8. In- Vivo studies

Animal models of *Mycobacterium tuberculosis* infection are used to predict if new candidate vaccines have any potential for safe and efficacious use in human beings. Animal model resemble one or other way to host response to disease and changes in immuno-pathology during disease progress and hence, animal studies results can be extrapolated to humans. The major advantages of using animal models for preliminary screening of vaccine efficacy include: these animals are easily infected by pulmonary route which is realistic route for TB infection in human, the disease progression in animals are as similar as human and various stages of disease progression similar and distinguished. The symptoms of the disease which are commonly in observed in human like fever, weight loss, granuloma formation and cavity formation, are also visible in animal models. Validation of animal models with respect to immuno-pathological response is critical aspect for selection of animal models (Orme 2005).

8.1 Protocol for animal studies for TB vaccine evaluation

Orme and Izzo (2004) reported that the peak immunity for TB in the lungs attained at around day 30 of an aerosol infection. Because of this, candidate vaccines are compared for their protective activity at this time point so as to identify those that have established a state of immunity in which the expression of acquired specific resistance has been accelerated. The protocol for animal testing mainly consider type of animal used, inclusion of number of animal groups, number of animal per group, route of vaccine administration, volume of vaccine administration, dose of vaccine, infection route, infection procedure and method of animal sacrifice and finally method to determine bacterial load in test organs.

For the better comparison of the efficacy of test vaccine and to eliminate interference of immune response due to NPs (which are also believed to be adjuvant in the vaccine formulation), we included six groups of animals. BCG vaccine was kept as positive control and administered as the recommended SC route. For all other groups, I.N. administration was done. To keep animal need at minimum though to get sufficient

information, four animals per group was decided. The outline of the groups is given in the table 8.1.

The quantity/ volume of material employed in nasal vaccines are very important. Eyles 1999 reported in his paper that when 50μL of particle suspension was administered through nasal route to the mice, a significant volume (~ 40%) entering to the lung. However, in contrast, most particles were found in nasal passage when 10 μL was administered. Larger than 50 μL volumes caused respiratory arrest in mice and survival rate decreased after administration.

The dose of vaccine to be administered through I.N. route is again a critical parameter. To determine the effective dose, we went through with various literatures (Olsen 2001, Yadav 2001, Brookes 2001, Dietrich 2005, Girard 2005, Orme 2006) where ESAT-6 and Ag-85B were used as antigens either alone or in combination for the studies and we found the range of doses used was lying in between 10μg to 50μg as one shot of vaccine or multiple shots at various time intervals. With all data, we discussed with our collaborator Eric Nuemberger, M.D., Associate Professor of Medicine, division of Infectious Disease at Centre for TB Research Laboratory, Johns Hopkins Medical Institutions, Baltimore, MD and decided 30μg of the individual antigens as the dose.

The route of microbial infection is another important criterion in the experiment design. Aerosol challenge is the preferred model for such assays, but intravenous challenge can also provide useful data. Intra-tracheal instillation is a reasonable alternative, but it involves a surgical procedure and a large bolus of fluid gets deposited in the upper bronchial tree and most does not reach the alveoli. I.N. challenge is far less efficient, because only a small percentage of the inoculum reach the lower lung. Intraperitoneal challenge is impossible to control and is not recommended (Orme 2005).
The chamber for the Aerosol infection is as shown in figure 8.1 and 8.2.

Figure 8.1 Chamber for the Aerosol infection

Figure 8.2 Chamber for the Aerosol infection

After microbial challenge with a low dose of aerosol of virulent *M. tuberculosis* mice were sacrificed at pre-determined time interval as shown in table 8.1 and infected organs (the lungs and the spleen) were collected for the evaluation of *M.Tb* infection.

Sr. No	Group of Animal study/ Title of Group	Animals Sacrifice after microbial challenge	
		Day 1	Day 28
1	Un-Treated mice	4	4
2	Adjuvanted Empty NPs	0	4
3	BCG vaccinated mice	0	4
4	Test Antigens in Pure form in PBS, Intra Nasal Route	0	4
5	Antigen conjugated- adjuvanted- NPs	0	4
6	Antigens encapsulated PLGA NPs	0	4
	Group of Animal study/ Title of Group	Animals Sacrifice after microbial challenge*	
		Day 29	Day 56
1	Un-Treated mice	4	4
2	Adjuvanted Empty NPs	0	4
3	BCG vaccinated mice	0	4
4	Test Antigens in Pure form in PBS, Intra Nasal Route	0	4
5	Antigen conjugated- adjuvanted- NPs	0	4
6	Antigens encapsulated PLGA NPs	0	4

Table 8.1 Animal study plan

*: Microbial infection was done at 2 time point, one was 4 weeks after vaccination and another was 8 weeks after vaccination.

