Title: Developing solid particulate vaccine adjuvants - surface bound antigen favouring a humoural response, whereas entrapped antigen shows a tendency for cell mediated immunity.

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Abstract

This present study compares the efficacy of microsphere formulations, and their method of antigen presentation, for the delivery of the TB sub-unit vaccine antigen, Ag85B-ESAT-6. Microspheres based on poly(lactide-co-glycolide) (PLGA) and chitosan incorporating dimethyldioctadecylammonium bromide (DDA) were prepared by either the w/o/w double emulsion method (entrapped antigen) or the o/w single emulsion method (surface bound antigen), and characterised for their physico-chemical properties and their ability to promote an immune response to Ag85B-ESAT-6. The method of preparation, and hence method of antigen association, had a pronounced effect on the type of immune response achieved from the microsphere formulations, with surface bound antigen favouring a humoural response, whereas entrapped antigen favoured a cellular response.

KEY WORDS Adjuvant, DDA, ESEM, Microspheres, PLGA, Subunit vaccine.

Introduction

Biodegradable polymers commonly contain chemical linkages such as anhydride, ester or amide bonds. These polymers degrade *in vivo* either enzymatically or non-enzymatically to biocompatible and non-toxic by-products. Biodegradable polymers not only have been extensively used in controlled delivery systems, but also extended to medical devices [1]. Synthetic biodegradable polymers have gained more popularity than natural biodegradable polymers. The major advantages of synthetic polymers include high purity of the product, more predictable lot-to-lot uniformity, and reduced concerns of immunogenicity [2]. In particular, the thermoplastic aliphatic poly(esters) like polylactide (PLA), polyglycolide (PGA), and especially poly(lactide-co-glycolide) (PLGA) have generated interest [3], due to their ability to control the release of bioactive macromolecules, such as some peptides or proteins. PLGA is approved by the US FDA and European Medicine Agency (EMA) in various drug delivery systems in humans [4] such as in sutures[5], bone implants [6] and screws [7], as well as implants for sustained drug delivery [8]. The polymers are commercially available and appropriate selection, depending on the molecular weight and copolymer ratio, allows the degradation time to be varied from several months to several years [9, 10].

When used in the form of polymeric microspheres, PLGA can increase the potency of a vaccine formulation [11-14]. As particulate delivery systems, polymeric microparticles can promote uptake, transport and/or presentation of the antigen to antigen presenting cells (APCs) (particularly in the sub-10 μm size range [15]) and PLGA microparticles have been shown to exhibit an adjuvant effect for both humoural [16, 17] and cell-mediated immunity [18]. In addition, Kanchan et al (2009) [19] carried out studies designing PLGA particles with different release kinetics and suggested that slow and continuous release from polymer particles is critical in eliciting improved memory antibody responses from single point immunisation. However, studies have indicated that immune responses from micron-sized particles generally promotes humoral (Th2) responses [20], while particles (<1000 nm) tend to promote cellular (Th1) responses [21, 22].

A comparison of humoural responses from a range of particle sizes was also carried out by Katare et al (2005) [23] after administration of very large particles (50-150 μm), microparticles optimal for

phagocytosis (2-8 μm) and small particles (<2 μm). The authors found an improvement in the antibody response for particles in the size range of 2-8 μm, in particular compared to the very large particles. Furthermore, Kanchan and Panda (2007) [24] showed that HBsAg-loaded polylactide microparticles (2-8 μm) elicited higher and long-lasting antibody titers, and although not taken up by macrophages, were on their surface. In addition, microparticles promoted IL-4 secretion and upregulation of MHC class II molecules and favoured Th2 immune response. On the other hand, the administration route of particles may influence the immune response elicited. Mohanan et al (2010) [25] have studied the bias of the immune response in mice when immunised by different routes, such as the subcutaneous, intradermal, intramuscular, and intralymphatic routes with ovalbumin-loaded liposomes, N-trimethyl-chitosan nanoparticles (NPs) and PLGA microparticles, all with and without immune-response modifiers. This study has demonstrated that the IgG2a response, associated with Th1 immune response, is sensitive to the route of administration, whereas IgG1 response, associated with Th2 response, was relatively insensitive to the administration route of particulate delivery systems.

In terms of using microspheres as vaccine adjuvants, microspheres are commonly prepared by the double emulsion solvent evaporation method (w/o/w): the initial primary w_1/o emulsion is formed by dispersion of an aqueous antigen solution (w_1) into an organic polymer solution. This primary emulsion is then mixed by high-speed homogenisation into a secondary water phase (w_2), often containing an emulsion stabiliser or surfactant such as poly(vinyl alcohol) (PVA) or chitosan, in order to form a secondary $w_1/o/w_2$ emulsion. The organic solvent is then allowed to evaporate to facilitate the formation and hardening of the microparticles. This formulation technique, originally developed by Vranken and Claeys (1970) [26] and modified by Ogawa et al (1988) [27] , prevents the partition of hydrophilic antigens into the aqueous phase, thereby achieving efficient and reproducible entrapment. On the other hand, a variation of w/o/w process is the single oil-in-water process (o/w), whereby the initial formation of the w_1/o emulsion is omitted, microparticles are formed and then antigen is adsorbed to their surface following harvesting [28-30]. This alternative process eliminates exposure of antigen to organic solvents during the formulation process and results in a different spatial location of the antigen compared to formulations prepared by the double emulsion method. In this study, 0.75% (w/v) chitosan (low molecular weight) was used as the emulsion

stabiliser in the external aqueous phase. The concentration was chosen due to previous reports of the use in microsphere formulation [31-33]. Chitosan is a hydrolysed (deacetylated) derivative of chitin, a biopolymer widely distributed in nature and biologically safe [34]. Chitosan has been shown to stimulate macrophage function [35,36] and cytokine production [37] and facilitate adjuvant activity [38].

