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Immunogenicity and Protective Efficacy of a Attenuated Salmonella-Vectored Vaccine Expressing Esat6 Against Tuberculosis in Mice

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Abstract: It is a need to develop protective vaccines against Tuberculosis (TB) that elicit full immune responses including mucosal immunity. Here an attenuated Salmonella-vectored vaccine expressing Esat6 of the *Mycobacterium tuberculosis* (Mtb) were constructed. These vaccine were assessed for their immunological efficacy on Specific Pathogen-Free (SPF) mice challenged with Mtb. The experimental data demonstrated that this SL(E6) vaccine and BCG could induce the stronger TB Ag-specific mucosal, humoral and cellular immune responses and exerted high protective efficacy in mice against virulent Mtb H37Rv challenge compared to the other vaccinated groups (mice immunized with Esat6, a DNA vaccine or BCG only). These data may represent a novel promising Salmonella vaccine candidate for the prevention of TB.

Key words: *Mycobacterium tuberculosis*, Salmonella-vectored vaccine, Esat6, efficacy, cellular immunity

INTRODUCTION

Tuberculosis (TB) is still one of the most important infectious diseases leading to 2 million deaths and 8 million new cases annually which is caused by an intracellular bacterium *Mycobacterium tuberculosis* (Sala and Hartkoorn, 2011; Golub *et al*., 2008). Especially the advent of *M. tuberculosis* and Human Immunodeficiency Virus (HIV) copandemic and the emergence of multidrug-resistant TB make the TB situation even worse. Although, the current TB vaccine, *Mycobacterium bovis* BCG has been widely used for decades its efficacy has been shown to be highly variable in several well-controlled clinical trials (Kuo *et al*., 2012). While BCG immunization is generally protective against miliary and menigial TB in children the inadequacy of BCG results primarily from its inability to protect against primary lung infections including the most prevalent form of the disease, adult pulmonary TB (Colditz *et al*., 1994; Fine, 2001). These data indicate that the BCG vaccine lacks the immunogenicity required to generate protective immunity in the immunized adult populations. Thus, the development of safe, efficacious and live TB vaccines that can confer potent protection in control TB field.

Comparative genomic analyses have identified >100 coding sequences that are missing from BCG but are present in *Mycobacterium tuberculosis* (Mtb) (Behr *et al*., 1999) including the 6 kDa early secreted antigenic target Antigen (Ag) (Esat6) protein which has been shown to be a protective Ag in several animal studies (Brandt *et al*., 1996; Pym *et al*., 2003). ESAT-6 and MPB64 have been identified as two of the most promising vaccine candidates which are strongly recognized by T-lymphocytes. There are many potential vaccines which have been introduced these two antigens and one of them is recombinant protein vaccine ESAT-6 which has entered into clinical trial (Brandt *et al*., 2000; Olsen *et al*., 2001).

Oral vaccination with live attenuated Salmonella vectors can result in the generation of both Salmonella and heterologous antigen specific humoral and cellular immune responses, normally biased towards TH1 which can be used both as amore effective typhoid vaccine and for delivery of heterologous antigens (Khan *et al*., 2007; Kirkpatrick *et al*., 2006). Therefore, an orally delivered and attenuated *S. typhimurium* vaccine strains, SL(ESAAT6), that harbor the Mtb H37Rv *Esat6* gene carried by a plasmid pVAX1, respectively were constructed in this study. Comparisons and analysis of the immuneresponses and protective efficacy of the SL(E6) vaccines the DNA vaccine pVAX1-Esat6 (pV-E6) and the BCG vaccine against Mtb were assasyed.

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MATERIALS AND METHODS

Bacterial strains, animal: *S. typhimurium* 7027 which harbours an attenuating mutation in the *aroA* gene was used as the base vector for all live vaccine studies and was routinely grown in LB-broth supplemented with L-phenylalanine, L-tryptophan, ltyrosine (40 μ gmL^{-1} each) along with p-aminobenzoic acid and 2,3-dihydroxybenzoic acid (10 μg mL^{-1}).

Pathogen-free C57BL/6 female mice were obtained from the Inner Mongolia Medical University. The mice were maintained under barrier conditions and fed commercial mouse chow and water *ad libitum*. The mice were 6-8 weeks old at the time of the vaccinations.