8.2 Materials and methods

Animal

Specific-pathogen free 4-6 week old, female BALB/c mice were used as animal model for these studies. All mice were housed in cages contained within Animal Bio-

Safety Laboratory, level 3 (ABSL-3) laminar flow safety enclosures. Animals used were allowed free access to water and standard mouse food.

Bacteria

M. Tuberculosis strain was grown at 37°C in modified M7H11 agar medium enriched with 0.5% sodium pyruvate, 0.5% glucose and 2- Thiophene Carboxylic Acid Hydrazide (TCH) 4 µg/ mL. BCG-Pasteur was strain was also grown on similar plates without adding TCH. The TCH containing modified MH711 agar plates were prepared at laboratory under aseptic conditions. The culture was then diluted in a way that it gave optical density at 600 nm about 0.06 with M7H11 medium.

Chemicals and Reagents

Modified 7H11 enriched plates were purchased from MiddleBrooke, USA, 2-Carboxylic Acid Hydrazide, Polymyxin B, Carbenicillin, Cycloheximide and Trimethoprim were purchased from Sigma Aldrich, USA. OADC was also purchased from Sigma, USA. Modified MH711, sodium pyruvate, glucose, 2- Thiophene Carboxylic Acid were purchased from Sigma Aldrich, USA. Petriplate (sterile, single use) were purchased from BD diagnostic, USA.

Instrument

Inhalation exposure system from Glas- Col, USA, model 4212, Glas- Col type of tissue homogenizer and Binder C-150 Air-Jacketed CO_2 Incubators, from Cole Parmer, USA were used in the study.

8.3 Experiment

8.3.1 Immunization with the BCG and the test vaccine

Mice were immunized with the BCG vaccine (2 x 10^2 *M.Tb Bovis*) and the test vaccine. Mice were lightly anaesthetized with an inhaled gaseous mixture of 2% (v/v) Isofluroprene in oxygen for Intra Nasal (I.N.) dosing procedure. For I.N. dosing, ESAT-6 and Ag 85B in pure form 30µg each in 50 µL volume of PBS and antigen conjugated- adjuvanted NPs suspension were administered using a micropipette attached with narrow tip. This method of I.N. administration was previously demonstrated by Eyles 1999 who showed that large portion of dose was deposited in the lungs. The standard BCG dose (2 X 10^2 CFU) was administered subcutaneously to one group of mice considered as a positive control. Four more groups were also

considered for the given study for comparison of efficacy of the vaccine and these were: adjuvanted NPs, adjuvanted proteins in pure form, antigen encapsulated in DDAB adjuvanted PLGA NPs and PBS. In all cases, 50 µL were administered intra-nasally.

8.3.2 Aerosol infection to the animals

Mice were infected with *M.Tb* (Pasteur) by aerosol route in an Inhalation Exposure system (model no A4212). 10 mL of culture (OD_{600} ~ 0.06) was transferred to sterile nebulizer aseptically and fixed to the Inhalation aerosol machine. The manufacturer's guidelines were followed for the operation of the machine and as SOP for the instrument was followed which delivered approximately 100-125 unit bacteria to each mouse. These numbers were tested by the sacrifice of one group of animals on the following day of infection. As shown in figure 8.3, experiment outline, animals were sacrificed 4 weeks and 8 weeks after infection.