The ability of microspheres to effectively stimulate appropriate immune responses requires more than effective delivery. Therefore, to potentiate immune responses, immunostimulatory agents are often employed within the formulations [13]. For example, a surfactant currently being investigated as an adjuvant is dimethyldioctadecylammonium bromide (DDA) [39-44]. DDA is a synthetic amphiphilic lipid, comprising a hydrophilic positively charged dimethylammonium headgroup attached to two hydrophobic 18-carbon alkyl chains [45]. DDA acts as a delivery vehicle serving to promote uptake and presentation of the vaccine antigen in the relevant subset of antigen-presenting cells (APCs). DDA is known to induce cell-mediated immunity and, along with its cationic nature and surfactant properties, has been shown to be an effective adjuvant in numerous applications including microspheres [13-14]. The adjuvant activity of DDA has been previously reviewed by Hilgers and Snippe (1992) [46] who assessed DDA to be a moderate/strong Th2 inducer and a strong Th1 inducer, and the mechanism of action behind the adjuvant effect of DDA has been attributed to its positive surface charge and its ability to associate with antigens [47]. Therefore, in this study the immunostimulatory agent DDA was investigated and included within PLGA microspheres stabilised with chitosan. PLGA, as the base polymer, will form the main matrix of the microspheres, with DDA likely interspersed throughout (although certainly some of it is on the surface, which aids protein binding). Since chitosan is used as an emulsion stabiliser, it is intended to both aid formulation, and imparts a positive charge to the particle by being located (predominantly) on the external surface. However given the cationic nature of both DDA and chitosan, there is the potential for electrostatic interactions between PLGA and DDA and/or chitosan. The impact of the method of preparation on the structural attributes is proposed in Figure 1.

Given the ability of microspheres to enhance antigen delivery and, in combination with an adjuvant, enhance immunogenicity of antigens, this present study considers two key aspects of microsphere adjuvant

formulation 1) antigen presentation by the delivery system, by directly comparing microspheres formulated with antigen incorporated within their polymer matrix core and those with surface adsorbed antigen and 2) the impact of using the immunostimulatory agent DDA.

Materials and methods

Materials

Poly(DL-lactide-co-glycolide) (PLGA) (75:25) (Mw 90,000-126,000), Chitosan (Low molecular weight), Sephadex® G-75, Phosphate buffered saline (PBS) and Chloroform were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Tris base (ultra pure) was from ICN Biomedicals (Aurora, OH). Dimethyl dioctadecylammonium bromide (DDA) was obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was > 99% by HPLC. Non his-tagged protein Ag85B-ESAT-6 was produced in *Escherichia coli* as described previously for the His-tagged version [48], purified by column chromatography and dissolved in 10 mM Tris-buffer, pH 7.4, at a concentration of 0.5 mg/ml. Iodo-gen® pre-coated iodination tubes were purchased from Pierce Biotechnology (Rockford, IL). ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Bucks, UK).

Preparation of PLGA (75:25) microspheres

Double emulsion solvent evaporation (w/o/w)

PLGA (75:25) microspheres were prepared using a modified w/o/w double emulsion solvent evaporation process, similar to that described elsewhere [13, 27]. Briefly, an aqueous solution of Ag85B-ESAT-6 was emulsified with an organic solution of PLGA (3 % (w/v)) and DDA (0.6% (w/v)) in chloroform by vortex mixing for 1.5 minutes. In order to try and maintain protein integrity and reduce shear forces, vortex mixing, rather than the more commonly used high-speed homogenisation, was employed at this stage. The primary w/o emulsion was then transferred to an aqueous solution of Chitosan (0.75%, w/v in 3% (w/v) acetic acid), and a secondary w/o/w emulsion was produced using high speed homogenisation (Silverson SL2 homogeniser at 6000 rpm), before being left under magnetic stirring for 12-18 hours at ambient conditions to allow for the evaporation of the organic solvent. Chitosan has previously been employed in

the formulation of particulate delivery vehicles [33, 49, 50], initiating enhanced Th1 immune responses [51], and therefore appears to be a viable alternative to PVA in the formulation of PLGA based microspheres. The microspheres were then harvested by centrifugation (20 minutes at 10000 x *g*), and washed three times with 10 ml of double distilled water. Harvested microspheres were either resuspended in ddH_2O for physico-chemical characterisation, or freeze-dried in the presence of 10% (w/v) sucrose for immunological investigation and then resuspended in ddH_2O prior to immunisation with the final concentration of Ag85B-ESAT-6 and DDA being fixed at 0.04 mg/ml and 1.25 mg/ml, respectively.

Single emulsion solvent evaporation (o/w)

For comparison, PLGA (75:25) microspheres were also prepared using an o/w single emulsion solvent evaporation process. Briefly, an organic solution of PLGA (3%, w/v) and DDA (0.6%, w/v) in chloroform was emulsified with an aqueous solution of chitosan (0.75%, w/v in 3% (w/v) acetic acid) using high speed homogenisation (Silverson SL2 homogeniser at 6000 rpm), before being left under magnetic stirring for 12-18 hours at ambient conditions to allow for the evaporation of the organic solvent. The microspheres were then harvested by centrifugation (20 minutes at 10000 x *g*), and washed three times with 10 ml of double distilled water.

The resultant microspheres were then resuspended in 2 ml double distilled water, and mixed with an aqueous solution of Ag85B-ESAT-6 (20.35 µl, 0.98 mg/ml) in order to facilitate surface adsorption of the antigen to the microspheres. For immunological investigations, formulations were freeze-dried in the presence of 10% (w/v) sucrose, and then resuspended in double distilled water prior to immunisation, with the final concentrations of Ag85B-ESAT-6 and DDA being fixed as before at 0.04 mg/ml and 1.25 mg/ml, respectively.

Particle size distribution analysis

Low angle laser light scattering was used to determine particle size and size distribution of microspheres with a Sympatec Helos (Sympatec, Germany). Samples were added to a magnetically stirred cell containing filtered double distilled water. The mean particle size in this case represents the De Brouckere mean

diameter, otherwise referred to as the volume or mass moment mean (D[4,3]), which avoids any need for particle counting.

Zeta potential analysis of microspheres

Surface charge on the microspheres was measured indirectly as zeta potential. The measurements were performed at 25 °C using a ZetaPlus instrument (Brookhaven Instrument Corporation, NY) by appropriately dispersing the microsphere dispersion in 2 ml 0.01M PBS solution. The reported measurements were the mean values of three independent samples, each of which was the mean value of 10 readings.

