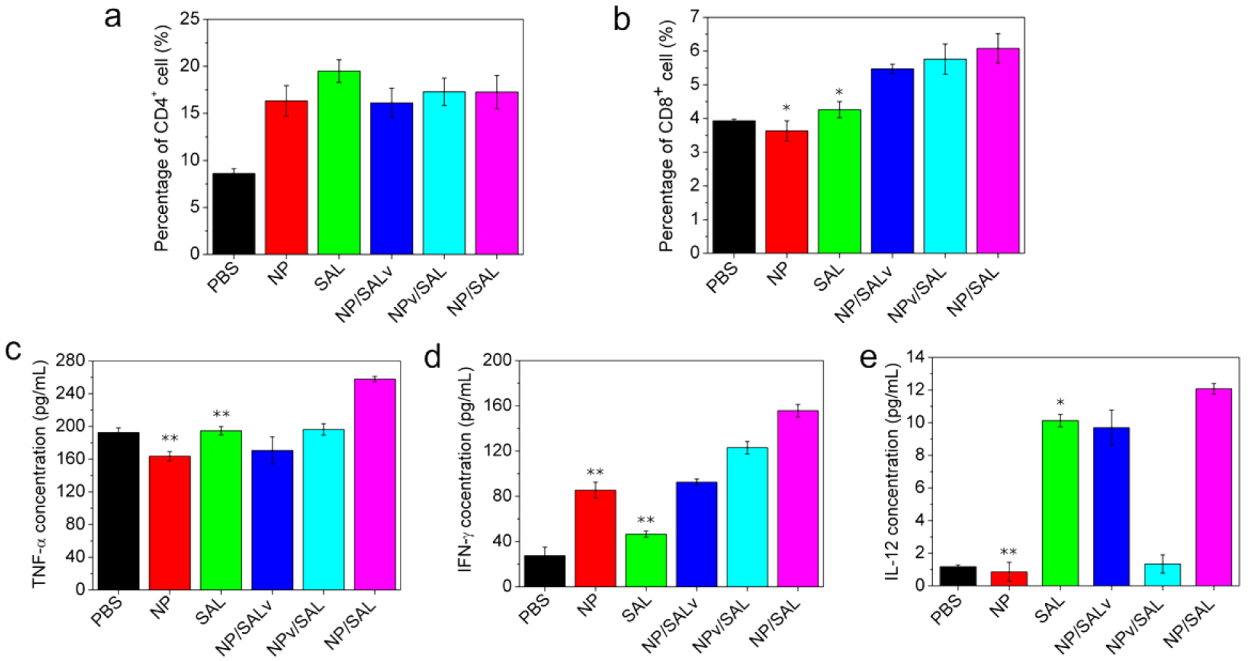

Figure 3. Induction of $CD4^+$ (a) and $CD8^+$ (b) T cells in C57BL/6J mice immunized with PBS, CP/pcDNA3.1-VEGFR2 (NP), *Salmonella*-pcDNA3.1-VEGFR2 (SAL), (CP/pcDNA3.1-VEGFR2)/*Salmonella*-pcDNA3.1 (NP/SALv), (CP/pcDNA3.1)/Salmonella-pcDNA3.1-VEGFR2 (NPv/SAL), and (CP/pcDNA3.1-VEGFR2)/*Salmonella*-pcDNA3.1-VEGFR2 (NP/SAL) on the B16 melanoma tumor model. The cells were isolated from the spleens and stained with PE-CD4 and FITC-CD8 antibodies. Quantification of cytokines TNF-α (c), IFN-γ (d), and IL-12 (e) harvested from the spleen cells after 3 weeks of successive immunization with different formations through the ELISA assay. NP/SALv represents hybrid of CP/pcDNA3.1-VEGFR2 complexes coating on *Salmonellae* loaded with pcDNA 3.1 (empty vector), whereas NPv/SAL stands for hybrid of CP/pcDNA3.1 complexes coating on *Salmonellae* loaded with pcDNA3.1-VEGFR2. NP/SAL stands for hybrid of CP/pcDNA3.1-VEGFR2 complexes coating on *Salmonellae* loaded with pcDNA 3.1-VEGFR2. The doses of pcDNA3.1-VEGFR2 in all formulations are equal. All data represent mean ± SD ($n = 3$, Student's t test, $^*P < 0.05$, $^{**}P < 0.01$, NP (or SAL) vs NP/SAL).

