Journal of Immunological Methods 472 (2019) 7–15

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Self-destructing *Salmonella via* temperature induced gene *E* of phage PhiX174 improves influenza HA DNA vaccine immune protection against H1N1 infection in mice model

Nitin Machindra Kamble[a], Amal Senevirathne[a], Hong Bum Koh[b], Jae Il Lee[b], John Hwa Lee[a,*]

[a] College of Veterinary Medicine, Chonbuk National University, Iksan Campus, 54596, Republic of Korea
[b] College of Veterinary Medicine, Chonnam National University, Gwangju 61186, Republic of Korea

ARTICLE INFO

Keywords:
Gene *E*
Salmonella delivery
Immune protection, influenza
H1N1

ABSTRACT

The delivery of DNA vaccines is the principle impediment for implementation of DNA vaccination on a mass scale. In this study, we report a temperature induced conditionally expressed phage PhiX174 gene *E* mediated lysis of *Salmonella* under *in vivo* conditions that can increase the immunogenicity of a DNA vaccine delivered *via Salmonella* carrier system. We electroporated gene *E* encoding lysis plasmid pJHL187 along with the pcDNA-HA plasmid encoding H1N1 HA into attenuated *Salmonella* Typhimurium, strain JOL1893. Using C57BL/6 mice as the model, we showed that the mice intragastrically vaccinated with JOL1893 induced significant production of HA-specific humoral and cell mediated immune responses compared to the JOL1837, which carry pcDNA-HA plasmid alone. Furthermore, mice vaccinated with JOL1893 vaccine were fully protected against the lethal H1N1 challenge compared to the JOL1837 strain, which showed 90% protection only. However, none of the animals survived treated with either the PBS or the *Salmonella* carrying empty vector. Taken together, our results indicate that mucosal immunization with conditional lysis enabled live attenuated *S.* Typhimurium as a DNA vaccine carrier can induce efficient systemic and mucosal immune responses, and improves immune protection against a highly pathogenic H1N1 infection in mice model.

1. Introduction

Unpredictable nature of influenza virus and difficulty to early predict the health-related threat of new isolates present a major challenge for health planners. Novel strains of influenza viruses are reported at regular intervals worldwide, while vaccination remains as the most effective method to protect both humans and animals (Chen et al., 2008; Rajapaksha and Petrovsky, 2014; Soema et al., 2015). Conventional vaccine development is a tedious process which involves viral propagation that requires a large supply of specific-pathogen-free (SPF) embryonated eggs and a long timeline that could be a serious impediment during an influenza pandemic. Further, the influenza viruses have the ability to continuously evolve either gradually through antigenic drift (point mutations) or rapidly through re-assortment with another divergent virus (antigenic shift) (Nicholson et al., 2003). Consequently, the immunity generated against one vaccine strain is only protective against another strain that shares antigenically related proteins or otherwise, immunity becomes outdated. Therefore, influenza vaccines needed to be annually updated pertaining to the antigenic changes of the circulating field strains. In accordance with this notion, many researchers have focused on developing efficient control measures which can be developed and implemented as quickly as possible against the strain matched virus (Lambert et al., 2016). In this aspect, DNA vaccine technology has proved to be an effective solution for mass production of vaccine agents. Unlike subunit protein-based vaccines, DNA vaccines induce immunogenic protein production within the cells of the targeted host species (Jorritsma et al., 2016; Koday et al., 2017). It may provide a better opportunity for essential post-translational modifications that viral antigens naturally undergo. Previous DNA vaccine investigations have shown that, the injection of DNA from the hemagglutinin (HA) (Krammer et al., 2013), neuraminidase (NA) (Job et al., 2018), nucleoprotein (NP) (Lee et al., 2019) or matrix (M2) (Tompkins et al., 2007) proteins of the influenza virus were able to induce immune responses against influenza infections (Stachyra et al., 2014) and various DNA based influenza vaccines are currently under clinical trials (Ledgerwood et al., 2012). The hemagglutination inhibition (HI) responses which are generally low in DNA vaccines compared to the conventional inactivated influenza vaccines, that can be improved

* Corresponding author.
E-mail address: johnhlee@jbnu.ac.kr (J.H. Lee).

https://doi.org/10.1016/j.jim.2019.06.008
Received 16 March 2019; Received in revised form 22 May 2019; Accepted 4 June 2019
Available online 06 June 2019

through various approaches including administering increased dosages of DNA vaccines, addition of additional immunogens (NA, NP, or M2), combining gene-based vaccines with electroporation, or by incorporating adjuvants (Beran et al., 2010; Brady et al., 2009; Laddy et al., 2008; Ryea et al., 2008). However, routine implementation of such approaches in animals and humans still do not seem to be feasible. The low efficiency of the naked DNA vaccine is partly attributed to the fact that these vaccines are not efficiently targeted to the antigen-presenting cells (APCs) (Yang et al., 2006). Efficacy of this vaccine may be enhanced by direct targeting into APCs by employing various synthetic and live strategies. The use of *Salmonella* as a nucleic acid delivery system has been emerged as a promising alternative to overcome many of the drawbacks associated with DNA vaccine delivery (Gahan et al., 2007). Being an intracellular pathogen, *Salmonella* can multiply within APCs rendering specific delivery of DNA constructs directly into APCs. The effectiveness of *Salmonella* Typhimurium as a vaccine delivery agent for DNA vaccines can be further enhanced by introducing lysis strategy. Timely lysis upon administration may not only increase plasmid delivery efficacy, but also the safety of the carrier strain. In order to achieve this objective, in the present study, we constructed a lysis plasmid comprised of gene *E* of bacteriophage ϕX174 (Hajam et al., 2015) that is stringently controlled by a convergent promoter system containing sense λpR promoter with repressor cI857 and antisense ParaBAD promoter with araC regulatory element. Lysis plasmid suppresses gene *E* expression at temperatures below 30 °C due to the λpR promoter with thermolabile repressor cI857 (Bernhardt et al., 2002). Simultaneous activations of the convergent promoters in the ghost plasmid can effectively prevent the translation of mRNA encoding the lysis gene, and the subsequent expression of the lysis gene during the cell growth. To enable the conditional lysis of the bacteria, the mid-log cultures of the bacteria are grown at 37–42 °C without the addition of the arabinose that prevents leaky expression allowing *Salmonella* to grow exponentially. Under a physiological temperature (around 37 °C) with conditions deprived with L-arabinose, gene *E* expression may switch on the lysis process resulting in pores in the bacterial membrane. Due to leaking out of life-supporting cellular content such as ATP, DNA, and essential proteins, the bacterium will die releasing it DNA cargo, however, the integrity of the bacterial structure will be retained. Moreover, the DNA vaccine is physically confined to the intracellular compartment of the *Salmonella* system, thus this strategy might inhibit antibody responses against the DNA vaccine backbone.

Considering the advantages of attenuated *Salmonella*-based delivery of cargo, in this study, we hypothesized that enabling the attenuated *S.* Typhimurium to lyse conditionally while delivering H1N1 (Influenza A Puerto Rico/8/34) HA based-DNA vaccine can increase the vaccine efficacy over the conventionally non-lysed approach. Here we report that attenuated *S.* Typhimurium harboring the lysis plasmid along with the pcDNA-HA1 DNA plasmid improves the HA-specific humoral and cellular immune responses, and provides effective immune protection against the highly pathogenic H1N1 virus infection.