^{125}I radio labelling of Ag85B-ESAT-6

Radiolabelling of Ag85B-ESAT-6 was performed using the Iodo-gen® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). Briefly, Ag85B-ESAT-6 was diluted with 50 µl Tris-buffer (25 mM, pH 8) and added to the pre-coated iodination tube. A pre-determined activity of ^{125}I (3.7 MBq) was then diluted up to 30 µl with 25 mM Tris-buffer and added to the iodination tube. This mixture was then left for 15 minutes, with intermittent shaking, to facilitate radio labelling of Ag85B-ESAT-6. Removal of the unlabelled Ag85B-ESAT-6 was performed by Sephadex G-75 gel column separation. In order to make the column, Sephadex G-75 (1%, w/v) was first soaked in double distilled water at 90 °C for 1 hour, with stirring. The swollen gel was then packed into a 5 ml column and equilibrated with the 25 mM Tris-buffer.

Prior to separation, the reaction mixture from the iodination tube was further diluted with the Tris-buffer, and then passed through the column with 25 mM Tris-buffer as mobile phase. Aliquots of the eluted solution (0.5 ml) were collected and measured for gamma radiation using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) and also for UV absorbance at 280 nm, so as to confirm the presence of radiolabelled Ag85B-ESAT-6. The appropriate aliquots were then pooled and stored at -20 °C until required for further use.

Determination of Ag85B-ESAT-6 entrapment

The degree of adsorption of Ag85B-ESAT-6 to the microspheres prepared by the single emulsion (o/w) technique was determined by ^{125}I radiation. Radiolabelled Ag85B-ESAT-6 was added to microspheres prepared as described above, mixed, and then allowed to stand for 10 minutes at ambient conditions. The formulation was then pelleted by ultracentrifugation (100,000 x *g* for 1 hour), resuspended, and then measured for gamma radiation. Adsorption of Ag85B-ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets. Similarly, microspheres were prepared by the w/o/w process as described above, with the addition of ^{125}I labelled antigen to the internal aqueous phase in order to spike the non-radioactive Ag85B-ESAT-6. To harvest the radioactive microspheres, Beckman Quick-Seal™ centrifuge tubes (Beckman Instruments inc., Spinco division, Palo Alto, CA) were used, and entrapment efficiency was calculated from the difference of measured gamma radiation emitted from both supernatant and resuspended microspheres.

Immunological analysis of formulations

Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. Groups of five female BALB/c mice, approximately six weeks old, received doses of microsphere vaccine formulations containing 2 µg of Ag85B-ESAT-6 in a 50 µl volume. Naïve groups received the appropriate volume of PBS. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter. Blood was drawn from the tail vein upon a small incision, obtaining 50 µl with micropipette capillary tubes lightly coated in heparin solution (0.1% w/v in PBS). The blood was subsequently added to 450 µl PBS (giving a final dilution of 1/10) and centrifuged using a micro centrifuge at 13,000 rpm for 5 minutes. The supernatants of each mouse sample was collected and transferred to a fresh eppendorf prior to storage at -20 °C for future analysis. As a result, assuming that the haematocrit or packed cell volume is approximately 50%, sera obtained from each mouse consisted of a final 20-fold dilution.

Analysis of Ag85B-ESAT-6 specific antibody isotypes

Sera samples obtained at different time intervals after immunisation were analysed for the presence of anti-Ag85B-ESAT-6 IgG, IgG1 and IgG2b antibodies (AbD serotec, Oxfordshire, UK) by enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with 60 µL of Ag85B-ESAT-6 per well (3 µg/ml) in PBS and incubated at 4ºC overnight. Unbound antigen was aspirated and residual washings were removed by blotting firmly onto paper towel. Plates were blocked with 0.2 ml per well of 4% w/v Marvel in PBS. Serially diluted serum samples (60 µl per well) were transferred to washed plates and incubated for 1 h at 37 ºC. Anti-Ag85B-ESAT-6 antibodies were detected by addition of horseradish peroxidase conjugated anti-mouse isotype specific immunoglobulin (goat anti-mouse IgG, IgG1 or IgG2b), and subsequent addition of substrate solution, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in citrate buffer incorporating 5 µl of 30% H_2O_2/50 ml following repeated incubation and washing with PBST buffer. Absorbance was measured at 405 nm.

Spleen cell culture preparation

Upon termination of experiments, mice were humanely culled and their spleens aseptically removed and placed into ice-cold sterile PBS. Spleens were treated as follows: A crude suspension of spleen cells in 10 ml working media (RPMI 1640 cell culture medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml) (Gibco-Invitrogen, Paisley, UK)) was prepared by gently grinding the spleen on a fine wire screen. After allowing the cell suspension to settle for approximately 5 minutes the liquid was transferred to sterile 20 ml 'Falcon' tubes, without disturbing the cellular debris at the bottom. The cell suspension was centrifuged at 200 g for 10 min. After centrifugation the supernatant was removed, the cell pellet resuspended in 10 ml fresh working media and the centrifugation procedure was repeated. These single cell suspensions were used to assess antigen specific cytokine production and antigen specific recall responses.

Analysis of spleen cell proliferation

For study of antigen specific proliferative responses, aliquots of 150 µl volumes of sterile media or antigen in sterile media (at the concentrations stated (0.5 or 5 µg/ml)) were seeded onto 96 well suspension culture

plates and 150 µL volumes of viable splenocytes (approximately 1×10^7 cells/ml) added to each well. As a positive control, cells were co-cultured with concanavalin A at a concentration of 3 µg/ml. Covered plates were incubated at 37 ºC for 72 h. After 72 h incubation, half a microcurie of [^{3}H] thymidine (Amersham, UK) in 40 µL volumes of freshly prepared sterile working media was added to each well, and the incubation continued for a further 24 h. The well contents were harvested onto plain filter mats (Molecular Devices Ltd., Wokingham, UK) using a cell harvester (Titertek). After drying, the discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe III, Fisher Scientific UK Ltd. Loughborough) and the incorporation of [^{3}H] thymidine into the cultured cells was measured using a Tri-carb 3100TR liquid scintillation analyser (Packard BioScience Co., Meriden, CT, USA) standard counting procedures.

Analysis of cytokine production

Cytokines were detected by taking cell culture supernatants after 48 hours incubation with 2.5 µg/ml Ag85B-ESAT-6 fusion protein. The cell medium was separated by centrifugation, collected in eppendorfs and stored at -70 ºC until analysed using DuoSet® capture ELISA kits (mouse IFN-γ, IL-2, IL-5) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. Briefly, ELISA plates were first coated with capture antibody, followed by washing and blocking. Samples of cell culture supernatants were then added and cytokines detected by addition of detection antibody, enzyme marker (Streptavidin-HRP) and substrate solution following repeated incubation and washing steps. Absorbance was measured at 405 nm.