Construction of plasmids and transformation into SL7027: The entire *Esat6* gene sequence from Mtb H37Rv (Beijing Biological Product Institute) chromosome DNA was amplified by PCR and subcloned into the eukaryotic expression vector pVAX1 (Invitrogen, USA). The primer sequences were (forward) 5'-GCAAGCTTATGACAGAGCAGCAGTGGAA-3'and (reverse) 5'-TTCCTAGGTGCGAACATCCCAGTGACGT-3' where the underlined oligonucleotides represent HindIII and BamHI sites, respectively to facilitate cloning. The amplified products were purified with the Quiaex II Gel Extraction kit (German), digested with BamHI and HindIII and ligated into the vector pVAX1 which was digested by BamHI and HindIII. The plasmids pVAX1-Esat6 (also called pV-E6) were confirmed by DNA sequence analysis.

The eukaryotic expression plasmids pV-E6 was electrotransformed into the attenuated *S. typhimurium* strain aroA SL7207 via the *S. typhimurium*-modifying strain LB5000 (r-m+) with ApR selection. Then, the attenuated Salmonella recombinant vaccine strains and SL(E6) were generated and confirmed by PCR.

Immunization: One hundred twenty, 7-8 weeks old female BALB/C mice were divided into six groups (n = 20). A groups of mice were orogastrically (o.g.) immunized with the attenuated Salmonella vaccine strains SL(E6) and the parental bacterium strain SL7207 by placing 100 μL of vaccine suspension containing 10^7 CFU into the lower esophagus using a gavage needle on days 0, 14 and 28. Another one DNA vaccination groups of mice that were preinjected with 10 μL 2% lidocaine were intramuscularly immunized with 100 μg DNA plasmid pV-E6 or the empty vector control pVAX1 per mouse on days 0, 14 and 28 by gene gun with an Electric Square Porator (Scientz Biotechnology). The BCG control group was vaccinated with 5×10^6 CFU of BCG Subcutaneously (SC) and a negative control group received equal volumes of subcutaneously administered phosphate-buffered PBS.

Evaluation of antibody responses by ELISA: Serum samples from mice post-immunisation were analysed for the presence of total IgG or IgA 2 weeks after each immunization using an ELISA Method. Briefly, ELISA plates were coated with recombinant Esat6 protein (5 μg^{-1} well) overnight at 4°C. Free binding sites were blocked by 1% Bovine Serum Albumin (PBS). Individual serum or tissues samples from immunized mice were analyzed in threefold dilutions. Horseradish Peroxidase (HRP)-conjugated rabbit anti-mouse IgG (DAKO) or IgA (DAKO) were added and incubation proceeded for 1 h at 37°C. The plate was then developed with substrate buffer. After 30 min of incubation at room temperature the reaction was stopped by adding 0.2 mol L^{-1} H_2SO_4 and absorbance was measured at 450 nm using a microplate reader. Titers are shown as the sample dilution resulting in an OD450 equal to twice the mean background of the assay.

Evaluation of IFN-γ responses by ELISPOT: The 2 weeks after the final immunization, mice were sacrificed. IFN-γ producing cells from splenocytes of immunized or control mice were quantified by a cytokine-specific Enzyme Linked Immunospot Assay (ELISPOT) kit (U-Cytech). In short, 96 well nitrocellulose-backed plates were coated with 100 μg of antimouse IFN-γ McAb overnight at 4°C the then washed with PBS (pH = 7.2) and blocked for1 h with 5% BSA. The splenocytes (2×10^5/well) were stimulated with Esat6 (5 μg mL^{-1}) or RPMI-1640 alone (negative control) in triplicate wells at 37°C thefor 16 h. Then, the cells were washed with PBS and incubated with biotinylated rabbit anti-IFN-γ antibodies for 1 h at room temperature followed by adding a streptavidin-HRP conjugate. Spots representing individual cytokine-producing cells were visualized using the HRP substrate and spots were then counted using an immunospot image analyzer.

Experimental infections: The 2 weeks after the final immunization, mice were divided into six groups (n = 6 each group) and were infected (i.v.) via the lateral tail vein or intransally (i.n.) with an inoculum of 5×10^6 CFU of Mtb H37Rv suspended in 0.1 mL PBS (Chen *et al*., 2007).

Statistical analysis: All the experiments were repeated three times. Data were statistically analyzed by one-way ANOVA followed by Tukey's test. The $p<0.05$ were considered to be statistically significant.