2. Materials and methods

2.1. Bacterial strains, virus and cell line

The bacterial strains used in this study are listed in Table 1. Influenza virus A/Puerto Rico/8/34 (H1N1) was used in this study. The virus was cultivated in the allantoic cavity of SPF embryonated eggs, titered in Madin Darby Canine Kidney (MDCK) cells, and expressed as 50% tissue culture infective dose ($TCID_{50}$). The 50% egg infective dose (EID_{50}) of H1N1 was determined in SPF embryonated eggs by Reed and Muench method (Reed and Muench, 1938), before use in challenge experiment. The MDCK cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

2.2. Construction of lysis and pcDNA-HA1 plasmids

The lysis plasmid pJHL187 was constructed by cloning bacteriophage PhiX174 lysis gene *E* in between the face to face oriented temperature inducible *λpR* promoter with a *cI1857* regulatory element and the antisense *ParaBAD* promoter with *AraC* regulatory element (Fig. 1) (Chaudhari et al., 2012; Hajam et al., 2015). To construct the H1N1 HA1-based DNA vaccine, the optimized HA1 gene sequence of Influenza virus A/Puerto Rico/8/1934 (H1N1) was synthesized (Bioneer, Korea) and built into the pcDNA3.1 (+) (Invitrogen, USA) at *EcoR1/XhoI* site and propagated in DH5- α *Escherichia coli* strain as previously described (Hur and Lee, 2011a). The recombinant plasmid, pCDNA-HA1 was then electroporated into attenuated auxotrophic mutant *S.* Typhimurium strain JOL912 (*Δlon ΔcpxR Δasd*), constructed previously using the parent wild type strain JOL401(Hur and Lee, 2011b). The novel strain was named as JOL1837. Next, the same clone was electroporated with lysis plasmid pJHL187 and was designated as JOL1893. To visualize the expression of the HA1 gene in mammalian cell culture, the green fluorescent protein (GFP) was cloned downstream to the HA1 gene in pCDNA3.1 at *XhoI/XbaI* restriction sites. The HA1-GFP plasmid construct and the lysis plasmid construct were also introduced into ST JOL912 as described elsewhere and designated as JOL1838 and 1839 respectively (Hur and Lee, 2011b). Culturing of ST strain JOL1893 carrying lysis plasmid were done at 30 °C in LB broth supplemented with 0.2% L-arabinose with low agitation (80 rpm) until the culture reaches mid-log phase. To assess lysis efficacy, cells grown at 30 °C was harvested and grown without L-arabinose at 37 °C with vigorous agitation (150 rpm). Viability of cells was determined by plating of periodically collected sample aliquots on LB agar.

The HA1 gene was cloned into pET28a (+) expression vector (Novagen, San Diego, USA) and subsequently transformed into *E.coli* BL21 DE (Novagen, USA) for protein expression. Isopropyl β-D-1-thiogalactopyranoside IPTG induced cells were lysed and subjected to protein purification using Ni-NTA affinity chromatography. The expressed proteins from both in *S.* Typhimurium and *E. coli* were confirmed by Western blot analysis using either polyclonal HA antibody (#A01557; GenScript, USA) or His-Tag antibody (#AB-TA13002, AprilBio, Co, Ltd., Korea). Purified proteins were quantified by a Bradford assay (Bradford, 1976), filtered, and stored at −20 °C until use.

2.3. Plasmid stability assay

The stability of the lysis plasmid and pcDNA-HA1 plasmids was assessed inside the same bacterial host. Bacterial culture of JOL1893, harboring pJHL187 and pcDNA-HA1 plasmids, were passaged for 5 consecutive days in the absence of any antibiotics. Decimal dilutions of the overnight culture were plated on the LB agar on every consecutive day and single colonies were picked and subcultured for the plasmid isolations. The isolated pJHL187 and pcDNA-HA1 plasmids were confirmed by the colony PCR for gene *E* and HA1 genes, respectively.

2.4. Expression of viral antigens by Salmonella-mediated delivery in cultured cells

The expression of the HA1 gene delivered *via Salmonella* system was evaluated in the MDCK cell line. The MDCK cells were seeded on gelatin-coated coverslips at a density of 5×10^5 cells per well in a 6-well culture plate. The near confluent MDCK cells were infected with JOL1822, JOL1837, JOL1838, JOL1839 or JOL1893 at a multiplicity of infection (MOI) of 40–100 bacteria per cell. The infected cultures were then incubated at 37 °C in 5% CO_2 for 1 h for the infection. Cells were five times washed with 1× phosphate-buffered saline (PBS), and incubated with fresh medium containing 50 μg/ml of gentamicin for 72 h incubation. For positive control, the pcDNA-HA1 plasmid was transfected into MDCK cells using transfection reagent LyoVec (Invivogen,

Table 1
List of bacterial strains and plasmids used in this study.

Strains/plasmids	Description	References
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Lab stock
BL21(DE3)	139(*ara-leu*)7697 *galUgalKrps*L (Str^r)*end*A1 *nup*GF$^-$*ompThsdSB*(rB$^-$mB$^-$)*dcmgalλ*(DE3) pLysSCmr	Lab stock
X232	*E. coli Δasd* strain, used for cloning of genes into *asd*$^+$ plasmid	Lab stock
JOL401	*Salmonella* Typhimurium wild type, SPI-1 *invAE*$^+$*hilA*$^+$*avr*$^+$; SPI-2, amino acid permease$^+$; SPI-3, *mgtC*$^+$; SPI4, ABC transporter; SPI5, *pipB*$^+$; antigen preparation	Lab stock
JOL912	*Δlon, ΔcpxR and asd* mutant *of S.* Typhimurium	(Hur and Lee, 2011)
JOL1822	JOL912 pcDNA3.1	This study
JOL1837	JOL912 with pcDNA3-HA1plasmid	Lab stock
JOL1838	JOL912 with pcDNA3-HA1-GFP plasmid	This study
JOL1839	JOL912 with pcDNA3-HA1-GFP/pJHL187 lysis plasmid	This study
JOL1893	JOL912 with pcDNA3-HA1/ pJHL187 lysis plasmids	This study
Plasmids		
pET28 (+)	IPTG-inducible, T7 expression vector, C-terminal 6× His tag, Kan^R	Novagen, USA
pJHL187	*asd* + pBRori plasmid carrying ghost cassette	(Hur and Lee, 2011)
pcDNA3-HA1	pcDNA3 harboring H1N1 HA1 gene	This study
pMMP65	*asd* + pBRori plasmid	(Hur and Lee, 2011)

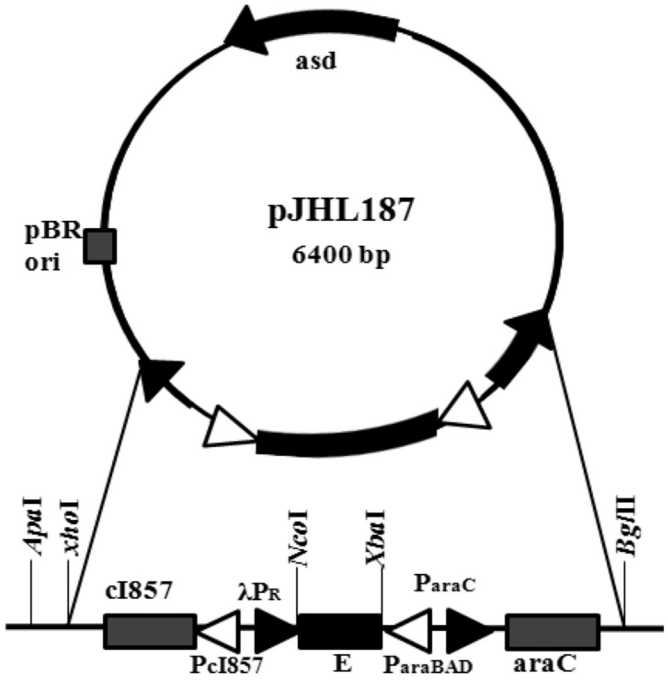

Fig. 1. Components of the lysis plasmid pJHL187. The *asd*$^+$ plasmid with pBR ori carrying the constitutive expression system of the lysis gene E regulated by the convergent promoter elements, sense λpR promoter with repressor cI857 and an anti-sense ParaBAD promoter with the araC regulatory system.

USA) as per the manufacturer's instructions. To analysis the expression of the HA1 protein, cells were washed once with the PBS and subsequently lysed with the eukaryotic cell lysis solution. The cell lysate was subjected to Western blot analysis using rabbit anti-H1N1 HA polyclonal antibody (Catalog #A01557, GenScript, USA). To further confirm the HA1 expression, MDCK cells infected with JOL1822, JOL1838 and JOL1839 were fixed with 4% paraformaldehyde. Then the cells were observed for green fluorescence using a fluorescent microscope (Leica, Germany).