Environmental Scanning Electron Microscopy (ESEM) of microspheres

ESEM analysis was performed using a Philips XL30 ESEM-FEG (Philips Electron Optics (FEI), Eindhoven). Ag85B-ESAT-6 loaded PLGA microspheres, incorporating DDA, were prepared as described above. Following harvesting and resuspension, microsphere suspensions were loaded onto gold-sputtered mica plates in order to yield high resolution ESEM images. Gradual reduction of pressure in the sample chamber of the ESEM instrument resulted in the controlled dehydration of the sample environment (Perrie et al 2007; Mohammed et al 2004).

Statistical Analysis

Statistical analyses were performed using GraphPad Instat 3 software (Version 3.06, GraphPad Software). For *in vitro* investigations, analysis of variance (ANOVA) followed by Tukey test was performed to compare the mean values of different groups. For *in vivo* data, Kruskall-Wallis' non-parametric rank sum test followed by Dunn's post test was used for differences in humoural and cellular immune responses. Statistical significance was considered at $p< 0.05$ in all the studies.

Results and discussion

To investigate the effect of antigen location when PLGA microspheres were employed as vaccine adjuvants, microspheres were prepared by the double emulsion solvent evaporation method (w/o/w), and compared to those prepared via the single oil-in-water emulsion solvent evaporation method (o/w). Table 1 shows the particle size, zeta potential and Ag85B-ESAT-6 association efficiency of the microsphere delivery systems. Due to the presence of DDA, methods of preparation produced cationic particles of a similar diameter, although there is a slight increase and heterogeneity in measured size for those prepared by the single emulsion (o/w) method (3.0 μm and 4.7 μm for the double emulsion and single emulsion method, respectively; Table 1). The surface charge of the microspheres produced is also similar for both methods of preparation; however, the slight decrease seen for the o/w method, whilst not significant, may be due to the adsorbed layer of antigen masking the positive charge (39 mV and 34 mV for the double emulsion and single emulsion method, respectively; Table 1). This masking of the positive charge may explain the increase in mean diameter, through a reduction in electrostatic repulsion between the particles. Nevertheless, adsorption of the antigen to the surface of the microspheres does prove to be a more efficient method of association, with an increase of approximately three-fold when compared to the double emulsion method (Table 1). This result may be expected, since adsorption of the antigen to pre-formed particles adds the advantage of avoiding potential loss of antigen through migration from the internal aqueous phase during formation of the secondary emulsion, and also eliminates potential loss on washing.

The release of antigen from the microspheres formulated via the single emulsion method exhibits a notable burst release, particularly over the first 24 hours, followed by prolonged, sustained release (Figure 2), suggesting that the majority of the initial antigen load remains adsorbed to the microspheres, potentially facilitating enhanced delivery within antigen presenting cells (APCs). Following this, over the time period studied, approximately 15 - 18% of loaded antigen is released from the microsphere formulations. This delayed release may potentially be attributable to the presence of chitosan, since there is a possibility that due to its gel forming attributes and varying solubility at elevated pH, there may be a surface coating of chitosan inhibiting antigen release. However, this theory would require further investigation. For the microspheres prepared by the double emulsion method, over time a similar percentage of antigen release was found for the DDA alone formulation as to the single emulsion method.

ESEM analysis

ESEM analysis was undertaken to investigate any morphological differences between the microspheres produced by either the w/o/w or the o/w method (Figure 3). The average diameter of the particles imaged by ESEM is shown to be heterogeneous and correlated well to the volume mean diameters calculated by laser light diffraction (Table 1). Although the diameters of the individual particles appear to be similar for microspheres produced by both the w/o/w method (Figure 3A) and o/w method (Figure 3B), the location of the antigen seems to be different depending on the method of preparation, as can be expected theoretically. The presence of a surface coating, possibly of antigen, was distinguishable as a corona-like ring on the surface of the particles produced by the o/w method, which was then seen to bubble off i.e. was detached from the surface of the particle as the pressure in the sample chamber was reduced (Figure 3B). This phenomenon was only made visible by the nature of the microscopic technique, since ESEM not only allows visualisation of the sample in the hydrated state, but also allows for the alteration of the environment within the sample chamber, in this case pressure. Further investigations of antigen-free microspheres would, however, be needed to confirm this, although this was not evident for the microspheres with entrapped antigen produced by the w/o/w method (Figure 3A).

Antibody production

Analysis of the ability of the delivery systems to raise anti-Ag85B-ESAT-6 IgG, IgG1 and IgG2a antibodies was performed at regular intervals by enzyme-linked immunosorbent assay (ELISA) (Figure 4). In terms of microsphere formulation type, the location of the antigen has an influence on the type and level of antibody response achieved; considering IgG levels, the o/w formulation (antigen adsorbed) showed increased levels ($p<0.001$) of antibodies investigated as compared to microspheres with entrapped antigen (the w/o/w) formulation (Figure 4A) and, in general, the o/w formulation (antigen adsorbed) shows increased levels of all antibodies investigated compared to the w/o/w (antigen entrapped) microspheres of the same formulation (Figure 4A-E). In addition, the o/w formulation shows a mixed antibody response, with both Th1 and Th2 type antibodies showing increased levels as compared to the naïve control. For IgG1 levels, PLGA+DDA microspheres with entrapped antigen tended to show a slower onset of response (Figure 4C) and a more rapid decrease (Figure 4E) in response levels compared to PLGA+DDA microspheres with adsorbed antigen ($p<0.01$), with comparable levels only being achieved 38 days after immunisation (Figure 4D). For liposome based formulations, studies have demonstrated that formulations with surface-adsorbed antigens can be highly stable and elicit robust antibody and cell-mediated responses in mice and ferrets [52, 53], This has been suggested to be due to surface-conjugated antigen being available on the particle surface for antibody or B cell receptor (BCR) recognition, whereas encapsulated antigen requires some measure of processing or vesicle disruption to be accessible [54, 55].