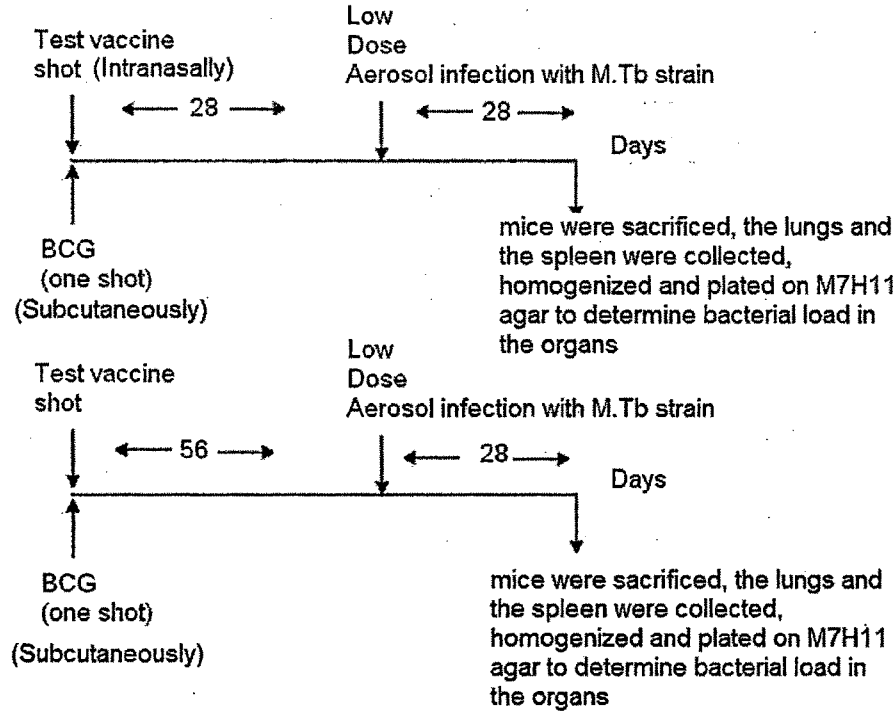

Figure 8.3 Experiment outline

After aerosol challenge of mice, on the second day (Day +1 of the infection), vaccine untreated mice were bled via cardiac puncture vein and about 750 µL blood collected and then they were sacrificed by euthanasia method and organs (the lungs and spleen) were collected aseptically and placed into ice-cold sterile PBS. Organs were homogenized manually with Glas- Col tissue homogenizer from Cole Parmer, USA, in sterile PBS aseptically. Serial dilutions (10^{-2} and 10^{-4}) were made and were plated onto Middlebrook 7H11 agar enriched with OADC (100 mL/ Liter of culture medium).

After 4 weeks, the similar procedure were followed for all six groups of mice and plated as described in the previous paragraph.

Organs collected from BCG vaccinated mice were placed on Middlebrooke 7H11 agar plates supplemented with Cycloheximide (50 µg/ mL), Polymyxin B (200 µg/mL), Carbenicillin (50 µg/mL), and Trimethoprim (µg /mL) and 4 µg/mL of 2-Thiophene-Carboxylic acid Hydrazide for selective inhibition of BCG strain.

All the plates were incubated over 3-4 weeks at 37^0 C and colonies were counted under the white light.

8.4 Results and Discussions

There is an increasing evidence that intra-nasal route is extremely effective, non-invasive means of vaccinations (Almeida 1993, Almeida 1996). Nasally applied antigens are capable of producing both mucosal and systemic immunological responses and able to provide protection against variety of infections (Eyles 1998). Surface presentation or encapsulation with biodegradable particles stimulates superior immunity to soluble antigens when given by I.N. route (Dietrich 2006). We took both the reports into consideration and formulated antigen conjugated NPs and administered them by nasal route. To determine the ability of nasal vaccination to generate immune response, groups of BALB/6 mice were lightly anaesthetized (with Isoflurane, USP 2% in oxygen) and given one dose of 30 µg of antigens (ESAT-6 and Ag85B) formulation in 50 µL of PBS through nostril with the help of a thin tip attached to micropipette. The antigen conjugated to adjuvanted NPs is a stable suspension formulation containing both the antigens ESAT-6 and Ag85B in 30 µg

each of antigens and 10% w/w of DDAB as an adjuvant. As a control group of mice, they received 50 µL of PBS (negative control), 50 µL of solution of both the antigens (ESAT-6 and Ag85B in 30 µg concentration and 200 µg of DDAB) (antigens in pure form) and plain NPs 20 mg/ 50 µL (adjuvanted NPs). Along with these control groups, one more group was also included for the comparison of surface presented antigens with encapsulated one. In this group, mice received 50 µL suspension prepared in PBS containing (10% w/w) DDAB adjuvanted PLGA NPs having 30 µg of each of ESAT-6 and Ag85B individually. All these groups of mice received respective dose through nasal route. As a positive control, one group of mice received BCG vaccine (2 x 10^2 *M.Tb Bovis*) subcutaneously.