damage the cell wall of bacteria (Figure 1b).[37,38] Furthermore, although low concentration of nanoparticle coating was found to facilitate cellular uptake of *Salmonellae*, uptake efficiency dropped when higher coating concentration was applied (Supporting Information Figure S1). Therefore, NP/SAL with the minimum concentration of the polyplex coating was used in subsequent experiments.

During intracellular delivery process, poor phagosomal escape is often considered as the major barriers inhibiting antigen presentation of *Salmonella*.[39] We hypothesize that the nanoparticle coating may facilitate intracellular delivery and upregulate antigen expression. Cationic CP polymer was demonstrated to possess a strong acidic buffering capacity in our previous study due to the protonable amines in its backbone. As a result, we expect coating CP nanoparticles will also initiate a "proton-sponge" effect to rupture the phagosome. As shown in Figure 1c, after incubating the coated *Salmonella* with RAW264.7 at multiplicity of infection (MOI) ratio of 50:1 for 2 h, the nanoparticle-coated *Salmonella* was able to enter the cells and was trapped in the phagosome. This suggests that when *Salmonellae* are recognized and internalized by macrophages, the cationic nanoparticles carried by *Salmonellae* can be simultaneously internalized by macrophages. Confocal laser scanning microscopy (CLSM) study indicates that intracellular trafficking of the coated *Salmonella* (labeled by FITC over PC polymers) colocalize and sequester with phago/lysosome (labeled by red lysotracker) and only a small portion of green fluorescence is observed to spread out from LysoTracker, indicating NP/SAL hybrids are trapped by phagosomes (Supporting Information Figure S2). Furthermore, we examined the phagosomal pH induced by the naked and coated *Salmonella* through LysoSensor Yellow/Blue DND-160. The blue fluorescence indicates the neutral pH of the endosome, whereas green fluorescence represents acidic pH. As shown in Figure 2d, the coated *Salmonellae* can induce stronger green fluorescence than the naked *Salmonella*, suggesting a more acidic phagosome environment where the coated *Salmonella* is trapped. The protonation property of the CP polymer is likely to result in an acidic environment to facilitate phagosomal escape. Further studies show that the nanoparticle-coated *Salmonella* integrated with EGFP is able to initiate stronger GFP expression in different cell lines compared with uncoated ones (Supporting Information Figure S3), thereby confirming the ability of nanoparticle coating to enhance the antigen expression of *Salmonellae*. To improve the safety of bacteria as oral vaccines, attenuated *Salmonellae* generally exhibit reduced virulence, thereby displaying lower infection activity.[18] Supporting Information Figure S4 indicates that coating *Salmonellae* with either nonviral "gold standard" PEI (25 kDa)[28,32] or biodegradable poly-D,L-succinimide (PSI)-based PSI-NN′$_{0.85}$-NN$_1$ nanoparticles[40] can mediate efficient infection and induce strong GFP expression in RAW 264.7 cells. These results also demonstrate the effectiveness and versatility of this general approach to engineer efficient nanoparticle/bacteria hybrid delivery system. Therefore, nanoparticle coating plays an important role in facilitating endosomal escape and nanoparticle/*Salmonella* (NP/SAL) hybrids are expected to mediate efficient gene expression in vivo.