2.5. In vitro and in vivo lysis of JOL1893 carrying pcDNA-HAI and pJHL187 plasmids

To assess the lysis efficacy of JOL1893, a culture was allowed to grow to mid-log phase (0.4 at OD_{600}) at 30 °C with 2% arabinose. Then, cells were harvested and washed twice with PBS and exposed to 37 °C without arabinose and 30 °C with arabinose. After 1 h of incubation at each temperature, 1 ml of each culture was harvested and washed twice with ice-cold PBS and subjected to staining with propidium iodide for flow cytometric analysis. Subsequently, due to the formation of clumps, cell lysis was assessed by plating on agar every 2 h for a total of 10 h duration. To investigate the JOL1893 lysis within the mammalian cells, confluent MDCK cells were infected with mid-log growth cultures of JOL1822, JOL1837 or JOL1893 at a multiplicity of infection (MOI) 10 without arabinose. After 1 h post-infection, cells were washed thrice with PBS and treated with gentamicin (100 μg/ml) to eliminate extracellular *Salmonella*. The infected MDCK cells were then further grown at 37 °C in 5% CO_2 for 24 h, and subsequently, the cells were washed and lysed with Triton-x. The bacterial load was enumerated by plating the lysate on BGA agar. The obtained *Salmonella* positive colonies were expressed as CFU/ml. Next, the *in vivo* lysis was assessed by intra-gastric immunization of mice (n = 4 per group) with JOL1837, JOL1893 and JOL1822 at 10^8 CFU/mice. After 7 days of post immunization, bacterial load in the spleen of immunized mice were enumerated as described previously (Yin et al., 2015).

2.6. Mice immunization

All animal experimentation work was approved by the Chonbuk National University Animal Ethics Committee (CBNU2015–00085) and was carried out according to the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with Animals). Four weeks old C57BL/6 mice were purchased (Samtako, South Korea) and maintained under standard conditions, and provided antibiotic-free food and water *ad-libitum*. One week later, the mice were randomly divided into four groups (n = 14) and were immunized intra-gastrically on day 0, 14 and 28. Groups 1 and 2 were immunized with JOL1893 and JOL1837, respectively, with 10^8 CFU of *Salmonella* bacteria in 100 μl volume of PBS using oral gavage. Groups 3 and 4 received 100 μl of JOL1822 containing an empty pcDNA vector and 100 μl of PBS using the same route of administration respectively. Serum and intestinal lavage samples were collected on the day of immunization (pre-immunization) and thereafter weekly to assess the HA-specific systemic and mucosal antibody responses. The animals were observed throughout the period of an experiment for any toxicity issues. Further, animals (n = 4) were sacrificed on day 14 post-immunization to assess the HA-specific IFN- γ, antigen recall responses splenocytes harvested from immunized mice.

2.7. Serum and mucosal antibody responses

Sera samples were drawn from the mice weekly after immunization and before challenge with the influenza virus strain A Puerto Rico/8/34 (H1N1). The intestinal lavage samples were collected to determine the

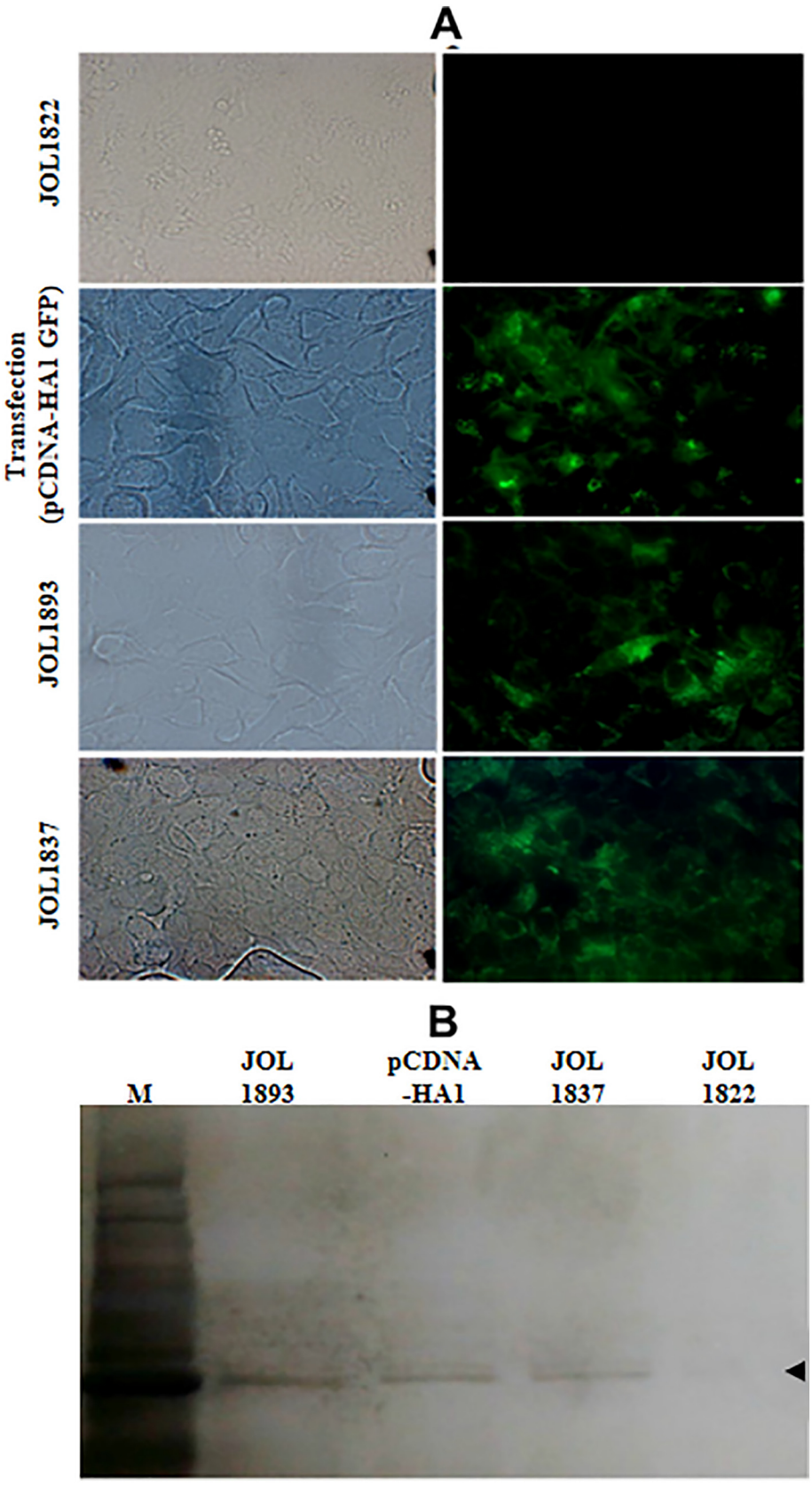

Fig. 2. Expression and characterization of the H1N1 HA1 protein by fluorescence microscopy and Western blot analysis. (A) Fluorescence microscopy for detection of the GFP tagged HA1 protein expressed in MDCK cell line. The MDCK cells (1×10^6/ml) were infected either with JOL1838, JOL1893 or JOL1822 at an MOI of 40–100, or transfected with a naked pcDNA-HA1-GFP plasmid. The cells were incubated for 48 h at 37 °C in 5% CO2 and then analyzed for the green fluorescence. The plasmid control JOL1822 showed no visible fluorescence, whereas JOL1837, JOL1893, and lipofected pcDNA-HA1-GFP plasmid showed green fluorescence. (B) Western blot analysis of the HA1 protein expressed transiently in MDCK cells. The MDCK cells expressing H1N1 HA1 protein were subjected to Western blot analysis using PR8-specific polyclonal sera. A protein band of 28 kDa corresponding to the size of the HA1 protein was found in case of pcDNA-HA1 lipofected cells, JOL1837, and JOL1893 infected cells while the same band was absent in case of cells infected with the JOL1822 carrying pcDNA3.1 vector alone. M; stands for the marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

secretory IgA (sIgA) concentrations as per the protocol described elsewhere (Won and Lee, 2016). An indirect ELISA was used to measure the systemic HA1 specific IgG and sIgA responses in sera and in intestinal lavage samples, respectively, as described previously (Won and Lee, 2016).

