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**PLGA nano/micro particles encapsulated with pertussis toxoid (PTd)
enhances Th1/Th17 immune response in a murine model**

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ABSTRACT:

25 Poly(lactic-co-glycolic acid) (PLGA) based nano/micro particles were investigated as a potential vaccine platform for pertussis antigen. Presentation of pertussis toxoid as nano/micro particles (NP/MP) gave similar antigen-specific IgG responses in mice compared to soluble antigen. Notably, in cell line based assays, it was found that PLGA based nano/micro particles enhanced the phagocytosis of fluorescent antigen-
30 nano/micro particles by J774.2 murine monocyte/macrophage cells compared to soluble antigen. More importantly, when mice were immunized with the antigen-nano/micro particles they significantly increased antigen-specific Th1 cytokines INF- γ and IL-17 secretion in splenocytes after *in vitro* re-stimulation with heat killed *Bordetella pertussis*, indicating the induction of a Th1/Th17 response. Also, presentation of
35 pertussis antigen in a NP/MP formulation is able to provide protection against respiratory infection in a murine model. Thus, the NP/MP formulation may provide an alternative to conventional acellular vaccines to achieve a more balanced Th1/Th2 immune response.

Keywords: Pertussis; PLGA nano/micro particles; Phagocytosis; Adjuvant

40

1. Introduction

The introduction of comprehensive vaccination programmes worldwide in 1950s has led to the global incidence of whooping cough (pertussis) falling dramatically (Roush et al., 2007). Acellular vaccines (ACVs) containing purified pertussis antigens have replaced the traditional whole cell vaccines (WCVs) in most developed countries of the world in the 1980s/1990s due to concerns over reactogenicity of WCVs (Greco et al., 1996). All marketed pertussis ACVs contain detoxified pertussis toxin (PTd) with most containing varying doses of detoxified filamentous haemagglutinin (dFHA) and pertactin (d69K) or fimbrial antigens (Fim2&3). However, despite efficient vaccination programmes in many ACV covering countries, the incidence of pertussis is increasing in both infants and vaccinated young adults with reported outbreaks in many countries including USA, Australia, Ireland and UK (Barret AS, June 2010 ; Nieves DJ, 2011; Spokes et al., 2010). In addition, current understanding on the mechanism of vaccine-induced immunity, especially the role of T-cell and cell mediated immunity (CMI), and pertussis infection models indicate that altering the immune bias to a Th1/Th17 response is beneficial for clearance of bacteria (Dunne et al., 2010; Mahon et al., 1997). This suggests that improving the efficacy and durability of the protection induced by current ACVs is of importance and development of new generation of vaccines with long-lasting immunity is needed for better control of this disease. There are several possible explanations for the short duration of protection offered by ACVs, such as incomplete antigen coverage and incorrect balance of antigens in the formulation. Most significantly, ACVs are widely acknowledged to be less protective than WCVs and require the inclusion of adjuvants that induce Th-1 cells to boost protective immunity. The immune response in humans induced by current ACVs with aluminium as an adjuvant is predominantly Th-2 type response (Gupta and Ahsan, 2011; Leroux-Roels,

2010). However, despite that aluminium can effectively induce a strong antibody response, it is a weak adjuvant for the induction of CMI (Mahon et al., 1997). The different profiles in cellular and humoral responses, macrophage activation and bacterial clearance of WCVs and ACVs indicated that different protective mechanisms are at work involving these two types of vaccine (Ausiello et al., 1997; Canthaboo et al., 2002; Mills et al., 1998; Warfel et al., 2014) . Therefore, the development of a vaccine which can mount both cellular and humoral responses to generate a more effective and long lasting immune response would be the key to improve the efficacy of the current ACVs.

75 Biodegradable micro/nanoparticles (NP/MP) generated from poly(lactic-co-glycolic acid) (PLGA) as a delivery system have attracted much attention due to their clinically proven biocompatibility and adjuvant activity during immunisation protocols (Danhier et al., 2012; Feczko et al., 2011). In particular, PLGA-based nano- and microparticles have been extensively investigated as a vaccine delivery system as well as used for controlled drug delivery for biopharmaceuticals (Abbas, 2008; Jiang et al., 2005; Kim et al., 2016; Santillan et al., 2011). Biodegradable particles with entrapped vaccine antigens, such as proteins, peptide or DNA, have recently been shown to possess significant potential as vaccine delivery systems. It is also known that size, charge and shape of particles play significant roles in antigen uptake. Both PLGA nanoparticles and microparticles have been investigated for vaccine delivery and demonstrated significant differences in modulating immune responses (Gregory et al., 2013; Hiremath et al., 2016; Johansen et al., 2000; Peyre et al., 2004; Singh et al., 2015). Nanoparticles are believed to promote cellular response whereas microparticles promote humoral one (Chong et al., 2005; Gutierrez et al., 2002). However, microparticles with a size less than 90 10 μm have shown efficient internalization by macrophages (Champion et al., 2008).