The ability of the vaccination protocols (as shown in figure 8.3) to generate immune response was assessed by determining the reduction in bacterial load. After immunization, mice were challenged with *M.Tb* (Pasteur strain) through aerosol route and they were sacrificed at 4 weeks and 8 weeks after infection. The lungs and the spleen were harvested and homogenate was prepared in 2.5 mL of ice cold sterile PBS aseptically with Glas- Col type of tissue homogenizer. From this, 10^{-2} and 10^{-4} serial dilutions were prepared and 0.5 mL was placed on 7H11 agar plates. Numbers of colonies were counted after 3 weeks incubation at 37^0C in 5% CO_2 incubator.

Table 8.2 and table 8.3 show the CFU data in the lungs at Day +28 days and Day +56 days after infection. Table 8.4 and table 8.5 shows data in the spleen at Day +28 days and Day +56 days after infection.

Figure 8.2, 8.3, 8.4 and 8.5 show the bar graph for the comparative studies of test vaccine with the controls.

Group of Animal study	Number of CFU in Lung Day +28	% log reduction in bacterial load
Untreated Mice D+ 1	2.5×10^2	
1: Untreated Mice	$5.1 \times 10^8 \pm 1.985 \times 10^2$	-
2: BCG vaccinated	$2.8 \times 10^4 \pm 2.19 \times 10^2$	4
3: Adjuvanted Nano Particles	$7.1 \times 10^6 \pm 9.425 \times 10^3$	2
4: Adjuvanted free antigens in PBS	$5.3 \times 10^5 \pm 2.63 \times 10^3$	3
5: Antigen encapsulated PLGA NPs	$9.1 \times 10^7 \pm 5.53 \times 10^2$	1
6: Adjvanted- antigen conjugated NPs	$1.25 \times 10^4 \pm 1.3 \times 10^2$	4

Table 8.2 Animal study Protection assay data on Day + 28in Lungs

* CFU: Colony Forming Units

Group of Animal study	Number of CFU in Lung Day +56	% log reduction in bacterial load
1: Untreated Mice	$3.75 \times 10^{12} \pm 2.46 \times 10^{10}$	-
2: BCG vaccinated	$1.53 \times 10^8 \pm 3.425 \times 10^6$	4
3: Adjuvanted Nano Particles	$5.12 \times 10^{10} \pm 1.96 \times 10^8$	2
4: Adjuvanted free antigens in PBS	$3.6 \times 10^9 \pm 2.88 \times 10^7$	3
5: Antigen encapsulated PLGA NPs	$5.5 \times 10^{11} \pm 3.22 \times 10^6$	1
6: Adjvanted- antigen conjugated NPs	$3.75 \times 10^4 \pm 3.33 \times 10^3$	8

Table 8.3 Animal study Protection assay data on Day + 56 in Lungs

Group of Animal study	Number of CFU* in Spleen Day +28	% log reduction in bacterial load
1: Untreated Mice	$7.7 \times 10^6 \pm 5.4 \times 10^4$	-
2: BCG vaccinated	$5.25 \times 10^4 \pm 1.12 \times 10^2$	2
3: Adjuvanted Nano Particles	$8.5 \times 10^5 \pm 12.72 \times 10^3$	1
4: Adjuvanted free antigens in PBS	$6.5 \times 10^5 \pm 12.83 \times 10^3$	1
5: Antigen encapsulated PLGA NPs	$3.23 \times 10^5 \pm 4.55 \times 10^3$	1
6: Adjvanted- antigen conjugated NPs	$7.75 \times 10^4 \pm 1.11 \times 10^2$	2

Table 8.4 Animal study Protection assay data on Day + 28 in Spleen

Group of Animal study	Number of CFU in Spleen Day +56	% log reduction in bacterial load
1: Untreated Mice	$1.67 \times 10^8 \pm 5.0 \times 10^6$	-
2: BCG vaccinated	$1.25 \times 10^6 \pm 8.33 \times 10^4$	2
3: Adjuvanted Nano Particles	$8.33 \times 10^7 \pm 10.33 \times 10^5$	1
4: Adjuvanted free antigens in PBS	$3.75 \times 10^7 \pm 8.35 \times 10^5$	1
5: Antigen encapsulated PLGA NPs	$6.4 \times 10^7 \pm 8.3 \times 10^5$	1
6: Adjvanted- antigen conjugated NPs	$6.75 \times 10^3 \pm 63.3$	5