Although investigation of the transfection efficiency in vitro can identify promising materials for transfection, overcoming the harsh environment in the stomach and intestine before arriving at

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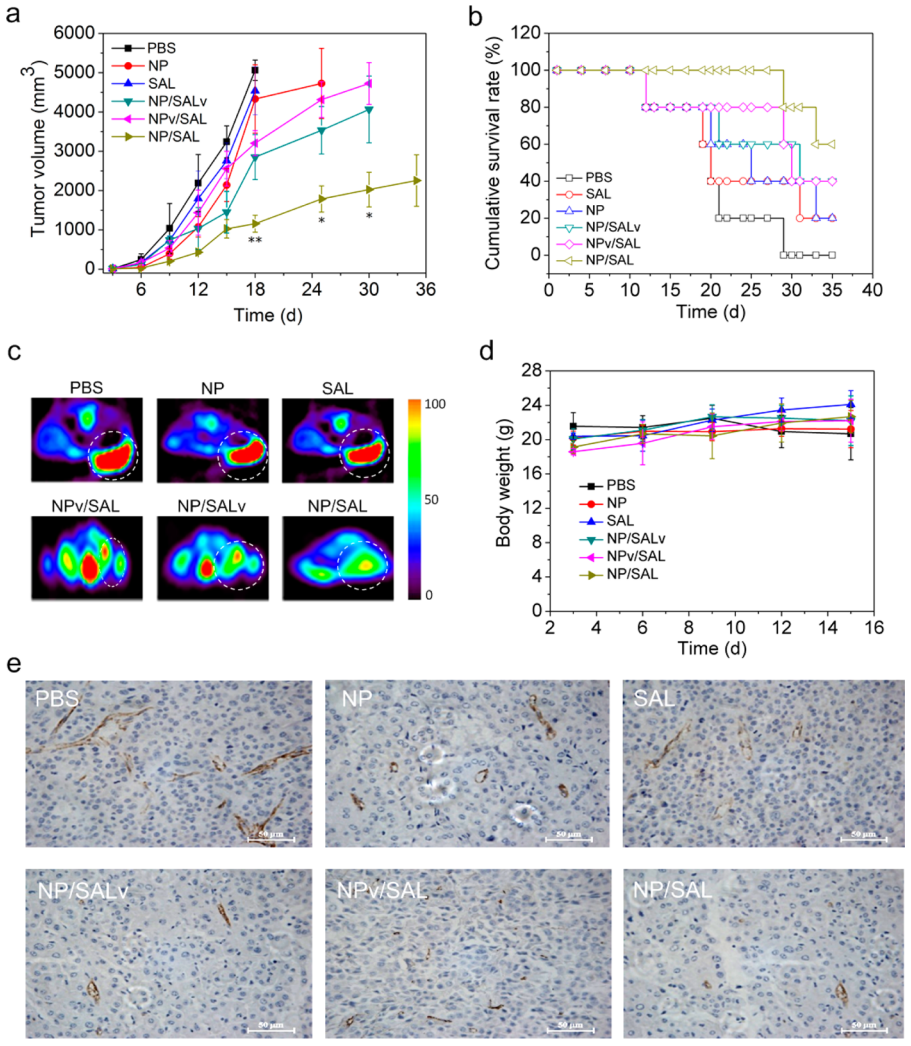

Figure 4. (a) Inhibition of tumor growth in C57BL/6-derived B16 melanoma tumor models orally vaccinated with PBS, NP, SAL, NP/SALv, NPv/SAL, and NP/SAL formulations. Data represent mean ± SD (n = 6, Student's t test, *P < 0.05, NP/SALv (or NPv/SAL) vs NP/SAL), **P < 0.01, NP (or SAL) vs NP/SAL). (b) Survival curves of tumor-bearing mice vaccinated with the different formulations. (c) PET images of in vivo tumor growth 18 days after the first treatment. The intensity is shown by the legend to the right. The circled area denotes the tumor position. (d) Average body weights of mice vaccinated with the different formulations in the B16 melanoma tumor model. Data represent mean ± SD (n = 6, Student's t test). (e) Immunohistochemical analysis of CD31 in the tumor slices of the B16 melanoma tumor model after immunization with different DNA vaccine formations. The scale bars represent 50 μm.