Adjuvants function through three primary mechanisms: (1) protect and stabilise the entrapped antigen, (2) deliver antigen more effectively to antigen presenting cells (APCs), and (3) act as an immunostimulant adjuvant to activate or enhance immunity. Particle-based antigen (Ag) delivery can potentially provide many levels of adjuvanticity that include: prolonging Ag residence in the body (depot formation); enhancing Ag uptake mediated by dendritic cell (DC); direct stimulation of DC and promotion of cross-presentation especially in cases when new vaccine applications require cell-mediated immunity (Rice-Ficht et al., 2010). Here we report a pilot study to investigate PLGA nano/micro particles as a candidate platform for the production of novel vaccines against pertussis that can enhance the efficacy of ACV.

2. Materials and Methods

2.1 Materials

The pertussis toxoid was kindly donated by Serum Institute of India. Poly (lactide-co-glycolide acid) (PLGA) (lactic acid:glycolic acid-50:50, Resomer RG 503H) was purchased from Evonik Industries, Germany. Albumin from bovine serum (BSA), polyvinyl alcohol (PVA, Mowiol® 4-88, Mowiol® 8-88, Mowiol® 18-88 with MW 31,000, 67,000 & 130,000 respectively), Sodium hydroxide, sodium phosphate monobasic monohydrate, foetal calf serum and L-glutamine were purchased from Sigma-Aldrich, UK. Albumin–fluorescein isothiocyanate conjugate (BSA-FITC), RPMI 1640 medium, penicillin, streptomycin and AlexaFluor594-conjugated phalloidin was obtained from Life Technologies, UK. Dichloromethane was received from Fisher Scientific, UK. Pierce® Bicinchoninic acid protein assay reagent A and B were purchased from Thermo Scientific, Germany.

2.2 Preparation of PLGA particles encapsulated with antigens

115 2.2.1 Formulation development using albumin from bovine serum (BSA)

PLGA micro/nanoparticles containing BSA was prepared using double emulsion (w/o/w) solvent evaporation method (Feczko et al., 2011). Briefly, appropriate amount of BSA (Table 1) was dissolved in deionised water and the resultant solution was emulsified in 5ml PLGA (200mg) dichloromethane solution by sonicating for 60
120 seconds (UP200S ultrasonic processor, Hielscher Ultrasound technology, Germany) at setting 37% (54.05W electric power input) in ice bath and the primary emulsion was obtained. 20ml polyvinyl alcohol (PVA) solution of appropriate concentration (second aqueous phase, Table 1) was added into the primary emulsion and was emulsified by sonicating at 37% for 3 minutes using the same sonicator under the same conditions to
125 obtain the second emulsion. The organic solvent in the emulsion was evaporated under magnetic stirring at 800 rpm for 2 hours in a fume cupboard under room temperature. The obtained BSA-loaded PLGA particles were separated from the liquid medium by centrifugation (T1250 Sorvall Ultracentrifuge, Thermo Fisher Scientific, Inc. USA) at 25,000 rpm for 30 minutes. The isolated BAS-loaded PLGA particles were washed with
130 deionised water and centrifuged again at 14,000 rpm for 15 minutes. The particles were then washed with deionised water for two more times without centrifugation and were freeze-dried (Micromodulyo freeze dryer, Mecha Tech Systems, UK) for 19 hours. The final particles were stored in a freezer at -20°C.

A range of BSA-loaded PLGA particle formulations were prepared by varying BSA
135 loading ratio (% w/w BSA loading based on PLGA), PVA molecular weight, PVA concentration in the aqueous phase and the phase ratio of the organic phase vs the second aqueous phase (Table 1).

2.2.2 Preparation of final BSA-FTIC and pertussis toxoid-containing PLGA particles

140 The final PLGA particles containing BSA-FTIC and pertussis toxoid (PTd) for use in the biological tests were prepared as described in Section 2.2.1. The BSA-FTIC orPTd loading ratio was 5 % (w/w); the PVA used has a molecular weight of 31,000 Da at a concentration of 1.75 % (w/v); and the phase ratio of the organic phase vs the second aqueous phase was 1:4.

145 2.3 Characterisation of antigen-loaded PLGA particles

2.3.1 Particle size and zeta potential measurement

Antigen (BSA-FTIC or PTd)-loaded PLGA particles (50 mg) were dispersed in 0.1% (w/v) PVA solution (molecular weight 31,000 Da). The particle size and zeta potential were determined using Zetasizer nanoseries (Malvern instruments, Malvern, UK) at 150 25°C. When multiple peaks were present at the particle size measurement, the particle size (mean \pm SD) and percentage intensity of each peak were reported.