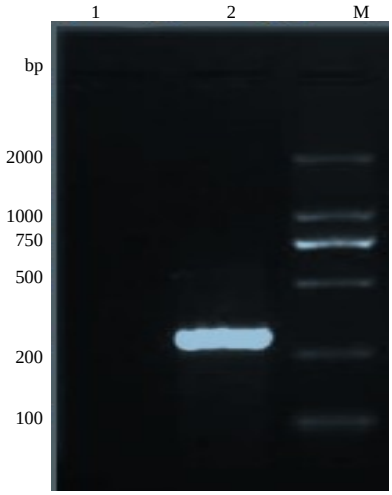

Fig. 1: Analysis of plasmid constructs in Salmonella. PCR analysis of recombinant plasmids pVAX1-Esat6, transformed to Salmonella (Lane 2); Lane 1 = Negative control; M = Marke

RESULTS

Construction of recombinant salmonella vaccine strains: To construct the recombinant plasmids the DNA fragment of the entire *Esat6* gene sequence from Mtb H37Rv was amplified and cloned into the eukaryotic expression vector pVAX-1. The recombinant Salmonella vaccine strain harboring the *Esat6* gene, transformed into the attenuated Salmonella strain (SL7207) also was itentified by PCR analysis. It was found that showed that the relative Molecular mass (Mr) of inserted DNA fragment was identical to the value predicted (Fig. 1).

Humoral and mucosal immuneresponses: Every 2 weeks after the immunization, levels of Esat6-specific IgG in the sera of immunized mice were determined by ELISA. Titers of Esat6-specific IgG increased in each immunized group as the number of immunizations increased (Fig. 2). After the third immunization the DNA vaccine group, recombinant Salmonella vaccine grop and BCG group had higher levels of IgG antibody production compared to the parental strain (SL7207) the empty vector group (pVAX-1) and the non-immunized mice (PBS controls) (Fig. 2 *$p<0.05$). In addition the antibody titers were similar in the SL(E6) group and the BCG group which induced the strongest IgG antibody responses among all groups.

To observe the mucosal immuneresponses against recombinant Salmonella vaccine strains, titers of Esat6 specific mucosal secretory IgA (SIgA) antibodies from the stomach, intestine and lung tissues of immunized mice

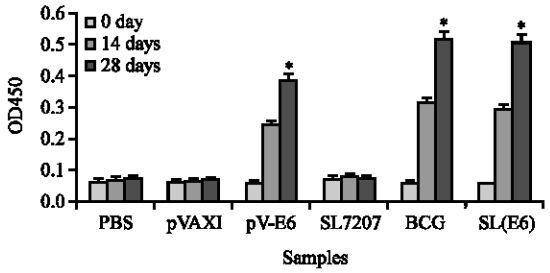

Fig. 2: Determination of Esat6-specific IgG responses of immunized mice. Total serum Esat6-specific IgG responses of immunized mice. Mice were injected i.m. with DNA vaccine pV-Esat6 or o.g. with SL(E6). The immunization was performed on days 0, 14 and 28 and Esat6-specific IgG titers were assayed by ELISA 2 weeks after each immunization. Six mice were used in each group. Data points represent means±SEM. *$p<0.05$ vs. parental vector control groups

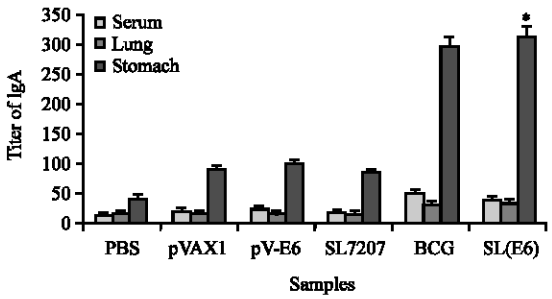

Fig. 3: Titers of IgA in different tissue.*$p<0.05$ vs. the DNA vaccine and the parental vector control groups

were determined by ELISA. Mice immunized with SL(E6) elicited much higher levels of gastric and lung SIgA and serum IgA than BCG the DNA vaccine and the parental vector control groups (Fig. 3).

Cellular immune reaction: The production of IFN-γ by splenocytes was detected for all immunized mouse groups by the ELISPOT technique. After stimulation with the Esat6 protein the numbers of IFN-γ producing cells in the SL(E6B) group the BCG group were significantly greater than the other group (Fig. 4). All of the vaccine groups had higher numbers of IFN-γ-producing cells than the parental SL7207 strain group the parental vector pVAX1 group and PBS group (Fig. 4, *$p<0.05$). The results indicated that recombinant Salmonella vaccines induced a Th1-biased immune response.