2.8. HI assay

Hemagglutination inhibition (HI) assay was performed to assess the HI titers in the sera of immunized and control mice as described previously (Huleatt et al., 2008).

2.9. Cytokine responses by qRT-PCR assay

Spleenocytes stimulated (S1) with recombinant HA protein were harvested after 24 h, and the total RNA was isolated by RNeasy Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The cDNA was prepared from an equal quantity of RNA (1 μg) using SuperScript™ III Reverse Transcriptase kit (Invitrogen, San Diego, California, USA) as previously described (Kamble et al., 2017), and stored at −20 °C until use. Real-time PCR assay (qRT-PCR) for gene expression studies was performed with the ABI applied biosystems using Power SYBR Green PCR Master Mix (#4367659, Applied Biosystems, USA) as described previously (Won and Lee, 2016). The relative amounts of cytokine mRNA present (normalized with GAPDH) was determined by $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

2.10. Protective efficacy

For viral challenge experiments, mice ($n = 10$, each group) were first anesthetized with sevoflurane and then challenged intranasally with 50 μl of PBS (25 μl per nostril) containing a dose of 10^6EID50 of lethal influenza virus strain A Puerto Rico/8/34 (H1N1) 6 weeks after vaccination as previously described (Kamble et al., 2017). After infection, mice were daily monitored for symptom development and body weights were daily recorded for 14 days. Humane endpoints were used during the survived animals. Animals were considered gravely ill and were sacrificed by an overdose of chloroform if they lost > 30% of their body weight or exhibited lethargy, ruffled hair coat or hunched posture.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.00 program (San Diego, CA, USA). Data were analyzed by one way ANOVA with Tukey's multiple comparison test was used between different groups. Data are represented as mean ± standard deviation. $p < .05$ were considered as statistically significant.

3. Results

3.1. Construction and characterization of an attenuated Salmonella lysis system

The synthetic HA1 gene of H1N1 was codon optimized for efficient expression in *S.* Typhimurium, strain JOL912 and cloned into pcDNA3.1 (+).The insertion of the HA1 gene into pcDNA3.1 vector was confirmed by digestion of the positive clones with *EcoR1* and *Xho1* to release a fragment of 675 bp size. Subsequently, the pcDNA-HA1 gene construct was electroporated into the host strain (JOL1837). To incorporate lytic ability in order to efficient release of HA1 DNA plasmid into the host cell cytoplasm, we transformed JOL1837 with the lysis plasmid pJHL187. The temperature inducible λpR-cI857 promoter enabled activation of lysis gene *E* while any leaky expression was avoided by antisense ParaBAD promoter with araC regulatory system (Fig. 1). The lysis of the JOL1893 only occurred under the upshifted temperatures 37–42 °C and devoid of arabinose (Fig. 3A). This finding was consistent with the earlier report (Curtiss et al., 2009).

To investigate the *in vivo* plasmid delivery efficacy, confluent MDCK cells were transfected with JOL1838, JOL1839 or naked plasmid pCDNA-HA1-GFP as a positive control and observed for GFP expression. Expression of green fluorescence was observed in cells infected with JOL1838 and JOL1839 while the maximum number of GFP expressing cells were observed with the pcDNA-HA1-GFP lipofected cells (Fig. 2A) showing the gross efficacy of plasmid delivery and protein expression within eukaryotic cells. To further confirm expression of HA1 gene, infected MDCK cells were infected with JOL1822, JOL1837 or JOL1893 and subjected to Western blot analysis revealing the specific immunoreactive band corresponding to the expected size of the HA1 protein. The intensity of the band obtained in case of JOL1837 and JOL1893 infected cells was comparable to that of the MDCK cells transfected with the naked pcDNA-HA1 alone (Fig. 2B).

Further, we evaluated the stability of the plasmids inside the JOL1837 (pcDNA-HA1) and JOL1893 (pJHL187 & pcDNA-HA1) strains. Our results demonstrated that respective plasmids were successfully isolated from 5 passages of JOL1837 and JOL1893 strains (Data not shown).

3.2. JOL1893 strain harboring pJHL187 and pcDNA-HAI plasmids efficiently lyse inside mammalian cells

The lysis efficacy of JOL1893 harboring pJHL187 and pcDNA-HA1 was investigated by exposing mid-log phage culture at 37 °C without L-arabinose. Early cell lysis was investigated using propidium iodide staining (Fig. 3A) which demonstrated lysis initiate as early as 1 h under sub-optimal temperature. Next, the reduction in viable cell number was investigated over a period of 10 h, which demonstrate gradual reduction with 2 log difference after 8 h of temperature shift (Fig. 3B). At the end of 30 h period, an almost complete destruction of cells could be observed (data not shown). Further, presence of pcDNA-HA1 plasmid in JOL1893 strain did not interfere with the lysis efficiency of the lysis plasmid pJHL187 while no lysis could be observed in case of JOL1837 strain that harbor the pcDNA-HA1 plasmid only, indicating stable growth of the bacteria.

Next, we analyzed the ability of JOL1893 strain to lyse inside the mammalian cells. In this regard, MDCK cells were infected with JOL1822, JOL1837 or JOL1893 for 48 h, keeping media treated cells as a negative control. The lysis efficiency was determined on the basis of recovery of CFU/ml from the infected cells. Our results demonstrated that bacterial recovery from JOL1893 infected MDCK cells was significantly ($p < .05$) lower compared to the JOL1822 and the JOL1837 infected cells. The JOL1822 and JOL1837 MDCK infected cells showed comparable bacterial counts (Fig. 3C). To further investigate the ability of JOL1893 strain to lyse inside the mammalian cells, 4 mice per group were immunized with either JOL1822 (10^8 CFU each/mouse), JOL1837 (10^8 CFU each/mouse) or JOL1893 (10^8 CFU each/mouse), and sacrificed at day 7 post-immunization for estimation of bacterial count in spleen. Mice immunized with JOL1822 and JOL1837 showed bacterial counts of 3 and 3.2 logs CFU/spleen, respectively, while no bacterial count was recorded from JOL1893 immunized mice (Fig. 3D).

3.3. JOL1893 vaccination stimulates efficient HA-specific humoral and mucosal immune responses

All mice were alive and healthy following immunization with *Salmonella* strains until day 42 pre-challenge. To examine the ability of Salmonella-based HA1 DNA vaccines to induce humoral and mucosal antibody responses in C57BL/6 mice, antigen-specific ELISA was carried out to assay the levels of IgG in immunized sera and sIgA in intestinal lavage at 0, 1, 2, 3, 4, 5 and 6-days post immunization (wpi). The kinetics of HA-specific IgG and sIgA responses are shown in Fig. 4. Our results demonstrated that the mice immunized with JOL1893 or JOL1837 displayed significantly ($p < .05$) higher HA-specific IgG responses compared to the JOL1822 and PBS controls. The IgG levels were detected at day 14 post-immunization, which peaked at day 42 (Fig. 4A). However, the mice immunized with JOL1893 strain showed significantly ($p < .05$) higher IgG responses at day 35 and 42 post immunization as compared to the mice vaccinated with JOL1837 strain. This finding clearly demonstrates that conditionally enabled *Salmonella*