2.3.2 Surface morphology using scanning electron microscopy (SEM)

The surface morphologies of the antigen-loaded PLGA particles were examined using SEM (JEOL JSM-35 Scanning Microscope, JEOL (UK) Ltd, UK), with an electron 155 energy at 5-10 keV.

2.3.3 Calculation of yield and encapsulation efficiency

The final weight of the antigen-loaded PLGA particles after freeze-drying was weighed and the yield (%) of the preparation was calculated using Equation 1.

Equation 1.

160 $Yield (\%) = \text{Actual weight of the particles} / \text{The cortical weight of the particles} * 100\%$

The encapsulation efficiency (%) was calculated using Equation 2.

Equation 2

*Encapsulation efficiency (%) = Actual concentration of the antigen in the particles /
The cortical concentration of the antigen in the particles*100%*

165 **2.3.4 Quantification of antigen loading**

The antigen loading in the PLGA particles were quantified using BCA assay. The loaded particles (10 mg) were suspended in 1 ml 1M sodium hydroxide (NaOH) and agitated at 250 rpm using an electronic shaker for 3 hours at room temperature (RT) to hydrolyse the polymer. 3 ml of PBS (pH 7.4) and 1ml 1M hydrochloride acid (HCl)
170 were added to the PLGA-NaOH mixture. The resultant sample (0.1ml) was mixed with 2 ml BCA reagent A and B (50:1). The absorbance of the above solution was measured using UV-Vis spectroscopy at 557 nm. Each measurement was repeated in triplication. A calibration curve was prepared at BSA concentrations of 20, 40, 80, 120, 160 and 200 mg/ml.

175 **2.5 Culture of J774.2 cells**

Murine monocyte/macrophage J774.2 cells were maintained at 37°C under the presence of 5% CO₂ in complete RPMI (cRPMI) comprising RPMI 1640 medium, 10% foetal calf serum, 10 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.6 Uptake of PLGA nano/micro particles by J774.2 cells

180 **2.6.1 Flow cytometry**

A quantity of 10⁶ J774.2 cells was seeded in each well of a 24-well tissue-culture plate and incubated for 2 h at 37°C in 5% CO₂. Subsequently 1 ml of fresh cRPMI was added to each well to replace the medium. Particles loaded with 50% BSA-FITC or

soluble BSA-FITC were dispersed in cRPMI to prepare a 10mg/ml suspension . A 0.5
185 ml aliquot of the suspension was added to each well and incubated for 60 min at 37°C,
in 5% CO₂ whilst protected from light. The uptake was terminated by washing the cells
twice with ice-cold PBS. The cells were then re-suspended in ice-cold PBS (1 ml) and
centrifuged for 10 min at 118 g. The resultant pellet was re-suspended in 4 ml fixing
solution (1% formaldehyde in PBS) and samples were stored at 4°C under protection
190 from light. A FACS Canto II flow cytometer (BD Biosciences) was used to determine
the uptake of fluorescent particles.

2.6.2 Confocal laser-scanning microscopy

Two mililiter of 10⁶ J774.2 cells in cRPMI was added to each well of a 24-well plated
containing 0.2% gelatine (in PBS) coated sterile glass coverslips and incubated for 3h
195 at 37°C in 5% CO₂. Following incubation, appropriate antigen formulation was added
and incubated for a further hour. After washing the cells twice with PBS-A (without Ca
and Mg), a fixing solution (4% paraformaldehyde in PBS-A, 300 µl/well) was added
and the cells were incubated for 20 min at room temperature (RT). Cells were washed
as before. To permeabilise the cells, 300µl of PBS-A containing 0.2% BSA and 0.2%
200 TritonX-100 was added to each well and incubated for 15 min at RT without shaking.
Following washing, to block non-specific binding, 300µl of blocking reagent (PBS-A
containing 5% BSA) was added to each well and incubated for 1 h at RT without
shaking. Once washed, AlexaFluor594-conjugated phalloidin was used to stain the
actin cytoskeleton for 5 min and subsequently 4,6-diamidino-2-phenylindole (DAPI)
205 were applied for nuclear staining for 3 min. The fluorescent cells were visualised using
an NA 1.25 oil immersion lens on a laser-scanning confocal microscope (Leica SP2
AOBS) . Images were analysed using IMARIS software v7.4.2 (Bitplane, Switzerland).