Cell proliferation

Each formulation was also investigated for its ability to initiate antigen-specific spleen cell proliferation (Figure 5). Cells undergoing proliferation increase their rate of protein and DNA synthesis. The increase in DNA synthesis can be measured by adding [3H] thymidine, a radioisotope-labelled DNA precursor, to the cell culture medium. The amount of tritium taken up by the dividing cells is correlated to the level of cellular proliferation. When comparing the microsphere formulation type, in contrast to the antibody responses, the results show very little positive immunological effect for the microspheres prepared with surface adsorbed antigen, with PLGA+DDA microspheres formed using the w/o/w process (and hence antigen incorporated within the microspheres) promoting significantly higher levels of proliferation

($p<0.05$). For the w/o/w formulation this suggests an increased ability to facilitate clonal expansion in response to re-stimulation with Ag85B-ESAT-6.

Cytokine production

The formulations were also investigated for Ag85B-ESAT-6 specific cytokine production, with indicators for Th1 (IFN-γ and IL-2) and Th2 (IL-5) type immunity (Figure 6A-C). The antigen incorporated w/o/w DDA formulation showed significantly enhanced production of IFN-γ and IL-2 cytokines studied compared to the o/w formulation ($p<0.05$; Figure 6), which showed little effect immunologically, with no significant difference to the control group in terms of INF-γ, IL-2 and IL-5.

This study acts to compare the method of preparation and, hence, method of antigen association and presentation of microsphere systems as subunit vaccine delivery vehicles, both in terms of physico-chemical characteristics and immunological efficacy. A common factor for the systems investigated is their associated cationic charge (Table 1), which is considered advantageous in terms of interacting with the cells of the immune system [56-58], a process deemed as the rate-limiting step for the uptake of both drug and particulate carrier [59, 60]. Chitosan was chosen as the emulsion stabiliser for the microsphere formulation due to the relatively high associated cationic charge, which would not only allow for effective adsorption of antigen, but also inherent Th1 biased adjuvanticity, potentially allowing for stimulation of macrophages and cytokine production [37, 42, 51, 61].

In terms of the ability of the formulations to initiate antigen specific antibody production, the apparent difference in immune response between the two microsphere preparation techniques may be attributable to several factors, including size and zeta potential [57, 58, 62, 63], although the most probable cause is the way in which the antigen is released and presented to the cells of the immune system. As revealed by the *in vitro* release profiles of the systems (Figure 2), the microsphere formulation with adsorbed antigen (o/w) shows an initial burst of antigen and it is this immediate accessibility to the cells of the immune system and persistence of antigen that may explain the enhanced antigen specific antibody responses.

With regards to the cell mediated response initiated by the formulations, the microsphere preparations show the converse result to the antigen specific antibody production, with those produced by the w/o/w method achieving greater levels of cell proliferation (Figure 5) and cytokine production (Figure 6A-C) as compared to the o/w method. Again, this is likely to be related to the release kinetics of the antigen from the particulate delivery system, with the burst release of antigen likely to be the cause of the high antibody responses, whereas the low levels of cell proliferation and cytokine production initiated by the o/w microsphere preparation intimate that such rapid release systems are not ideal for promoting cell mediated immunity.

Conclusion

The particulate nature of microspheres can lead to recognition and recruitment of cells of the immune system and the consequent immunological cascade [15, 64]. However, the ability of these systems to retain and control the delivery of antigens is an important consideration. The results from the above studies demonstrate that the choice of manufacturing protocols for particulate vaccines can be used to control the physical location and release kinetics of antigens from microsphere adjuvants, with surface binding of an antigen promoting the burst release of antigen, which could promote its efficient recognition and processing, however in a soluble antigen format rather than in combination with an adjuvant. In contrast, for both antigen and adjuvant uptake, particle size is a key attribute [65], and may play a part in the immune response initiated by the various formulations [59, 66]. In our studies, the PLGA+DDA microspheres prepared using the o/w or w/o/w method were of similar size, but gave notably different results, suggesting that, in this study, the release kinetics and localisation of the antigen were the controlling factor in the immune responses. Overall, the results presented here underline the importance of considering formulation parameters and physico-chemical attributes of delivery systems to their ability to act as effective adjuvants for sub-unit vaccine antigens. In terms of microsphere preparations, the location of antigen plays a significant role on the type of immunity induced, with surface bound antigen favouring a humoural response, whereas entrapped antigen shows a propensity for cell mediated immunity.

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Figures and Tables

Table 1. The effect of preparation method on the physico-chemical characteristics of PLGA+DDA microspheres produced. Microspheres composed of PLGA were prepared by the double emulsion solvent evaporation (w/o/w) and the single emulsion solvent evaporation (o/w) method. Size was measured using a Sympatec Helos (Sympatec, Germany). Zeta potential was measured using a Brookhaven Zetaplus (Brookhaven, NY). Ag85B-ESAT-6 entrapment was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent mean ± SD of triplicate experiments.

Figure Legends

Fig. 1. Schematic representation of microsphere formulation by emulsion solvent evaporation processes.
A. water-in-oil-in-water double emulsion solvent evaporation process ($w_1/o/w_2$). Initially, an aqueous solution of antigen is emulsified with an organic, polymer containing phase by vortex mixing to form a primary water-in-oil (w_1/o) emulsion (a). This is then transferred to an external, surfactant containing aqueous phase (w_2) under homogenisation to yield the water-in-oil-in-water ($w_1/o/w_2$) emulsion (b). Solvent is then allowed to evaporate, and hardened microspheres are harvested by centrifugation (c).
B. oil-in-water single emulsion solvent evaporation process (o/w). A polymer containing organic phase is first emulsified with a surfactant containing aqueous phase under homogenisation, to yield an oil-in-water emulsion (o/w) (a). Solvent is then allowed to evaporate, and hardened microspheres harvested by centrifugation. Microspheres are then resuspended, and mixed with antigen solution by vortex mixing (b) to facilitate surface adsorption of antigen (c).

Figure 2. Cumulative antigen release (%, w/w) vs time. PLGA + DDA (o/w), PLGA +DDA (w/o/w) were incubated in Tris-HCl, pH 7.4 at 37°C. Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± SD of triplicate experiments.