the targeted site of action remains a great challenge for effective oral DNA vaccine in vivo. An unavoidable reduction in the number of viable bacteria occurs during this process and only 10 to 25% of the ingested *Salmonella* can survive and reach the gut to initiate infection.[41] In order to be effective, it is essential to protect the *Salmonellae* from degradation when they pass through the stomach and upper intestine before reaching the gut in a sufficient amount. To mimic the stomach pH, we first analyze the effect of acidic pH on the viability of naked and coated *Salmonellae*. As shown in Figure 2a and c, there is almost no difference in terms of viability between the naked and coated *Salmonellae* at pH 7.4. In contrast, the relative viability of the naked *Salmonellae* dramatically drops to 10% at pH 3.0, whereas the relative viability of the coated *Salmonella* is about 58% at the same pH. We further measured the concentration of *Salmonellae* in blood after oral vaccination with the naked or coated *Salmonellae* (Figure 2b). Initially, slightly fewer coated *Salmonellae* were detected in the blood as compared with naked ones 30 min after oral administration. Afterward, the concentration of naked *Salmonellae* decreased significantly after another 30 min, becoming negligible after 2 h. In contrast, coated *Salmonellae* were found to have significantly higher blood concentration than uncoated *Salmonellae* in both time points (1 and 2 h). The above results demonstrate that whereas the tolerance of the *Salmonella* in the acidic environment can be significantly improved by surface coating with polyplex nanoparticles, the viability of uncoated *Salmonellae* remains extremely sensitive to gastric pH. We speculate that on one hand, strong buffering capacity of CP could partially protect bacteria from acid attack, thereby improving the tolerance of the *Salmonella* in the acidic condition. On the other hand, the coated NP layer may become more hydrophobic after nanoparticle coating, serving as a protective layer over the bacterial surface. In such a circumstance, the increased surface roughness of *Salmonellae* may reduce the direct contact between NP/SAL and acid fluids by forming a large contact angel between coated *Salmonella* surface and acid fluids. After oral administration, the higher blood concentration of viable bacteria with nanoparticle coating

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strongly suggests that surface coating promotes the dissemination of the bacteria into blood, thus making the lymphoid tissue and systemic sites more accessible to the coated *Salmonella* to induce broad-based immune responses.[19] Meanwhile, the intestine toxicity results reveal that the small intestine treated with nanoparticle-coated *Salmonellae* exhibits well-defined heterochromatin and nucleoli in the epithelial cells and plasma cells, indicating the safety of the nanoparticle-coated *Salmonella* in small intestine in vivo (Figure 2d).