Table 8.5 Animal study Protection assay data on Day + 56 in Spleen

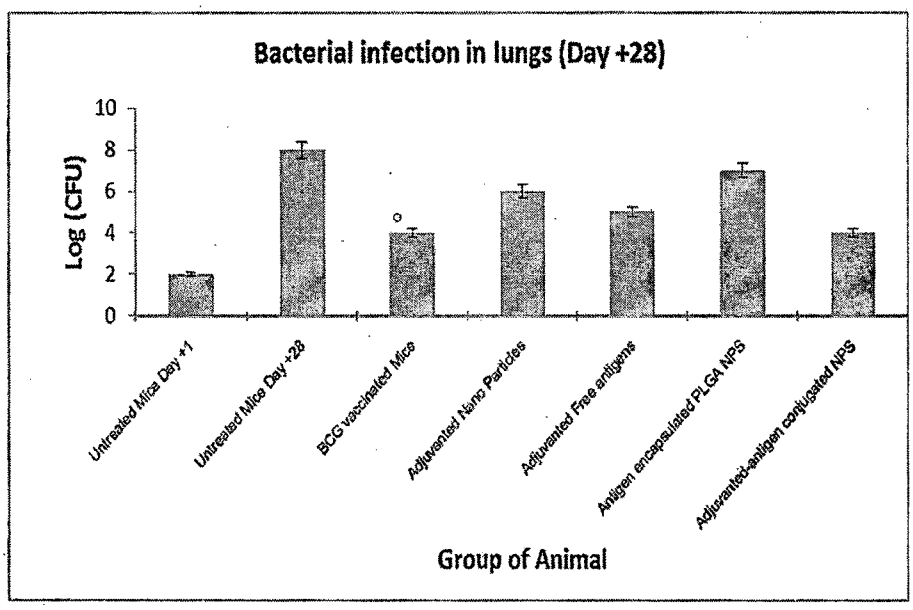

Figure 8.4 Comparative CFU studies in Lungs at Day 28

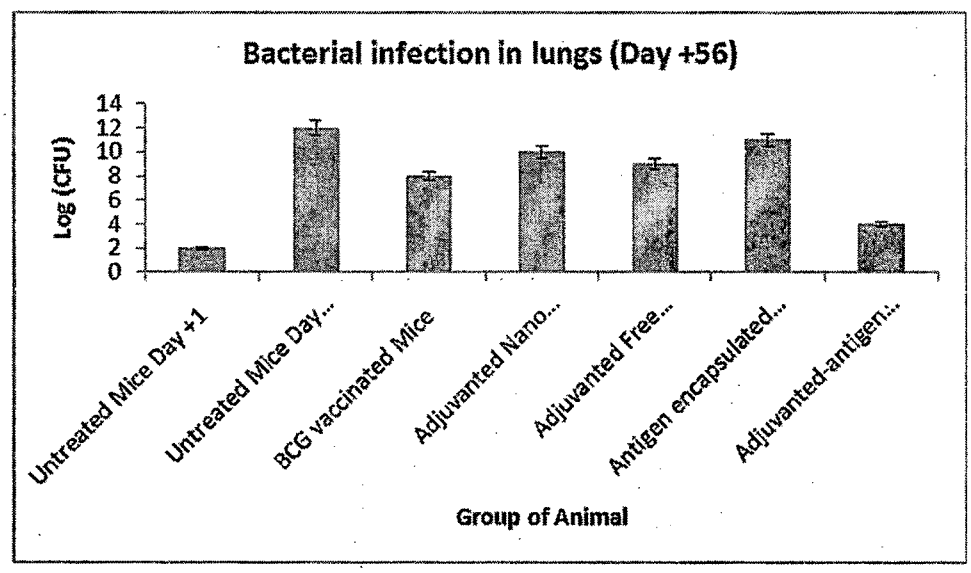

Figure 8.5 Comparative CFU studies in Lungs at Day 56

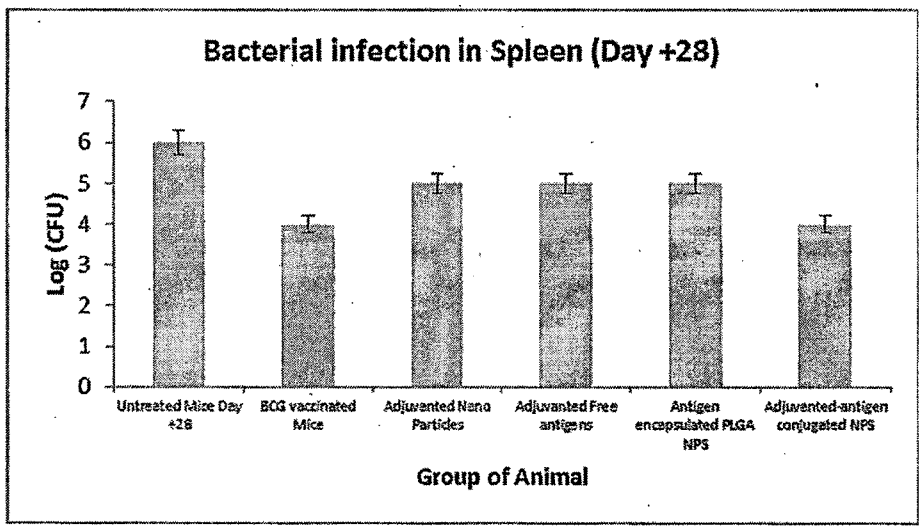

Figure 8.6 Comparative CFU studies in Spleen at Day 28

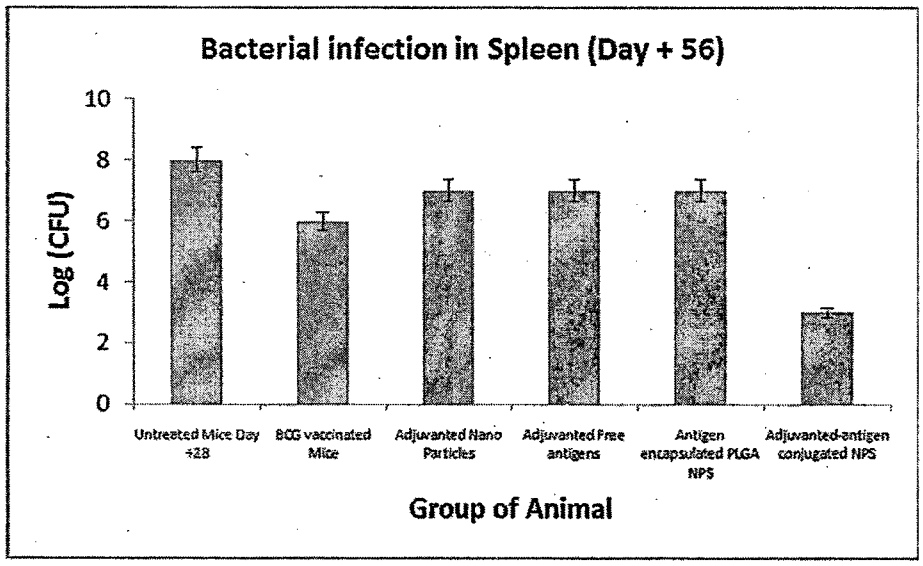

Figure 8.7 Comparative CFU studies in Spleen at Day 56

The results shown in tables 8.3 indicated that the bacterial load reduction in the lungs at Day 28 in the case of vaccine formulation containing Ag85B and ESAT-6 conjugated DDAB adjuvated B-PEG-PLA NPs and the BCG vaccine was equal (%

log reduction was 2 in both the case). A comparative efficacy was found. This is mainly attributed to the strong immnuno-dominant nature of these two proteins, presence of adjuvant DDAB and to a certain extent NPs (NPs also contribute in eliciting non-specific immune response to some extent). Actual TB infection produces these two proteins in copious amount while BCG is able to produce Ag-85 complex only. However, BCG fails to produce ESAT-6 antigen which plays vital role in the disease protection mechanism (Pym et al 2003).

At Day 56, the bacterial load reduction in lungs was found more in the case of antigen conjugated DDAB adjuvanted B-PEG-PLA NPs compared to the BCG vaccine. In 8 weeks of the time, the bacteria were grown in the lungs to 3.75×10^{12} in number of CFU which was reduced to 1.53×10^{8} number of CFU in BCG treated group and to 3.75×10^{4} number of CFU in the antigen conjugated DDAB adjuvanted B-PEG-PLA NPs. It indicates that at Day 56, the % log bacterial load reduction was about two times better in the case of antigens conjugated DDAB adjuvanted B-PEG-PLA NPs than the BCG vaccine. B-PEG-PLA, a PEGylated PLA degrades in body over 30 days (Kulkarni 1966), which may have allowed antigens to get free from the NPs for complete interaction with APCs which may have enabled them to provide longer and stronger immunity against the disease.