Figure 3. ESEM micrographs of PLGA+DDA microspheres formulated via the w/o/w process (A) and o/w process (B). Arrow indicates presence of an adsorbed layer, possibly of antigen, as a corona-like ring associated with the surface of the microspheres (**B**), which was seen to "bubble off" at reduced pressures within the sample chamber.

Figure 4. Ag85B-ESAT-6 specific antibody titres. Groups of five female C57BL/6 mice, approximately six weeks old, received doses of vaccine formulations containing 2 µg of Ag85B-ESAT-6 in a 50 µl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Sera samples obtained at **A:** IgG antibodies, **B:** after day 12, **C:** after day 26, **D:** after day 40 and **E:** after day 54 for the antibody subsets of IgG1 (white bars) and IgG2b (black bars) antibodies by enzyme-linked immunosorbent assay (ELISA). * denotes significantly increased proliferation in comparison to naïve controls ($n=5$, $p<0.05$) ** denotes significantly increased levels in comparison to naïve controls ($n=5$, $p<0.01$) *** denotes significantly increased levels in comparison to naïve controls ($n=5$, $p<0.001$).

Figure 5. Spleen cell proliferation in response to stimulation/re-stimulation with Ag85B-ESAT-6 antigen. Cell proliferation was measured by incorporation of ^{3}H into cultured splenocytes.
** denotes significantly increased proliferation in comparison to naïve controls ($n=5$, $p<0.01$)
*** denotes significantly increased proliferation in comparison to naïve controls ($n=5$ $p<0.001$)

Figure 6. Ag85B-ESAT-6 specific cytokine production. Cytokines were detected using DuoSet® capture ELISA kits (mouse IFN-γ (**A**), IL-2 (**B**), IL-5 (**C**)) purchased from R&D systems, Abingdon, UK, according to the manufacturers instructions. * denotes significantly increased levels in comparison to naïve controls ($n=5$, $p<0.05$) ** denotes significantly increased levels in comparison to naïve controls ($n=5$, $p<0.01$) *** denotes significantly increased levels in comparison to naïve controls ($n=5$, $p<0.001$)

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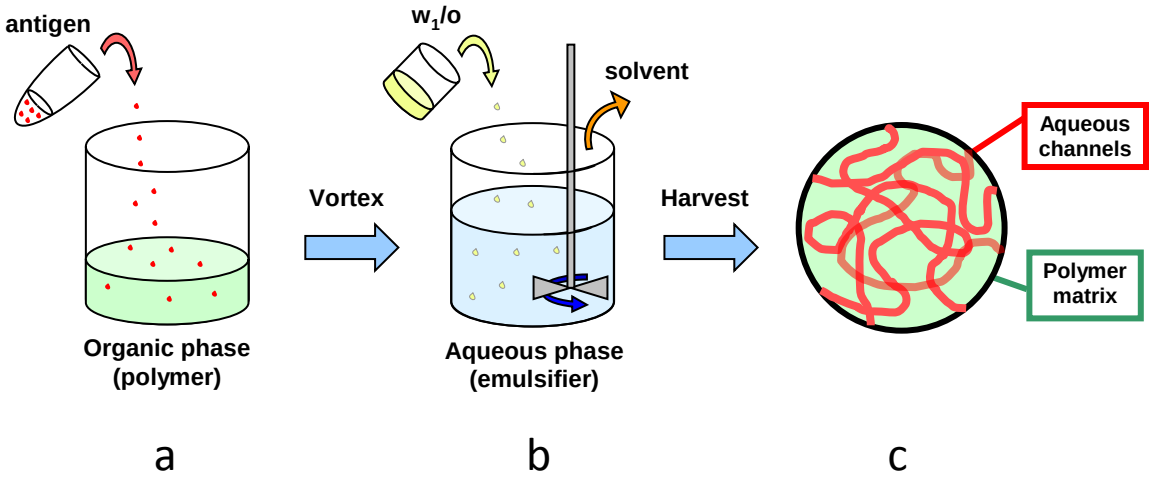
Preparation	Volume mean diameter (μm)	Zeta potential (mV)	Ag85B-ESAT-6 entrapment efficiency (%)
DDA o/w	4.7 ± 1.1	34.2 ± 2.3	77.4 ± 6.5
DDA w/o/w	3.0 ± 0.1	39.1 ± 1.6	24.2 ± 4.2

Table 1.

A. water-in-oil-in-water double emulsion solvent evaporation process ($w_1/o/w_2$)



B. oil-in-water single emulsion solvent evaporation process (o/w)

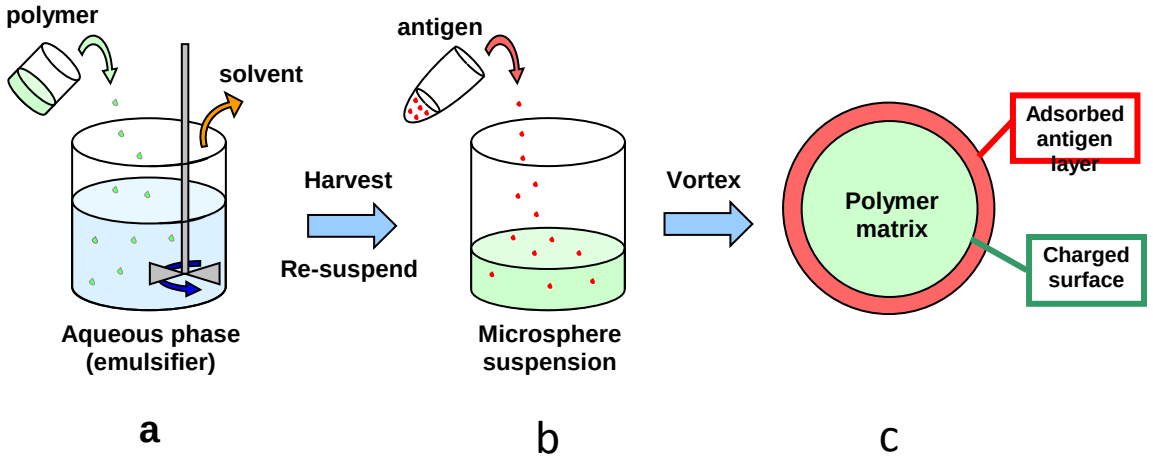


Figure 1.