2.7 Immunisation of mice

The required doses of PTd NP/MP formulations were re-suspended in sterile PBS
210 immediately prior to immunisation. Groups of 5 female NIH mice (6-8 week old,
Envigo, UK) were injected subcutaneously with 0.5 ml desired formulation at days 0
and 28. A further group of five mice were immunised with PBS as negative control.
Mice were sampled for sera at four weeks after primary immunisation and two weeks
post-boost.

215 2.8 Antigen-specific serum IgG titres

Antibody response of the formulations was determined using antigen specific ELISA as
described previously (Cheung et al., 2006) . Total antigen-specific IgG titres were
determined relative to First International Reference Serum for pertussis (97/642,
NIBSC, UK).

220 2.9 Protection studies by respiratory challenge

Two weeks after the second dose of immunisation, groups of 10 mice were subjected to
aerosol challenge with a suspension of 10^8 cfu/ml *B. pertussis* 18.323 in 1% (w/v)
casamino acids solution containing 0.6% w/v NaCl as described previously (Xing et al.,
1999). Successful infection was confirmed in 5 mice from the negative control group at
225 2 h post-infection. At 7 d post-infection, the lungs and trachea from remaining mice
were removed and homogenized in 1 ml casamino acids solution. Serial dilutions of the
homogenate were plated onto charcoal agar (Oxoid) plates containing 10% (v/v)
defibrinated horse blood (Oxoid) to perform viable counts. The numbers were
expressed as CFU/lungs and trachea with a detection limit of 50 CFU/mouse.

230 2.10 In vitro assays for cytokines from immune cells

Spleenocytes from immunised mice were placed in 24-well tissue culture plates (4×10^6 cells/well) and cultured with or without heat-killed *B. pertussis* cells (5×10^7 /well)(Xing et al., 1998). Cell supernatants were collected and screened for cytokine responses. Initial cytokine screening was done by BD Cytometric Bead Array for 7
235 cytokines: interleukin-6 [IL-6], IL-2, IL-4, IL-17 and IL-10, tumor necrosis factor alpha [TNF-alpha], gamma interferon [IFN-gamma](BD Biosciences) according to the manufacturer's recommended procedure. After initial screening, for further experiments, selected cytokine (IL-17 and IFN- γ) responses in cell supernatants were determined by an ELISA method using DuoSet kit (R&D Systems).

240 **2.11 Statistical analysis**

Statistical analysis was performed using Combostat software Version 4.0, EDQM. Student's t-tests were performed to compare two groups with a statistical significance defined as $p < 0.05$.

2.12 Ethical approval

245 Ethical approval was obtained for all animal experiments from the Ethical Committee of National Institute for Biological Standards and Control, UK. All experiments were regulated according to the Animal Scientific Procedures Act (1986), UK.

3. Results

250 **3.1 Characteristics of antigen-loaded PLGA particles**

The yield and encapsulation efficiency of BSA-containing PLGA particles produced during the formulation development are shown in Table 1. F9 has achieved the highest yield, antigen loading, and encapsulation efficiency and was selected as the formulation

to produce the final BSA-FITC and PTd loaded PLGA particles. The characteristics of
255 the final BSA-FITC and PTd loaded PLGA particles are shown in Table 2. The
obtained antigen-loaded PLGA particles are a mixture of nanoparticles and
microparticles exhibiting particle sizes in two size ranges (Table 2). Fig. 1 shows that
the BSA-loaded PLGA particles are spherical with smooth surfaces.

3.2 Antigen phagocytosis by J774.2 cells

260 FITC-BSA was used as a model antigen to investigate NP/MP uptake by both
Fluorescence-activated cell sorting (FACS) and confocal microscopy. J774.2 murine
macrophages were incubated with 120ug/ml FITC-BSA in soluble or particle
formulation for 3h and 24h for analysis by confocal microscopy and FACS
respectively. Fig.2 A-C shows the uptake of fluorescent antigen using confocal laser-
265 scanning microscopy quantified by flow cytometry (panels D-E). Fig.2 A and D serve
as control. It is evident that J774.2 cells contained low levels of fluorescence indicating
soluble BSA-FITC was poorly phagocytosed (Fig. 2B). In contrast, punctate regions of
green fluorescence were displayed in cells treated with NP/MP formulation suggesting
enhanced FITC-BSA uptake by macrophages (Fig.2 C). These observations were
270 confirmed by flow cytometry. The cells that were exposed to BSA-FITC NP/MPs
exhibited enhanced phagocytosis with an increase in both the number of fluorescent
cells and fluorescence intensity (Fig. 2D and 2E), showing an increase in MedFI of the
P2-gated population (Fig.2E).