When we compared the results with antigen encapsulated PLGA NPs with surface presented NPs, we got better result in the later case (% log bacterial load reduction was 1 in the case of antigens encapsulated PLGA NPs and 4 in the case of antigen conjugated B-PEG-PLA NPs). This must have been mainly contributed to the surface presentation of antigens which would have helped immediate interaction of the antigens with the APCs. When antigen is encapsulated in the NPs, the antigens are not accessible to the APCs for the interaction until they get got released from the NPs. In such cases, immune response can be elicited when polymer degrades completely and antigens get released from it. However, we got better result at Day 56 in the case of surface presented NPs. The results are supported with the work reported by the Hanes group. Hanes group have reported in their earlier work on transport of PLGA NPs v/s mucus penetrating NPs and showed that conventional NPs got trapped in the mucus

(Lai 2008), failed to reach to the epithelia where antigens are supposed to interact with antigen presenting cells. As reported by Hanes and co-workers, that nanoparticles coated with low molecular weight (MW) poly(ethylene glycol) (PEG) possess hydrophilic and near neutrally-charged surfaces that minimize mucoadhesion by reducing hydrophobic or electrostatic interactions. Low PEG MW and high (dense) PEG surface coverage are both required for rapid mucus penetration of coated particles, and however high MW PEG can increase mucoadhesion (Wang 2008). The in-vitro mucus penetration studies showed that over 75% of the antigens conjugated B-PEG-PLA particles were travelled through the mucus when 20 mS time was given for the penetration when compared with the polystyrene particles. Hence, we can conclude that more numbers of particles was reached to epithelia to elicit better immune response.

Surface presentation of antigens was also attempted successfully and reported by Sloat et al (2010) with model protein bovine serum albumin and the *Bacillus anthracis* protective antigen protein. As described in their paper, model protein antigens, bovine serum albumin (BSA) and *Bacillus anthracis* protective antigen (PA) protein were covalently conjugated onto the nanoparticles. They showed that when mice immunized with the BSA-conjugated nanoparticles had developed strong anti-BSA antibody responses comparable to that induced by BSA adjuvanted with incomplete Freund's adjuvant and 6.5-fold stronger than that induced by BSA adsorbed onto aluminium hydroxide. Immunization of mice with the PA-conjugated nanoparticles elicited a quick, strong, and durable anti-PA antibody response that afforded protection of the mice against a lethal dose of anthrax lethal toxin challenge.

From the lungs, the TB infection spreads to the spleen and hence we conducted bacterial load in the spleen too. There also we found that the % log bacterial load reduction at Day 28 was similar in the cases of the BCG and surface presented NPs (it was 2 for both) while in the case of antigen encapsulated PLGA NPs, % log bacterial load reduction was 1. At day 56, the bacterial load in untreated mice was increased to 1.67×10^8, which was found 1.25×10^6 in the BCG treated mice, 6.4×10^7 in antigens encapsulated PLGA NPs and 6.75×10^3 in the antigens conjugated DDAB adjuvanted

B-PEG-PLA NPs. This indicates that the surface presented NPs showed better disease protection at Day 56 compared to BCG vaccine and antigens encapsulated PLGA NPs.

We justify our results on the basis of three facts:

(1) **Intranasal administration** of the test vaccine which enables antigens to enter as similarly to pathogen entry to in the host and is also helpful for the generation of mucosal and systemic immunity;

(2) **Surface presentation of the antigens** for provides easy access to the APCs for interaction to elicit disease specific immune response and

(3) **Mucus penetrating nanoparticles** help to avoid mucus adhesion of particles as well as of antigens and thus helps the antigens to reach epithelia for the immune response.

However, detailed studies on immune-profiling can provide supportive data on level of cytokins produced during the immune response. Some more animal studies work can also be conducted on this topic which may include:

- ✓ Role of protein based vaccine in "Long term memory of immune response" where studies may be continue for one year.
- ✓ Determination of long term immunity when given with BCG vaccine as prime-boost techniques
- ✓ Multiple administration of protein based vaccine at various time interval and its effect on long term immune response
- ✓ In-vivo stability studies
- ✓ Preventive- curative combination therapy in the cases of latent infection, and reactivated infection for the control of active tubercle infection.

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Reviewer's Comments