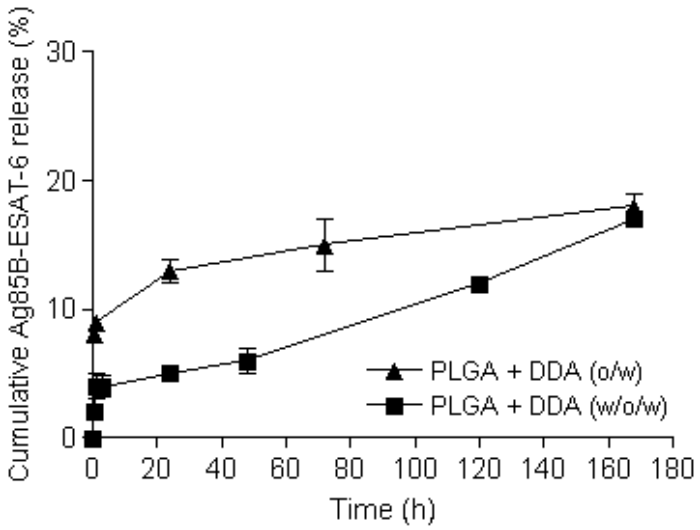

Cumulative Ag85B-ESAT-6 release (%)
30
20
10
0
0
20
40
60
80
100
120
140
160
180
Time (h)
PLGA + DDA (o/w)
PLGA + DDA (w/o/w)

Figure 2.

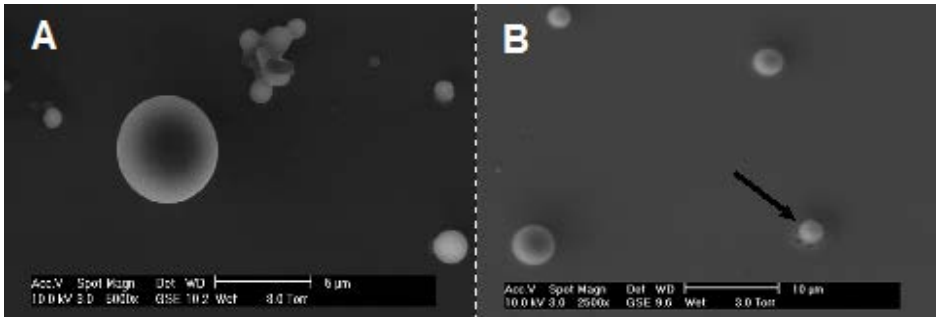


Figure 3.

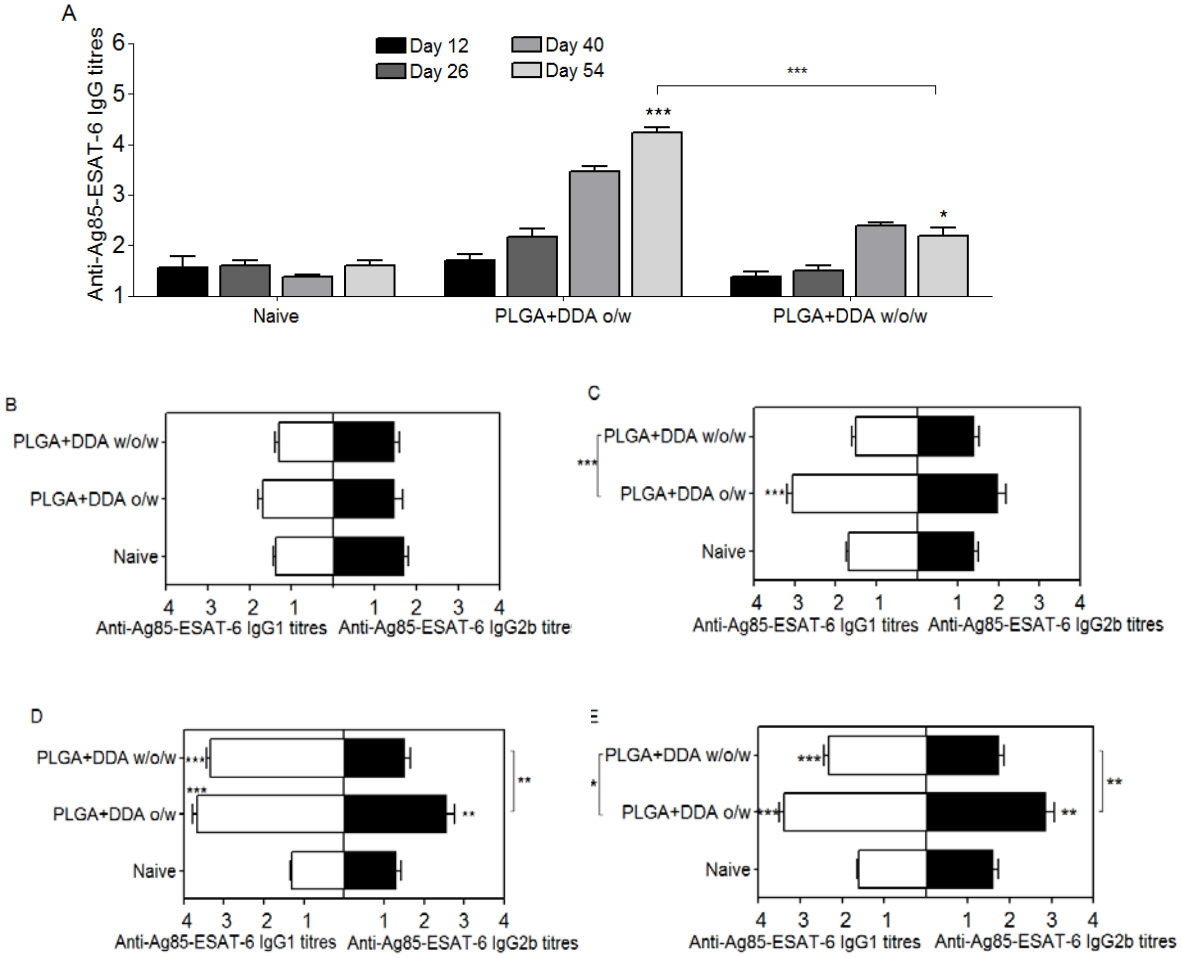

A
Anti-Ag85-ESAT-6 IgG titres
Day 12
Day 26
Day 40
Day 54
Naive
PLGA+DDA o/w
PLGA+DDA w/o/w
B
PLGA+DDA w/o/w
PLGA+DDA o/w
Naive
Anti-Ag85-ESAT-6 IgG1 titres
Anti-Ag85-ESAT-6 IgG2b titre
C
Anti-Ag85-ESAT-6 IgG1 titres
Anti-Ag85-ESAT-6 IgG2b titres
D
Anti-Ag85-ESAT-6 IgG1 titres
Anti-Ag85-ESAT-6 IgG2b titres
E
Anti-Ag85-ESAT-6 IgG1 titres
Anti-Ag85-ESAT-6 IgG2b titres

Figure 4.