3.3 Antibody responses

275 Subcutaneous injection of mice with PTd encapsulated NP/MP formulation induced
similar or slightly lower anti-PT IgG titres compared with the soluble antigen at
equivalent dose at both 28 and 42 d (Fig.3). Immunisation with both the soluble and the

NP/MP formulations gave good booster response which indicates memory response. However, neither the antigen-specific IgG titres nor the duration of antibody response were altered by the NP/MP formulation indicating that it did not have adjuvant effect on PT antibody responses *in vivo*.

3.4 Antigen-specific cytokine profile

To investigate the type of cytokine produced in the spleen cell supernatants, mice were immunised with soluble PTd or PTd-NP/MP formulations. Cytokine production profile after *in vitro* stimulation with heat killed cells (HKC) was first screened using BD Cytometric Bead Array for 7 cytokines: IL-6, IL-2, TNF-alpha, IFN-gamma, IL-4, IL-17 and IL-10. In general, the spleen supernatants from mice immunised with soluble PTd and PTd-NP/MP induced similar levels of TNF-alpha, IL-10. Less significantly increased induction of IL-4 and IL-6, and a slightly decreased in IL-2 level was observed in the NP/MP group (data not shown). The most significantly increased levels in Th1 type cytokines IFN-gamma (γ) and IL-17 cytokines were observed in the PTd - NP/MP immunised group. To further confirm this, in latter experiments, sandwich ELISA was used to determine the levels of IFN- γ and IL-17 in spleen supernatants after *in-vitro* re-stimulation with HKC. Fig 4 shows spleen cell supernatants from mice that received PTd-NP/MP formulation had significantly higher levels (>4 folds, $p < 0.05$) of IFN- γ and IL-17 production than the soluble antigen group.

3.5. Protective effect of PTd NP/MP formulation in an aerosol infection model

The protective effect of PTd NP/MP formulation against the infection was investigated in an aerosol challenge model. Under the current immunisation dose, an approximately

10% reduction in CFU was found in the soluble PTd group in comparison to PBS control group. PTd in NP/MP formulation group showed similar results with a further ~5% reduction in CFU although this did not reach statistical significance (Fig.5) between these two groups.

305

4 Discussion

In the last two decades, pertussis has resurged in different countries. In particular significant mortality in infants were caused by large outbreaks in US, Australia, UK and The Netherlands in the 2010–2013 period,(Black RE, 2010; Cherry, 2010). The epidemiological situation of pertussis is possibly to be related to suboptimal or waning immunity induced by ACV (Klein et al., 2012) . This points out the urgent need to design and develop new vaccine formulation with high effectiveness in conferring protective immunity. In this study, we used biodegradable polymers as vaccine carriers to explore their ability to promote both Th1 and Th-17 cells immune responses for pertussis.

315

The yield and encapsulation efficiency of the PLGA particles are extremely important for incorporating valuable protein antigens such as pertussis toxoid (PTd), and the capacity of antigen loading determines the volume of the final vaccine product. Formulation parameters, including PVA molecular weight, PVA concentration and the phase ratio, were found to affect the loading capacity, yield and encapsulation efficiency of the final particles using the double emulsion solvent evaporation method, in agreement with published studies (Bilati et al., 2005; Feczko et al., 2011). The optimised formulation was able to successfully incorporate PTd with high loading, yield and encapsulation efficiency. The PTd-loaded PLGA particles showed dual-sized

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325 characteristics, as a mixture of nanoparticles (size range 200-300 nm) and
microparticles (size ~5 µm). Both PLGA nanoparticles and microparticles as vaccine
delivery have been reported to show differences in modulating cellular or humoral
immune responses (Gutierrez, Hernández et al. 2002, Chong, Cao et al. 2005, Champion,
Walker et al. 2008). An *in vitro* study demonstrated that PLGA microparticles (5-7 µm)
330 were attached to cell membrane and required more time to be engulfed by the
macrophages compared to nano-sized particles, which led to more potent inflammatory
stimulus after the uptake process (Nicolette et al., 2011). Therefore, it is difficult to
predict the immune response by using either micro- or nanoparticles of PLGA as
antigen carriers. In this pilot study, the combination of nanoparticles and microparticles
335 were applied for *in vitro* and *in vivo* investigations to demonstrate the potential of
PLGA based particles for enhanced PTd delivery. Further separation of the particle
sizes and investigation into their distinct effects will be the subject of future research.