T cells play a critical role in the protective immunity against tumors. $CD4^+$ helper T cells play several important roles during the development and maintenance of $CD8^+$ cytotoxic T lymphocyte. Bacterium-based vaccines can induce systemic T-cell responses including polyfunctional cytokine-secreting $CD4^+$ and $CD8^+$ T-cells. Therefore, evoking the secretion of circulating tumor-specific T cells would promote antitumor immunity.[42] As shown in Figure 3a and b, the population of the $CD4^+$ T cells among the spleen administered with the PBS buffer is only 8.62%. The $CD4^+$ T cells significantly proliferate to the range of 16.11−19.30% when the mice were vaccinated with CP/pcDNA3.1-VEGFR2 (NP), *Salmonella*-pcDNA3.1-VEGFR2 (SAL), and (CP/pcDNA3.1-VEGFR2)/*Salmonella*-pcDNA3.1-VEGFR2 (NP/SAL). There is no distinct difference among NP, SAL, and NP/SAL, reflecting the naturally protective immunity. In the case of the $CD8^+$ T cells, the upregulated $CD8^+$ level was clearly evident when the tumor-bearing mice were immunized with all nanoparticle-coated *Salmonellae*, namely (CP/pcDNA3.1-VEGFR2)/*Salmonella*-pcDNA3.1 (NP/SALv), (CP/pcDNA3.1)/Salmonella-pcDNA3.1-VEGFR2 (NPv/SAL), and NP/SAL. Among these nanoparticle-coated *Salmonellae*, NP/SAL formulation is found to be most effective in activating $CD8^+$ T cells. These results strongly suggest that the nanoparticle coating can effectively stimulate the up-regulation of tumor-specific T cells thus boding well for the antitumor activity. Besides T-cell mediated antitumor immunity, different cytokines that are secreted in response to infection, inflammation, and immunity can exert inhibitory effects on tumor development and progression.[43] For example, interferon-γ (IFN-γ) is identified for its capacity to enhance tumor immunogenicity, promote mononuclear cell infiltration into the tumor tissue, and inhibit tumor angiogenesis.[44] Interleukin-12 (IL-12) is able to regulate the differentiation of naive T cells into TH1 cells,[45] and tumor necrosis factor-α (TNF-α) plays an important role for inducing hemorrhagic necrosis of solid tumors.[46] Thus, we quantify the cytokines level of IFN-γ, IL-12, and TNF-α in tumor-bearing mice after the administration of NP-coated *Salmonellae* by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 3d, the NP/SAL possesses the strongest ability to produce TNF-α, which shows significantly higher level than NP or SAL, thereby suggesting that higher immune activation achieved by NP/SAL. The similar trends are also observed from IL-12 and TNF-α production (Figure 3c and e). Collectively, the results validate that the NP-coated *Salmonellae* are capable of improving antigen expression and antitumor immunity in an effective manner. Therefore, NP coating would exert positive effect on *Salmonellae* in activating antitumor immunity, which is expected to promote tumor suppression.

Angiogenesis is known as the formation of new blood vessels from pre-existing microvasculature, which is a complex and crucial process during tumor formation.[47,48] Many tumor masses secrete angiogenic factors such as vascular endothelial growth factor (VEGF) to promote blood vessel formation which eventually functions as a route for tumor metastasis. Among the VEGF receptor (VEGFR) family, VEGFR2 is essential for tumor angiogenesis and plays an important role in tumor growth, invasion, and metastasis.[49−51] Therefore, targeting the VEGFR2 pathway has become an attractive strategy for antiangiogenesis in cancer therapy.[52,53] In the current study, pcDNA3.1-VEGFR2 encoding autologous VEGFR2 was constructed and used as oral DNA vaccine for the study of tumor angiogenesis suppression (Supporting Information Figure S5). By preliminarily accessing antitumor activity on C57BL/6-derived B16 melanoma tumor models, we identified the formulation of NP/SAL (300 μg CP, 10^7 CFU bacteria) is most effective in inhibiting tumor growth and this formulation was then applied for the subsequent in vivo therapeutic experiments (Supporting Information Figure S6). As indicated in Figure 4a, tumor growth is greatly inhibited after oral immunization with NP/SAL hybrids loaded with pcDNA3.1-VEGFR2. The average tumor volume of the mice vaccinated with NP/SAL formulation is around 1000 mm^3 on the 18th day, which is at least 4 times and 3.7 times more potent than those vaccinated with naked *Salmonellae* (SAL) and polyplex nanoparticles (NP), respectively (Figure 4a, Supporting Information Figure S7). Interestingly, the intermediate inhibition effect was observed when the tumor-bearing mice were immunized with either NPv/SAL or NP/SALv formulation at the 18th day, and the average tumor volume of mice vaccinated with these formulations is more than two times larger as compared with those vaccinated with NP/SAL formulation at 30th day. Therapeutic outcomes can be maximized only when both nanoparticles and bacteria were loaded with DNA vaccine (NP/SAL), indicating a synergistic effect of two vectors. Furthermore, positron emission tomography (PET), which provides highly sensitive imaging modality for tumor diagnosis, is utilized to precisely measure the tumor volume. As shown in Figure 4c, the red color represents the highest intensity. The red color area decreases dramatically from uncoated *Salmonellae* to the coated ones. In the NP/SAL group, the weakest red fluorescence suggests the most effective vaccination against tumor growth. Generally, coated *Salmonellae* (NP/SAL, NPv/SAL and NP/SALv) are also more efficacious with respect to cumulative survival than PBS, SAL, NP formulations (Figure 4b). The survival study showed that 60% mice vaccinated with the formulation of NP/SAL survived entire 35-day study duration without tumor growth. Meanwhile, almost no body weight loss was observed over the mice vaccinated with the NP/SAL formulation, indicating its low systemic toxicity nature and minimum side effect (Figure 4d). These results strongly indicate the effectiveness of NP/SAL formulation as a safe oral DNA vaccine to inhibit tumor growth and to extend the survival time of tumor-bearing mice.