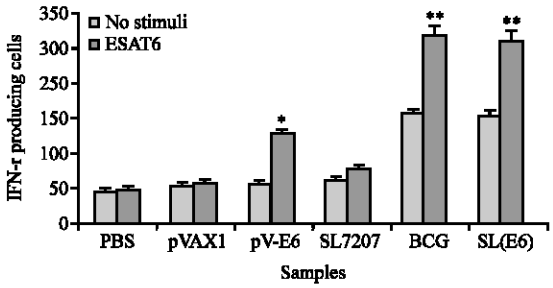

Fig. 4: Esat6-specific IFN-γ producing assay. Each group of mice (n = 6) was immunized three times and cytokine production was assayed by ELISPOT 2 weeks after the final immunization. IFN-γ producing cells per 10^6 splenocytes. *p<0.05, **p<0.01 vs. PBS group and parental vector control groups

Table 1: Comparisons of various vaccines-induced protection efficacy in mice infected with H37Rv. T50: time when 50% mortality

Groups	T_{50} (days)	Mortality (%) (50 days)	Samples
SL(E6)	>60	14.00	20
BCG	>60	19.00	20
PV-E6	39	33.33	20
pVAX 1	32	75.00	20
PBS	27	70.00	20
SL7027	30	80.00	20

Salmonella vaccines protective immunity against Mtb H37Rv infection: Challenge experiments were performed to evaluate whether the strong immune responses induced in mice would confer protection against *M. tuberculosis* challenge. The SL(E6B) group and the BCG group had longer 50% death times (T_{50}) and lower death mice compared to the DNA vaccine Esat6 group the pVAX1 group and PBS group (Table 1). This indicated that Salmonella vaccines can induce stronger protective immunity against Mtb than DNA vaccine.

DISCUSSION

As a delivery system, live attenuated Salmonella vaccines are capable of eliciting an immune response to guest antigens from bacteria, parasites and viruses (Khan *et al*., 2007; Tacket *et al*., 2000). As bacterial vectors, both *S. typhi* and *S. typhimurium* have been extensively studied in both animal models and humans (Angelakopoulos and Hohmann, 2000; Sirard *et al*., 1999). In addition, immunisation at one mucosal inductive site can lead to an immune response at another, anatomically remote, mucosal effector site (e.g., lungs) due to the subnetworks that exist within the mucosal immune system (Allen *et al*., 2000; Challacombe *et al*., 1997; Ruedl *et al*., 1994). Therefore, researchers have constructed recombinant Salmonella vaccine strains and evaluated their efficacy in this study.

Several studies have indicated that both oral and intravenous administration of recombinant Salmonella strains may be efficient routes of immunisation for a vaccine against tuberculosis. Earlier, Hess and colleagues used *S. typhimurium* to express and secrete the *M. tuberculosis* protein Ag85B. They transformed SL7027 with a plasmid encoding the HlyB/HlyD/TolC export machinery from (*E. coli*) and the *Ag85B* gene. Those mice orally vaccinated with the recombinant Salmonella strain were offered partial protection after intravenous (i.v.) challenge with *M. tuberculosis* (Hess *et al*., 2000). A further study also used *S. typhimurium* as a vector to export another *M. tuberculosis* antigen, Esat6, via the Haemolysin (HlyA) secretion system. Mice immunised intravenously with this Salmonella vaccine expressing Esat6 were found to have reduced numbers of tubercle bacilli in the lungs throughout the course of Mtb infection (Mollenkopf *et al*., 2001) which was complied with the results However, plasmid-based systems of this stuty have inherent problems with stability and antibiotic resistance genes are discouraged for incorporation into live vectors for approval for licensed clinical use (Bowe *et al*., 2003). In the present study, researcher cloned the tuberculosis fusion antigen Esat6 into the chromosomal *phoN* gene of SL7027. The *Esat6* gene is under the constitutive control of the lacZ promoter with strong Esat6 production readily detectable under *in vitro* growth conditions. So, this expression system would hopefully be a more realistic basis for an efficient Salmonella delivery system of Esat6 for use in humans based upon a *S. typhi* construct.

In the present study, researchers generated a recombinant Salmonella vaccine strains, SL(E6) which harbor the Mtb *Esat6* gene. The data strongly suggest that a novel recombinant Salmonella vaccine strain can protect against M.tb infection. Mice vaccined with SL(E6) contained high titers of East6 specific lgG in their serum and SlgaA in their stomach, intestine and indicating that vaccination with SL(E6) induced both a local and a systemic immune response which was compiled with previous results (Hess *et al*., 2000).

CONCLUSION

The results demonstrate that the most effective recombinant Salmonella vaccine strain in the study, SL(E6) would be a novel vaccine candidate for the prevention of TB.

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