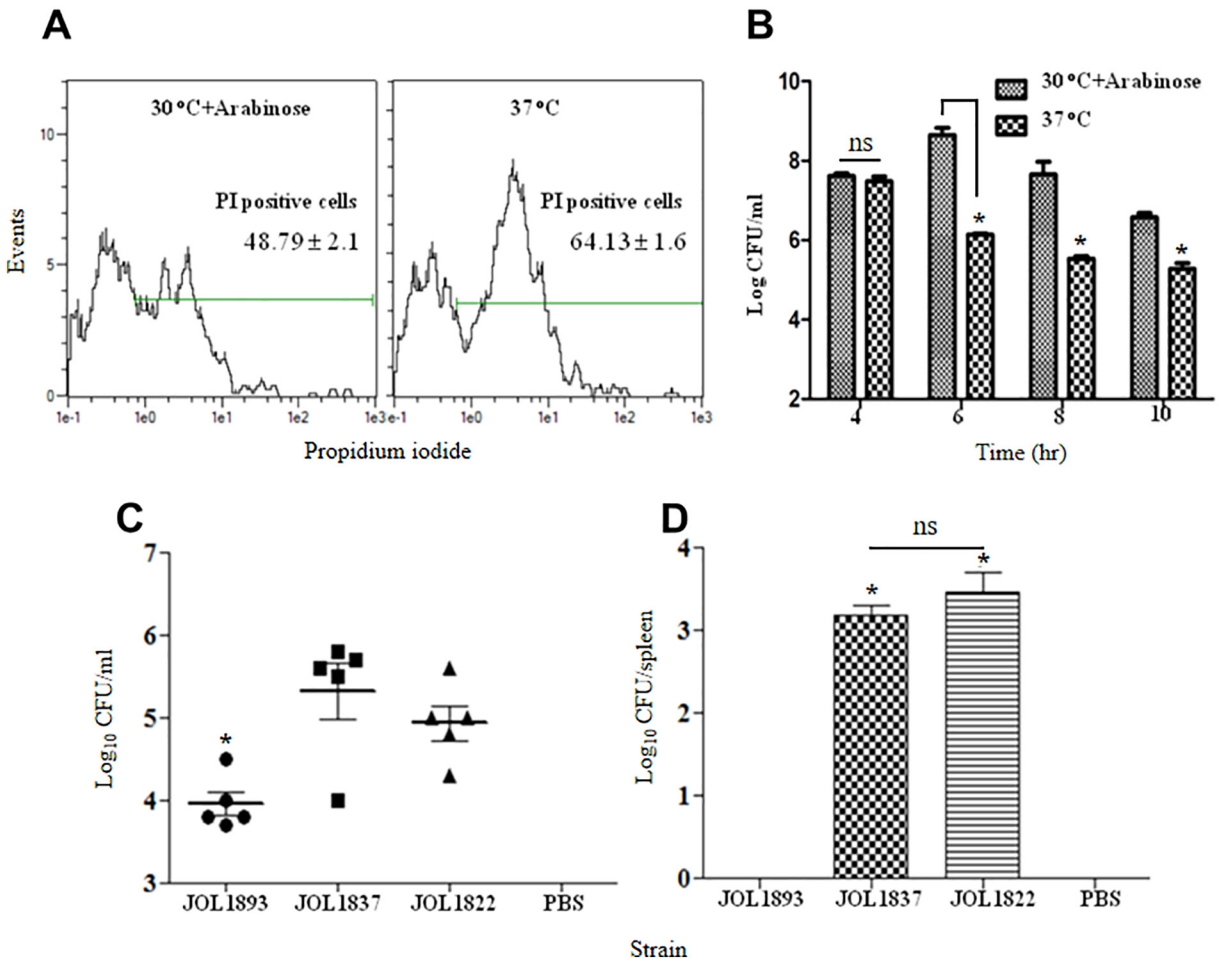

Fig. 3. *In vitro* and *in vivo* lysis analysis of the JOL1893 strain. *In vitro* lysis analysis of the JOL1893 strain harboring pcDNA-HA1 and pJHL187 plasmids. (A) Early initiation of lysis process was assessed using propidium iodide staining procedure and analyzed in a flow cytometer. (B) The reduction in viable JOL1893 bacterial count was investigated by plating on agar plates. (C) The MDCK cells (1×10^6/ml) were infected either with JOL1893, JOL1837 or JOL1822 at an MOI of 40–100 or left untreated as a negative control. After 48 h of incubation, the bacterial count was recorded and the results are expressed in $\log_{10}$ CFU per ml. Each data point represents the mean of two independent experiments. (D) *In vivo* analysis of lysis efficiency of the JOL1893 strain. Mice ($n = 4$) were inoculated with either JOL1893, JOL1837, JOL1822 or PBS and 7 days later, mice were sacrificed and the bacterial count was estimated in the recovered spleen. The results are expressed as $\log_{10}$ CFU/spleen. Each data point represents the mean of four animals.*$p < .05$. ns, non-significant, "PI" stands for propidium iodide.

lysis system has the potential to elicit efficient HA-specific systemic antibody responses in mice. The intestinal lavage sIgA responses exhibited a similar pattern of increase in titers following immunization, albeit, JOL1893 and JOL1837 immunized groups showed comparable sIgA responses (Fig. 4B).

The functional activities of the sera from the vaccinated mice were further investigated by determining the H1 titers against the influenza virus strain A Puerto Rico/8/34 (H1N1). Consistent with our results of the serum IgG and IgA responses, mice immunized with either JOL1893 or JOL1837 displayed significantly higher ($p < .01$) H1 titers as compared to the control JOL1822 and PBS groups (Fig. 4C). Although JOL1893 immunized displayed higher H1 titers compared to the JOL1837 immunized mice, but there was no statistically significant difference between the two immunized groups.

3.4. JOL1893 vaccination induces efficient IFN-γ recall responses

To investigate the ability of immunized splenocytes to respond to a recall HA antigen in the context of induction of IFN-γ mRNA transcription, splenocytes isolated from vaccinated mice ($n = 4$ per group) 14 days after immunization were stimulated with HA protein (10 μg/ml) for 24 h and then RNA was isolated for quantification IFN-γ mRNA level inductions by qRT-PCR assay. After stimulation with HA protein, splenocytes of JOL1837 and JOL1893 groups showed significantly higher ($p < .01$) mRNA induction levels of IFN-γ in comparison to the JOL1822 and the PBS control groups (Fig. 5). However, the IFN-γ mRNA levels were significantly higher ($p < .05$) in JOL1893 immunized splenocytes compared to the JOL1837, indicating that JOL1893 induces better recall responses and have the potential to efficiently stimulate T cell responses.

3.5. JOL1893 immunization improved clinical protection against the lethal H1N1 challenge

The benchmark of an influenza vaccine is protection against a lethal virus challenge. Therefore to evaluate the efficacy of JOL1837 and JOL1893 immunization, all vaccinated and control mice were challenged with a lethal dose of a highly pathogenic H1N1 virus at 42nd-day post-immunization. The clinical protection observed in the context of body weight and survival is shown in Fig. 6. After virus challenge, mice immunized with JOL1837 or JOL1893 showed resistance against the decrease in body weight and 90 and 100% protection was recorded against the challenge, respectively. The mice vaccinated with either JOL1822 or PBS alone, however, showed continued significant weight loss and exhibited no protection against the lethal H1N1 challenge (Fig. 6A & B).