Humoral immunity was shown to be critical for optimum protection against *B. pertussis*
infection together with cell-mediated immunity; long term immunity is effectively
340 maintained by memory T and B cells and the highest level of protection is associated
with the Th1 subtype (Mills et al., 1998). Antigens entrapped PLGA particles, when
delivered parenterally, was found to stimulate cell-mediated immune responses (Moore
et al., 1995) and PTd with another pertussis antigen FHA entrapped in biodegradable
polymer particles have been reported to elicit a potent type 1 T cell and antibody
345 response (Conway et al., 2001). In the present study, INF-γ level from mice immunized
with PTd NP/MP were greatly enhanced. The MP/NP incorporated antigen showed
ability to shift the T cell response from Th2 or mixed Th1/Th2 to Th1, which is
consistent with above literature reports. This is further supported by the uptake and
presentation of particulate antigen by phagocytic cells which are thought to act as

350 antigen presenting cells (APC) for Th1 response (Gajewski TF, 1991). In deed, great
enhancement of phagocytosis for NP/MP formulation by macrophages was observed in
this study and this was in agreement with previous reports that INF- γ producing cells
could activate macrophages to kill intracellular bacteria and thus play a critical role in
protection against *B. pertussis* during primary infection (Barbic et al., 1997; Mahon et
355 al., 1997)

While NP/MP formulations were found to have an effect on INF- γ producing cells, to
our knowledge, effect of PTd NP/MP on Th17 cells has not been addressed so far. It
was in recent years that the important role of Th17 cells in clearing pathogens during
host defense reactions have attracted much attention (Korn et al., 2009). In a non-
360 human primates study, it was found that ACV vaccinated animals exhibited a Th1/Th2
response; however, previously infected animals and animals that were vaccinated using
conventional whole cell pertussis possess strong *B. pertussis*-specific T helper 17
(Th17) memory as well as Th1 memory (Warfel et al., 2014). Th17 cells were also
reported to be involved in adaptive immunity to other pathogens e.g. *Pseudomonas*
365 *aeruginosa*, *Staphylococcus aureus* (Lin et al., 2009; Priebe et al., 2008). Here we
demonstrate that IL-17 secretion was significantly enhanced in the spleen cell culture
from mice immunized with PTd NP/MP formulation which indicate that the NP/MP
formulations were able to have enhancement effect on INF- γ as well as IL-17 producing
cells. According to previous report, IL-17, especially IL-17A, and IFN- γ can promote
370 macrophage killing of *B. pertussis* (Ross et al., 2013; Sarah C. Higgins, 2006) .

It has been reported that immunity of children who had received 5 doses of ACV wanes
substantially each year (Klein et al., 2012) . Biodegradable NP/MP could have
potential to avoid this problem by providing controlled antigen release from the

micro/nanoparticles which is resultant by efficient phagocytosis and transportation to
375 the lymph nodes (Gray and Skarvall, 1988).

In conclusion, these preliminary results suggested that NP/MP formulation could
promote protective immunity both Th1 and Th-17 responses of pertussis antigen, which
may enhance vaccine protective efficacy. NP/MP could be a potential candidate
platform for the development of novel vaccines against pertussis.

380

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Author's contribution:

The concept and design of the study was done by Dorothy Xing, Xiaoqing Wei, and
395 Bing Song. The experiments, acquisition of data, analysis and interpretation of data
were done by Pan Li, Catpagavalli Asokanathan, Fang Liu, Kyi Kyi Khaing, and
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Kong contributed to the manuscript writing. Deling Kong made the submission.

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Figure legends:

535 **Figure 1** The particle morphology of BSA-loaded PLGA nano/microparticles by SEM.

Figure 2 Effect of NP/MPs formulation on phagocytosis by macrophages. The figure is a representative result for confocal laser-scanning microscopy and flow cytometry of $n \geq 2$

independent experiments. J774.2 cells were incubated with 120 µg/ml soluble BSA-FITC antigen (panel B), BSA-FITC NP/MPs (panel C) for 1h at 37°C in an atmosphere of 5% (v/v) CO₂. Cells treated under identical conditions without incubation with antigen were used as negative controls (panel A) in all experiments. Uptake of fluorescent antigen was visualised by confocal laser-scanning microscopy (panels A-C, scale bars = 15 µm) and quantified by flow cytometry (panels D–F). Confocal image stacks of each sample were collected for individual emission/detection channels and a composite image formed from data from multi-channels. Images were analysed using IMARIS software v7.4.2 (Bitplane, Switzerland) [green, target protein; blue, nucleus; and red, F-actin of cytoskeleton]. The flow cytometry (panels D–F) shows the median fluorescence intensity (MedFI) of the P2 daughter population derived from alive cell gated parent population (P1). (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article).