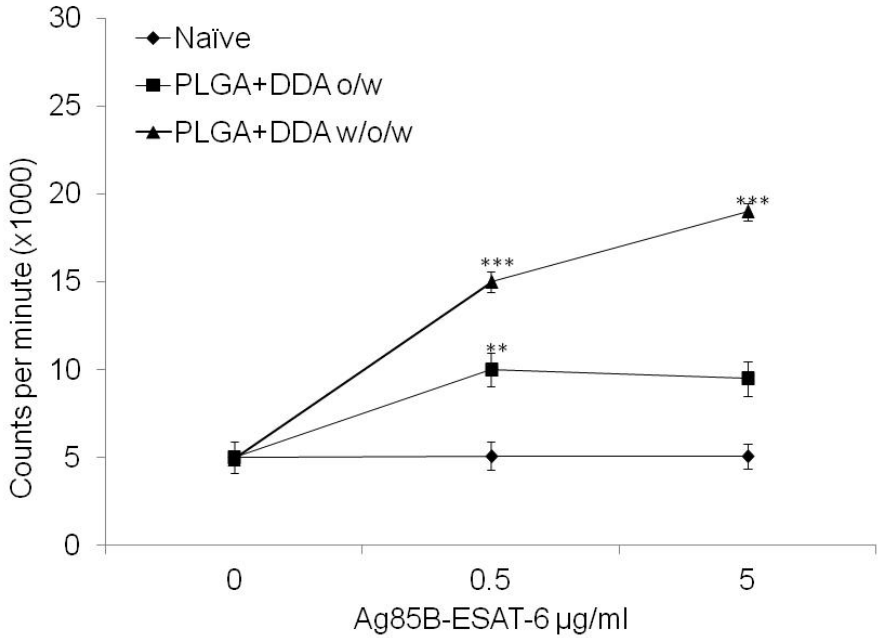

30
25
20
15
10
5
0
Counts per minute (x1000)
Naïve
PLGA+DDA o/w
PLGA+DDA w/o/w
***
***
**
0
0.5
5
Ag85B-ESAT-6 µg/ml

Figure 5.

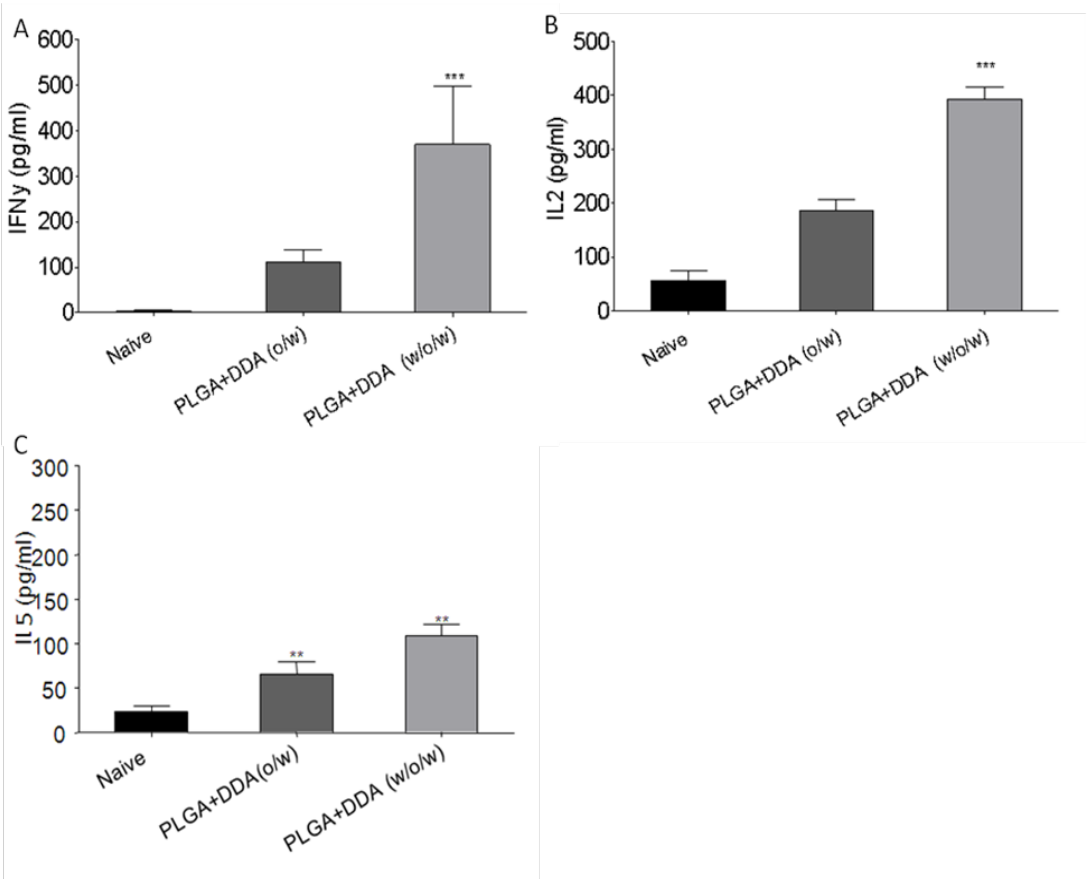

A
IFNy (pg/ml)
600
500
400
300
200
100
0
***
Naive
PLGA+DDA (o/w)
PLGA+DDA (w/o/w)
B
IL2 (pg/ml)
500
400
300
200
100
0
***
Naive
PLGA+DDA (o/w)
PLGA+DDA (w/o/w)
C
Il 5 (pg/ml)
300
250
200
150
100
50
0
**
**
Naive
PLGA+DDA(o/w)
PLGA+DDA (w/o/w)

Figure 6.