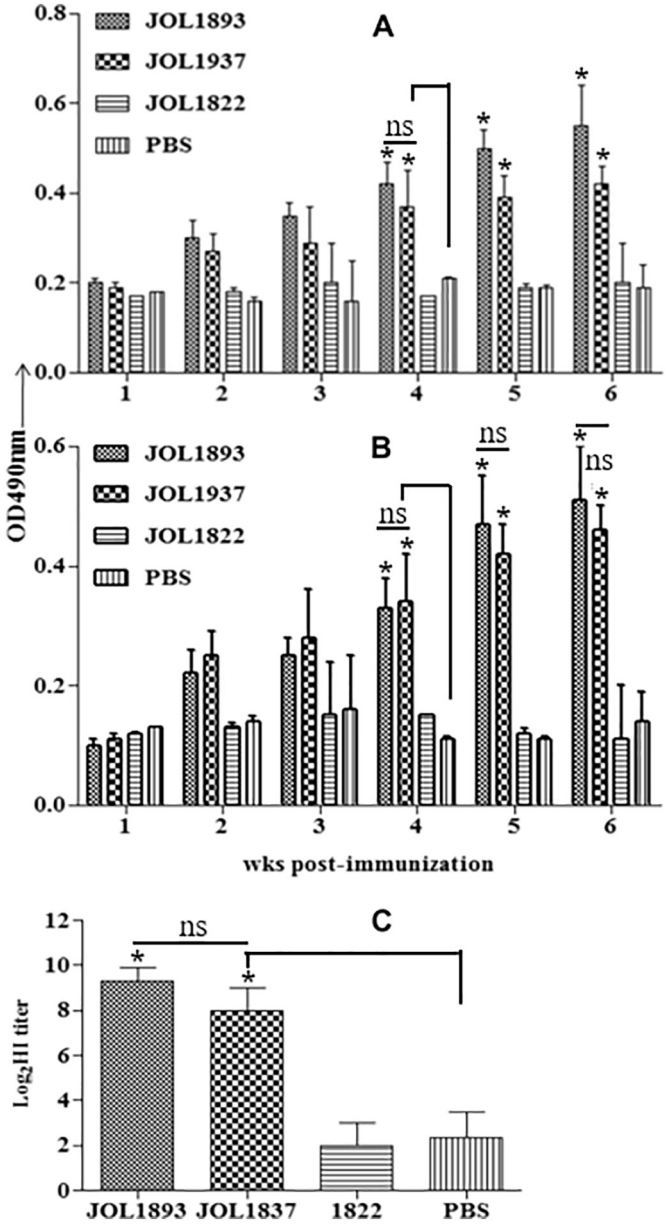

Fig. 4. Efficient humoral immune responses are stimulated in C57BL/6 mice ($n = 6$) after vaccination with JOL1893. Mice were either immunized intragastrically with the PBS only, JOL1822 carrying the empty vector pcDNA3.1, JOL1837 carrying pcDNA-HA1 plasmid or JOL1893 carrying pcDNA-HA1/pJHL187 plasmids. The IgG antibody responses and the H1 titers were measured in serum at different time points post-vaccination by indirect ELISA and HI assay, respectively. Further, sIgA responses were measured in intestinal lavage samples at different time points post-vaccination by indirect ELISA. (I) Kinetics of HA-specific IgG responses in sera from the vaccinated mice. (II) Kinetics of sIgA responses in intestinal lavage samples from vaccinated mice. (III) H1 titers at day 42 post-immunization. The assays are performed in duplicate and the data are presented as mean ± SD. $^{**}p < .05$.

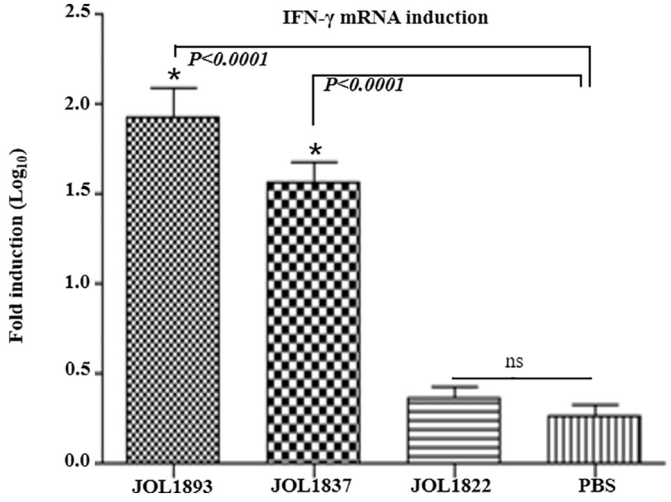

Fig. 5. Analysis of IFN-γ cytokine gene expression in splenocytes. Mice were either immunized intragastrically with the PBS only, JOL1822 carrying the empty vector pcDNA3.1, JOL1837 carrying pcDNA-HA1 plasmid or JOL1893 carrying pcDNA-HA1/pJHL187 lysis plasmid. Splenocytes (2×10^6, n = 4) were harvested from immunized mice after 14 days post-immunization and restimulated with HA antigen *in vitro* (10 μg/ml) for 24 h. Then the total RNA was extracted and gene transcription for IFN-γ was quantified by qRT-PCR assay. β-actin was used as an internal control and mRNA levels at 0 h were used as calibrator. Histograms represent mean cytokine levels and bars represent SD. $^{*}p < .05$. ns; indicates non significance.

4. Discussion

Despite numerous advantages, inefficient intracellular delivery of DNA vaccines by conventional immunization strategies generates poor immunogenicity. It is a considerable limitation for commercial implementation of DNA vaccine technology in human and veterinary fields. To overcome such impediment, in recent years intracellular bacteria based platforms have been emerged with encouraging outcomes (Bauer et al., 2005; Gahan et al., 2007)(Daudel et al., 2007). In the present scenario, we have developed a conditional *Salmonella* lysis strategy to further enhance plasmid DNA delivery for *in vivo* utilization. To incorporate an active cell lysis strategy into the *Salmonella* live vaccine system, here we have utilized a temperature inducible activation of phage lysis gene *E*. The divergent promoter system found in lysis plasmid pJHL187 (Fig. 1) prevents activation of gene *E* under permissible temperatures < 30 °C and with 2% arabinose containing culture conditions. Uplift of temperatures above 30 °C (ideally 37–42 °C) and absence of arabinose activate lysis gene *E* resulting lysis of the host *Salmonella* by promoting the release of the plasmid cargo into the host eukaryotic cell. Our results indicate, that the lysis process initiates as early as 1 h under nonpermissible conditions (> 30 °C. without arabinose) (Fig. 3A) and approximately two log reduction in bacterial numbers could be observed in 8 h of incubation under *in vitro* conditions (Fig. 3B). We further observed a significant reduction in bacterial numbers within the cultured eukaryotic cells as well as under *in vivo* conditions in mice model. Hundred percent lysis of bacteria could be observed in 30 h incubation period under nonpermissible conditions with 37 °C, without arabinose. Due to the fact that average body temperature of mice is around 35 °C and with conditions lacking arabinose could promote lysis of *Salmonella* carrier strain that may release the plasmid cargo into the host within about 8 h post inoculation. In addition, the mutations incorporated into the host bacterium, *lon* and *cpxR* significantly attenuate *Salmonella* enabling us to delivery at high dose (10^8 CFU/mice) *via* the intra gastric route. After inoculation, no mice undergo server sickness or death demonstrating the suitability as a vaccine candidate.

To explore the differences between the lysis enabled strain JOL1893 and the conventional JOL1837 strain, the immunogenicity, and protective efficacy parameters were investigated in immunized mice. Earlier studies show that inefficient intracellular delivery of DNA plasmid into the eukaryotic cells, particularly APCs, leads to low expression levels and consequently sub-optimal immune responses (Bauer et al., 2005). In this study, we observed that the DNA vaccine delivered through the lysis enabled JOL1893 strain produced significantly higher HA-specific humoral immune responses compared to the conventional JOL1837 *Salmonella*-mediated delivery of DNA vaccine. We also

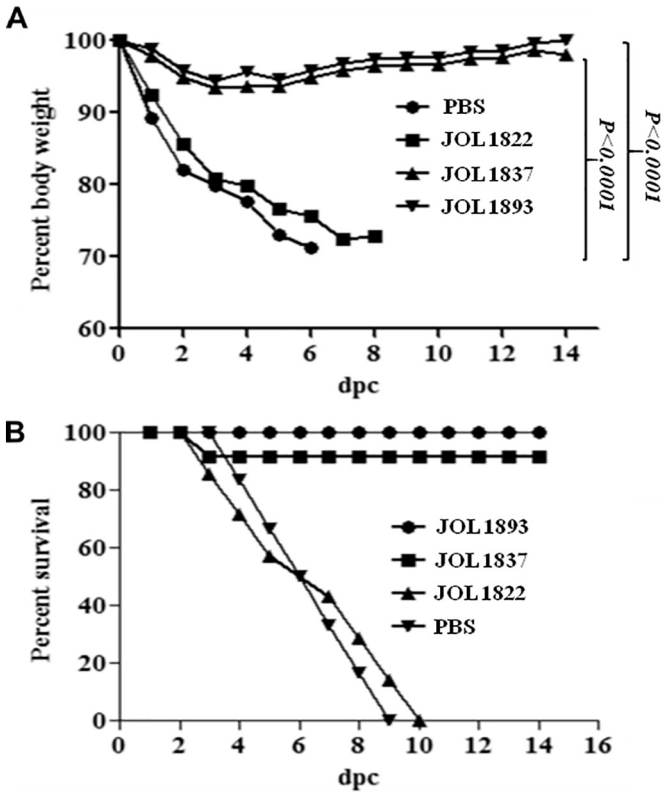

Fig. 6. Immune protection of mice against lethal influenza A/Puerto Rico/8/34 (H1N1) challenge ($n = 10$ per group). Mice were either immunized intragastrically with the PBS only, JOL1822 carrying the empty vector pcDNA3.1, JOL1837 carrying pcDNA-HA1 plasmid or JOL1893 carrying pcDNA-HA1/pJHL187 lysis plasmid. Mice were then challenged intranasally with 10^6EID50 of lethal H1N1 virus at 6 weeks after primary immunization. Mice were monitored for weight loss (I) and survival (II) throughout a 14 day observation period. The results are presented in terms of percent of body weight and percent survival, respectively.