550

Figure 3 IgG antibody response from mice immunised with soluble PTd and PTd NP/MP. Mice were injected subcutaneously at days 0 and 28 with 0.5 ml volumes of the desired formulation (5µg/dose). Mice injected with PBS served as negative controls. Sera for antibody determinations were obtained at four weeks after primary immunisation (grey) and two weeks post-boost (black). Results represent the geometric mean antibody for five mice per group plus the SEM (error bars). Insert shows individual response from groups of five mice

Figure 4 Production of (A) INF-γ and (B) IL-17 from spleen cells from mice at two weeks post boost after re-stimulation with heat killed *B.pertussis* for 48 h. Groups of mice were immunized with soluble PTd or PTd NP/MP (5µg/dose) or were given PBS only. Spleen cells were obtained on day 42, and the cells from five mice were pooled. Results represent the means for duplicate assays with SEM (error bars).

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Figure 5 Protection of mice against aerosol challenge with *B. pertussis*. Group of 10 mice were immunized intraperitoneally on days 0 and 28 with soluble PTd or PTd NP/MP (5
565 µg/mouse). Mice injected with PBS served as negative controls. Mice were challenged aerosol with *B. pertussis* 18.323 on day 42. Five mice from the PBS control group were sampled at 2 h post-challenge for enumeration of bacteria in lungs and tracheas. All remaining mice immunized with the desired formulation and mice from the PBS control group were sampled at 7 days post-challenge. Results represent percentage reduction in CFUs for immunised groups
570 compared with PBS group (100%).

Figure 1

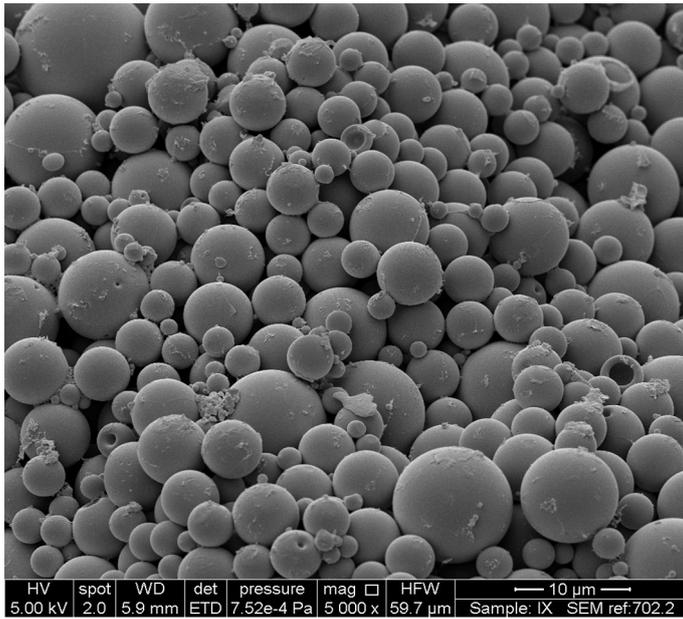
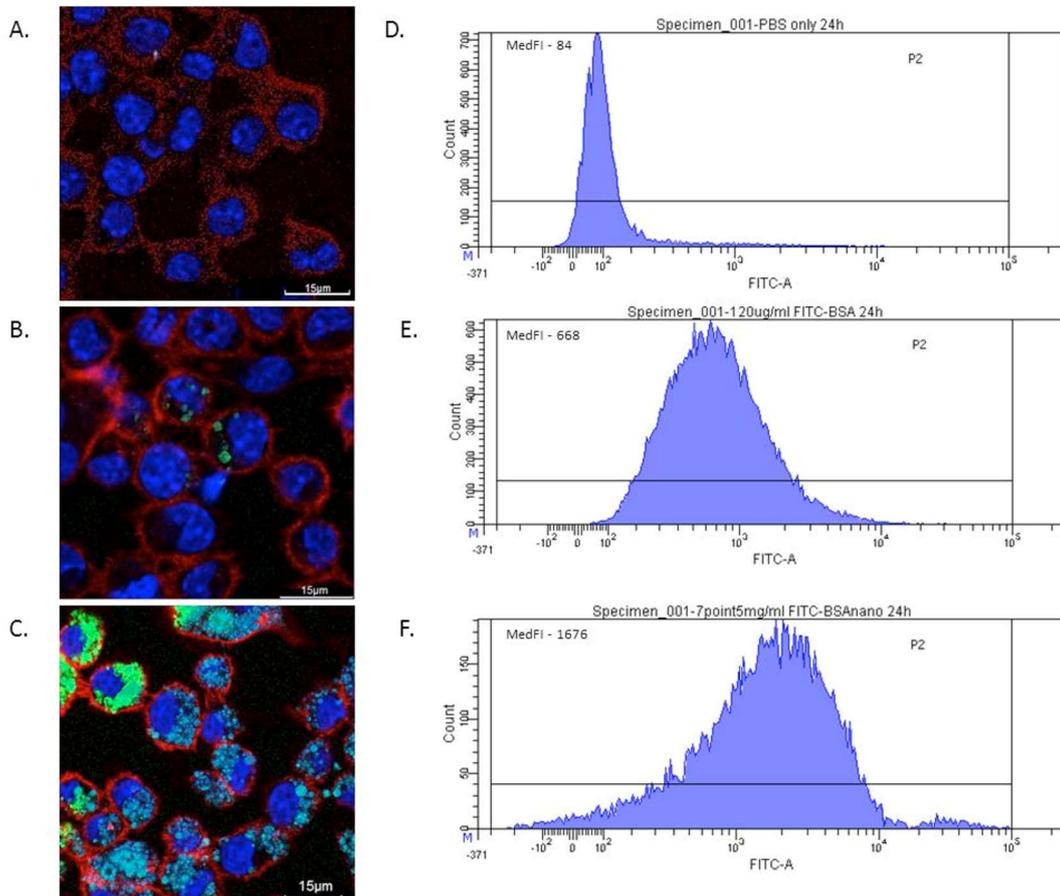