Cell apoptosis in the tumor tissues after treatment with various formulations were analyzed by H&E staining (Supporting Information Figure S8) and TUNEL assay (Supporting Information Figure S9). The H&E stained sections of tumor tissues from PBS and SAL groups appeared to be most hypercellular and exhibited nuclear polymorphism more obviously. Among these therapeutic groups, the tumor tissues from the vaccination with the NP/SAL formulation displayed the fewest tumor cells and the highest level of tumor necrosis. The TUNEL assay also showed that DNA vaccination with NP/SAL can induce much more TUNEL-positive cells. These results further validate the effectiveness of DNA vaccination through the oral delivery of pcDNA3.1-VEGFR2 by coated *Salmonellae*. Immunohistochemical analysis of CD31 reveals a distinct decrease in the vessel density of the tumor slice of the B16

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melanoma tumor model after NP/SAL oral administration, when compared with the NP, SAL, and other formulations (Figure 4e). These results also provide important evidence for the effective tumor inhibition mediated by NP/SAL.

In conclusion, a new oral DNA vaccination approach mediated by cationic nanoparticle-coated bacteria for efficacious cancer immunotherapy is developed in this study. By coating attenuated *Salmonellae* with cationic CP nanoparticles, the protective nanoparticle coating layer is able to facilitate *Salmonellae* to effectively escape phagsomes, remarkably promotes the dissemination of the bacteria into blood, and significantly enhance the acid tolerance of *Salmonellae* in stomach and intestines. Oral delivery of DNA vaccine encoding autologous VEGFR2 by the hybrid vector shows remarkable T cell activation and cytokine production. Most importantly, successful inhibition of tumor growth is also achieved by this delivery strategy due to angiogenesis suppression in the tumor vasculature and tumor necrosis. This work demonstrates coating live attenuated bacteria with polymeric nanoparticles represents a promising strategy to engineer efficient and versatile DNA vaccine vectors. With a wide spectrum of bacterium choices, nanoparticles can be further tailored and functionalized for different delivery applications. This approach to delivering DNA vaccine may become a very promising therapeutic modality for treating a wide spectrum of cancers in the dawning era of personalized nanomedicine.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, cellular uptake of coated or uncoated *Salmonellae* stained with SYTO9 in RAW 264.7 cells, intracellular distribution of coated *Salmonellae* after phagocytosis, GFP expression mediated by nanoparticle-coated *Salmonellae* and uncoated *Salmonellae*, GFP expression mediated by uncoated *Salmonellae* or *Salmonellae* coated with different nanoparticles, the plasmid structure of pcDNA3.1-VEGFR2, inhibition of tumor growth in C57BL/6-derived B16 melanoma tumor models orally vaccinated with PBS and nanoparticle-coated *Salmonellae* of different bacterium doses, dissected tumor tissues after successive 18 days of vaccination of different formulations in tumor-bearing mice, histological images of the tumor slices collected from the mice with H&E staining after oral DNA vaccinations, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥These authors contributed equally to this work.

Author Contributions

Q.H. and M.W. primarily performed the experiments. C.F., C.C., and W.F. assisted analysis of T cells and cytokines as well as evaluation of bacterial survival in vivo. M.Z. helped antitumor efficacy study in vivo. P.J.C. advised on the SEM study. Y.P. and G.T. designed the experiments, supervised the whole project, and wrote the paper. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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