observed higher cell-mediated immune responses in case of JOL1893 immunized mice. Our data demonstrate that there is an increment in IFN-γ expressions by HA restimulated splenocytes (Fig. 5). IFN-γ is a prototypic Th1-polarizing cytokine and is critical in the development of cellular immune responses, especially cytotoxic CD8+ responses, which are effective in clearance of viral infections (McMichael et al., 1983; Mills, 2008). The CD8+ T cell responses are essential to clear influenza viruses from the lungs and, having their broad antigen-specificity, can reduce disease symptoms caused by a potential pandemic influenza A virus outbreak (McMichael et al., 1983; Sridhar et al., 2013). A strong correlation could be observed between productive virus infection and cross-reactive cellular immunity against subsequent infection with heterologous influenza viruses based on the studies involving humans and other host-species (Kreijtz et al., 2007; Sridhar et al., 2013). DNA vaccines are particularly capable of inducing cellular immune responses essential for virus clearance, thus, enhanced delivery of plasmid DNA observed in JOL1893 should be advantageous over the conventional JOL1837 strain. According to our findings lysis strain JOL1893 resulted maximum protection. However the conventional strain JOL1837 also received comparable protection level against the lethal challenge. This observation may possibly occur due to *Salmonella* undergoing early lysis under *in vivo* condition. However the phenomenon needs further investigation.

A possible explanation for the observed augmentation in the humoral and cellular immune response to JOL1893 might lie in the fact that lysis enabled *Salmonella* increased the intracellular delivery of the DNA vaccine construct inside the mammalian cells and consequently, the improved DNA expression and immunogenicity (Tsen et al., 2007). The conventional intramuscular route of DNA immunization suffers from the drawback that muscle cells have a low number of APCs. In addition, DNA vaccine is exposed to the host immune responses that might increase chances of development of immunity against the DNA vaccine itself leading to poor immunogenicity of the vaccine (McCluskie et al., 1999). Contrary to the intramuscular injection, the *Salmonella* can infect and multiply inside APCs, thus increasing the possibility of DNA vaccine delivery and evade from the host immune response (Cheminay et al., 2016). In addition, due to mucosal nature of the *Salmonella*, the *Salmonella* mediated DNA vaccine delivery vehicle can easily be administered *via* mucosal route and have the potential to elicit mucosal immune responses (Abdul-Wahid and Faubert, 2007). Overall, the delivery of the pcDNA-HA1 along with the pJHL187 in JOL1893 produced significantly higher immune responses compared to the JOL1837 vaccine construct.

Taken together, our results indicate that mucosal immunization with gene *E* induced conditionally lysis enabled live attenuated *S.* Typhimurium as a DNA vaccine carrier can induce potent systemic and mucosal immune responses and effective immune protection against a highly pathogenic H1N1 infection in mice model. The strategy of employing conditionally lysis *Salmonella* system to deliver DNA vaccines holds a great promise, and further studies are warranted to test this method in other target species.

5. Conclusion

In the present study, we have built a temperature induced lysis strategy into *Salmonella* by utilizing bacteriophage PhiX174 gene *E.* The lysis gene *E* encoding pJHL187 plasmid demonstrated effective lysis even at suboptimal temperatures enhancing the immunogenicity of the DNA vaccine. Present *Salmonella* lysis system effectively induced protective immune responses in mice and provide significant protection against H1N1 experimental infection in mice. Further experiments can be accompanied to increase the lysis efficacy and antigenicity of the *Salmonella* system that brings protection against other strains of influenza viruses.

Competing interests

The authors declare that they have no competing interests.

Author contributions

IAH, AS, JHL, and NMK designed and performed the experiment. IAH and AS wrote the manuscript. JHL did the critical revision of the manuscript.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education (2019R1A6A1A03033084).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.008.

References

Abdul-Wahid, A., Faubert, G., 2007. Mucosal delivery of a transmission-blocking DNA vaccine encoding Giardia lamblia CWP2 by Salmonella typhimurium bactofection vehicle. Vaccine 25, 8372–8383. https://doi.org/10.1016/j.vaccine.2007.10.012.

Bauer, H., Darji, A., Chakraborty, T., Weiss, S., 2005. Salmonella-mediated oral DNA vaccination using stabilized eukaryotic expression plasmids. Gene Ther. https://doi.org/10.1038/sj.gt.3302423.

Beran, J., Abdel-Messih, I.A., Raupachova, J., Hobzova, L., Fragapane, E., 2010. A phase III, randomized, open-label study to assess the tolerability and immunogenicity of an H5N1 influenza vaccine administered to healthy adults with a 1-, 2-, 3-, or 6-week interval between first and second doses. Clin. Ther. https://doi.org/10.1016/S0149-2918(11)00024-5.

Bernhardt, T.G., Roof, W.D., Young, R., 2002. Genetic evidence that the bacteriophage phi X174 lysis protein inhibits cell wall synthesis. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.97.8.4297.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brady, R.C., Treanor, J.J., Atmar, R.L., Keitel, W.A., Edelman, R., Chen, W.H., Winokur, P., Belshe, R., Graham, I.L., Noah, D.L., Guo, K., Hill, H., 2009. Safety and immunogenicity of a subvirion inactivated influenza A/H5N1 vaccine with or without aluminum hydroxide among healthy elderly adults. Vaccine. https://doi.org/10.1016/j.vaccine.2009.06.057.

Chaudhari, A.A., Jawale, C.V., Kim, S.W., Lee, J.H., 2012. Construction of a Salmonella Gallinarum ghost as a novel inactivated vaccine candidate and its protective efficacy against fowl typhoid in chickens. Vet. Res. https://doi.org/10.1186/1297-9716-43-44.

Cheminay, C., Möhlenbrink, A., Hensel, M., 2016. Presentation by dendritic cells inhibit antigen Salmonella intracellular intracellular Salmonella inhibit antigen presentation by dendritic cells. J. Immunol. Ref. 174, 2892–2899. https://doi.org/10.4049/jimmunol.174.5.2892.

Chen, M.-W., Yu, A.L., Wong, C.-H., Cheng, T.-J.R., Ho, D.D., Jan, J.-T., Ma, S.-H., Huang, Y., 2008. A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.0806901105.

Curtiss, R., Wanda, S.-Y., Gunn, B.M., Zhang, X., Tinge, S.A., Ananthnarayan, V., Mo, H., Wang, S., Kong, W., Kong, W., 2009. Salmonella enterica serovar Typhimurium strains with regulated delayed attenuation in vivo. Infect. Immun. 77, 1071–1082. https://doi.org/10.1128/IAI.00693-08.

Daudel, D., Weidinger, G., Spreng, S., 2007. Use of attenuated bacteria as delivery vectors for DNA vaccines. Expert Rev. Vaccines 6, 97–110. https://doi.org/10.1586/14760584.6.1.97.

Gahan, M.E., Webster, D.E., Wesselingh, S.L., Strugnell, R.A., 2007. Impact of plasmid stability on oral DNA delivery by Salmonella enterica serovar Typhimurium. Vaccine. https://doi.org/10.1016/j.vaccine.2006.10.042.

Hajam, I.A., Dar, P.A., Appavoo, E., Kishore, S., Bhanuprakash, V., Ganesh, K., 2015. Bacterial ghosts of Escherichia coli drive efficient maturation of bovine monocyte-derived dendritic cells. PLoS One. https://doi.org/10.1371/journal.pone.0144397.