Figure 2



575

Figure 3

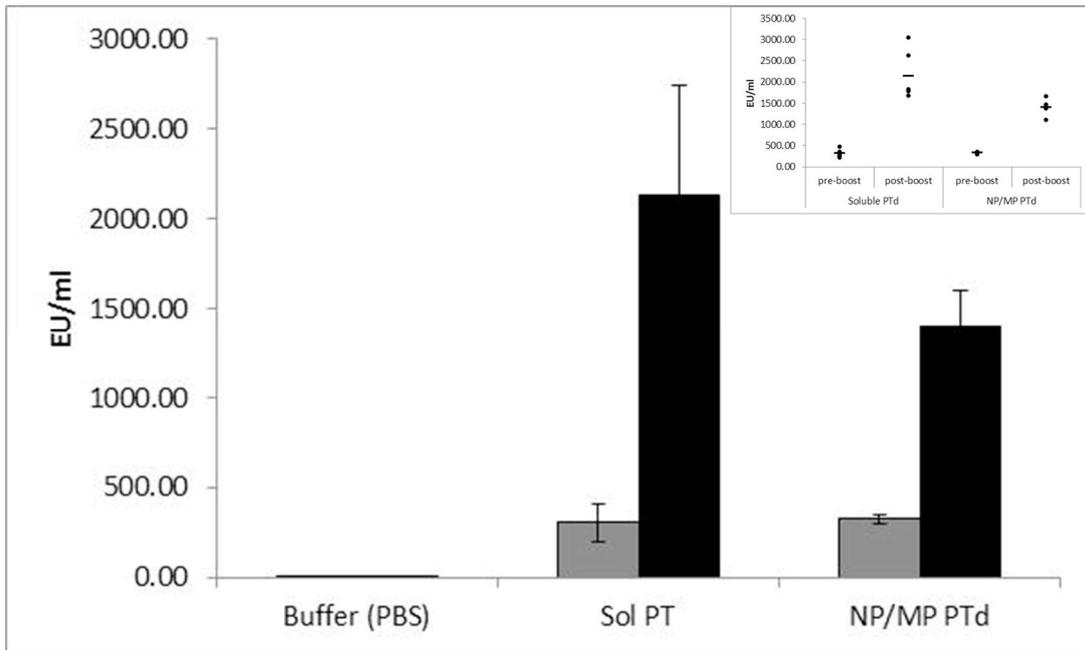
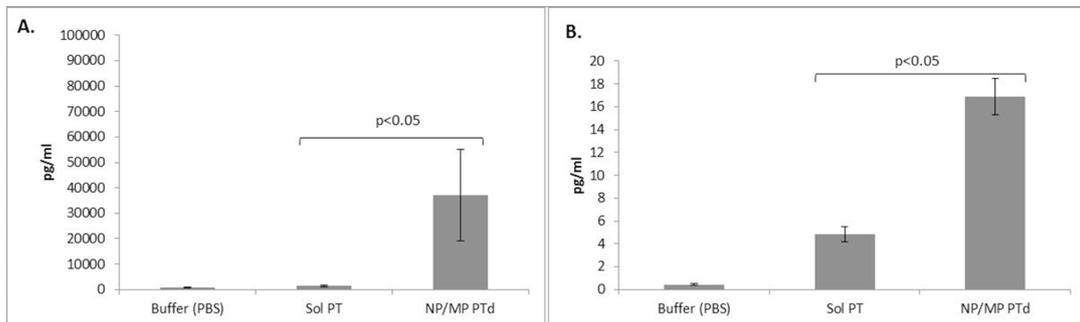


Figure 4



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Figure 5