Huleatt, J.W., Nakaar, V., Desai, P., Huang, Y., Hewitt, D., Jacobs, A., Tang, J., McDonald, W., Song, L., Evans, R.K., Umlauf, S., Tussey, L., Powell, T.J., 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 26, 201–214. https://doi.org/10.1016/j.vaccine.2007.10.062.

Hur, J., Lee, J.H., 2011a. Enhancement of immune responses by an attenuated Salmonella enterica serovar typhimurium strain secreting an Escherichia coli heat-labile enterotoxin B subunit protein as an adjuvant for a live Salmonella vaccine candidate. Clin. Vaccine Immunol. https://doi.org/10.1128/CVI.00407-10.

Hur, Jin, Lee, John Hwa, 2011b. Enhancement of immune responses by an attenuated Salmonella enterica Serovar typhimurium strain secreting an Escherichia coli heat-labile enterotoxin B subunit protein as an adjuvant for a live Salmonella vaccine candidate. Clin. Vaccine Immunol. 18, 203–209.

Job, E.R., Ysenbaert, T., Smet, A., Christopoulou, I., Strugnell, T., Oloo, E.O., Oomen, R.P., Kleanthous, H., Vogel, T.U., Saelens, X., 2018. Broadened immunity against influenza by vaccination with computationally designed influenza virus N1 neuraminidase constructs. npj Vaccines. https://doi.org/10.1038/s41541-018-0093-1.

Jorritsma, S.H.T., Gowans, E.J., Grubor-Bauk, B., Wijesundara, D.K., 2016. Delivery methods to increase cellular uptake and immunogenicity of DNA vaccines. Vaccine. https://doi.org/10.1016/j.vaccine.2016.09.062.

Kamble, N.M., Hajam, I.A., Lee, J.H., 2017. Orally administered live attenuated Salmonella Typhimurium protects mice against lethal infection with H1N1 influenza virus. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2017.01.006.

Koday, M.T., Leonard, J.A., Munson, P., Forero, A., Koday, M., Bratt, D.L., Fuller, J.T., Murnane, R., Qin, S., Reinhart, T.A., Duus, K., Messaoudi, I., Hartman, A.L., Stefano-Cole, K., Morrison, J., Katze, M.G., Fuller, D.H., 2017. Multigenic DNA vaccine induces protective cross-reactive T cell responses against heterologous influenza virus in nonhuman primates. PLoS One. https://doi.org/10.1371/journal.pone.0189780.

Krammer, F., Pica, N., Hai, R., Margine, I., Palese, P., 2013. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J. Virol. https://doi.org/10.1128/JVI.00641-13.

Kreijtz, J.H.C.M., Bodewes, R., van Amerongen, G., Kuiken, T., Fouchier, R.A.M., Osterhaus, A.D.M.E., Rimmelzwaan, G.F., 2007. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine 25, 612–620. https://doi.org/10.1016/j.vaccine.2006.08.036.

Laddy, D.J., Yan, J., Kutzler, M., Kobasa, D., Kobinger, G.P., Khan, A.S., Greenhouse, J., Sardesai, N.Y., Draghia-Akli, R., Weiner, D.B., 2008. Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. PLoS One. https://doi.org/10.1371/journal.pone.0002517.

Lambert, L., Kinnear, E., McDonald, J.U., Grodeland, G., Bogen, B., Stubsrud, E., Lindeberg, M.M., Fredriksen, A.B., Tregoning, J.S., 2016. DNA vaccines encoding antigen targeted to MHC class II induce influenza-specific CD8+ T cell responses, enabling faster resolution of influenza disease. Front. Immunol. https://doi.org/10.3389/fimmu.2016.00321.

Ledgerwood, J.E., Hu, Z., Gordon, I.J., Yamshchikov, G., Enama, M.E., Plummer, S., Bailer, R., Pearce, M.B., Tumpey, T.M., Koup, R.A., Mascola, J.R., Nabel, G.J., Graham, B.S., 2012. Influenza virus H5 DNA vaccination is immunogenic by intramuscular and intradermal routes in humans. Clin. Vaccine Immunol. https://doi.org/10.1128/CVI.05663-11.

Lee, S.-Y., Kang, J.-O., Chang, J., 2019. Nucleoprotein vaccine induces cross-protective cytotoxic T lymphocytes against both lineages of influenza B virus. Clin. Exp. Vaccine Res. https://doi.org/10.7774/cevr.2019.8.1.54.

McCluskie, M.J., Brazolot Millan, C.L., Gramzinski, R.A., Robinson, H.L., Santoro, J.C., Fuller, J.T., Widera, G., Haynes, J.R., Purcell, R.H., Davis, H.L., 1999. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Mol. Med. 5, 287–300.

McMichael, A.J., Gotch, F.M., Noble, G.R., Beare, P.A.S., 1983. Cytotoxic T-cell immunity to influenza. N. Engl. J. Med. 309, 13–17. https://doi.org/10.1056/NEJM198307073090103.

Mills, K.H.G., 2008. Induction, function, and regulation of IL-17-producing T cells. Eur. J. Immunol. https://doi.org/10.1002/eji.200838535.

Nicholson, Karl G., Wood, John M., Zambon, Maria, 2003. Influenza. Lancet 362, 733–745. https://doi.org/10.1016/S0140-6736(03)14854-4.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 45e–45. https://doi.org/10.1093/nar/29.9.e45.

Rajapaksha, H., Petrovsky, N., 2014. In silico structural homology modeling and docking for assessment of pandemic potential of a novel H7N9 influenza virus and its ability to be neutralized by existing anti-hemagglutinin antibodies. PLoS One. https://doi.org/10.1371/journal.pone.0102618.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Epidemiol. 27, 493–497.

Ryea, J., Zangwill, K.M., Treanor, J.J., Noah, D.L., Campbell, J.D., 2008. Evaluation of the safety and immunogenicity of a booster (third) dose of inactivated Subvirion H5N1 influenza vaccine in humans. J. Infect. Dis. https://doi.org/10.1086/526537.

Soema, P.C., Kompier, R., Amorij, J.P., Kersten, G.F.A., 2015. Current and next generation influenza vaccines: formulation and production strategies. Eur. J. Pharm. Biopharm. https://doi.org/10.1016/j.ejpb.2015.05.023.

Sridhar, S., Begom, S., Bermingham, A., Hoschler, K., Adamson, W., Carman, W., Bean, T., Barclay, W., Deeks, J.J., Lalvani, A., 2013. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat. Med. 19, 1305–1312. https://doi.org/10.1038/nm.3350.

Stachyra, A., Góra-Sochacka, A., Sirko, A., 2014. DNA vaccines against influenza. Acta Biochim. Pol. 61 (3), 515–522.

Tompkins, S.M., Zhao, Z.S., Lo, C.Y., Misplon, J.A., Liu, T., Ye, Z., Hogan, R.J., Wu, Z., Benton, K.A., Tumpey, T.M., Epstein, S.L., 2007. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg. Infect. Dis. https://doi.org/10.3201/eid1303.061125.

Tsen, S.-W.D., Paik, A.H., Hung, C.-F., Wu, T.-C., 2007. Enhancing DNA vaccine potency by modifying the properties of antigen-presenting cells. Expert Rev. Vaccines 6, 227–239. https://doi.org/10.1586/14760584.6.2.227.

Won, G., Lee, J.H., 2016. Multifaceted immune responses and protective efficacy elicited by a recombinant autolyzed Salmonella expressing FliC flagellar antigen of F18+Escherichia coli. Vaccine 34, 6335–6342. https://doi.org/10.1016/j.vaccine.2016.10.066.

Yang, M., Wu, T.C., Janet, B.L., Mark, S.W., Hong, S.L., Hung, C.-F., 2006. Modifying Professional Antigen-Presenting Cells to Enhance DNA Vaccine Potency, in: DNA Vaccines. https://doi.org/10.1385/1-59745-168-1:199.

Yin, J., Cheng, Z., Xu, L., Li, Q., Geng, S., Pan, Z., Jiao, X., 2015. Immunogenicity and protective efficacy of Salmonella enterica serovar Pullorum pathogenicity island 2 mutant as a live attenuated vaccine candidate. BMC Vet. Res. 11, 162. https://doi.org/10.1186/s12917-015-0497-3.