Activation of antigen presenting cells and induction of mucosal antibodies by *Chlamydia trachomatis* outer membrane peptide encapsulated within PLA-PEG nanoparticles

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ABSTRACT

In pursuit of a vaccine against *Chlamydia trachomatis*, the most reported bacterial sexually transmitted infection, we recently reported that encapsulation of M278, a peptide derived from the major outer membrane protein of *C. trachomatis*, within poly (lactic acid)-b-Poly (ethylene glycol) (PLA-PEG) nanoparticles triggered enhanced systemic adaptive immune responses in mice. Here in this study attempts were undertaken to begin to understand the mecahsnim(s) of PLA-PEG capacity to facilitate uptake of antigens by antigen presenting cells (APCs); the ability of encapsulated-M278 to trigger cytokine production by APCs and mucosal antibody immune responses in imminized mice. Our results revealed that *in vitro* exposure of mouse J774 macrophages and human THP-1 monocytes to varying concentrations of encapsulated-M278 induced marked production of the IL-6 and IL-12 Th1 cytokines, suggesting the ability of encapsulated-M278 to directly interact with and activate APCs. FITC-labelled PLA-PEG nanoparticles were localized in the cytoplasm of macrophages, confirming their phagocytic internalization. BALB/c mice immunized subcutaneously with encapsulated-M278 produced higher IgG and IgG2b M278-specific mucosal antibodies as compared to bare M278 immunized mice, suggesting that PLA-PEG potentiated the capacity of M278 to induce mucosal antibody responses in mice. Collectively, encapsulated-M278 trigerred and potentiated innate and mucosal adaptive immune responses in mice. Studies are ongoing to determine the mechanisms involved in uptake of PLA-PEG by APCs and for enhancement of adaptive immune responses in mice.

Key words: *Chlamydia*, bacteria, Major outer membrane protein, poly (lactic acid)-b-Poly (ethylene glycol), antibody, cytokines.

1.0 INTRODUCTION

Chlamydia is the most frequently reported bacterial sexually transmitted infection in the United States. In 2012, 1,422,976 cases of *Chlamydia* were reported to CDC from 50 states and the District of Columbia [1] with an estimated 2.86 million infections occurring annually. It is estimated that 1 in 15 sexually active females aged 14-19 years are infected with *Chlamydia* [2]. Therefore, development of a vaccine against *Chlamydia* is at the forefront of *Chlamydia* research since it is believed that this is the primary means of combating this bacterial infection.

Our laboratory is focused on development of a *Chlamydia* nanovacine employing biodegradable nanoparticles as delivery systems [3, 4]. Nano-particulate delivery systems belong to category of adjuvants that facilitate antigen uptake by antigen presenting cells (APCs) or by increasing the influx of professional APCs in to the injection site. PLA-PEG microparticles and nanoparticles are extensively studied as drug delivery vechicles. They offer the advantages of controlled, sustained drug release, sub-cellular size and biocompatibility. Di-block nanoparticles like Poly (D,L-lactide-co-glycoide) (PLGA) and PLA-PEG provide controlled/sustained release properties, subcellular size, and biocompatibility with tissue and cells [4, 5], and they are well established carrier systems with high potential for delivery of bioactive macromaolecules, including peptides, proteins, and nucleic acid vaccines. However limited studies have used PLA-PEG as delivery system for vaccines [6]. Moreover, very little is known about the uptake mechanism and subcellular bioavailibility of PLA-PEG nanoparticles.

Recently we reported that encapsulated-M278 (PLA-PEG-M278) induced heightened cellular and antibody immune responses as compared with bare M278 in immunized mice [4]. Here, we assessed the subcelleluar locaziation of PLA-PEG nanoparticles in APCs; the ability of PLA-PEG-M278 to interact with, and activate APCs, and to induce mucosal antibody immune responses in imminized mice.

2.0 MATERIALS AND METHODS

2.1 Preparation of Nanoparticles and FITC labelling

The M278 peptide was encapsulated in PEG-b-PLA Diblock polymer nanoparticles by a modified water/oil/water double emulsion–evaporation technique essentially as described [4]. Briefly 500 mg of PLA-PEG was emulsified in Ethyl acetate followed by addition of 2 mg of M278, homogenization and than addition of 1% Polyvinyl Alcohol (PVA). The resulting double emulsion was gently stirred overnight at room temperature (RT) to evaporate the organic solvents, harvested by ultacentrifugation, washed and lyophilized in the presence

of 5% trehalose. Sterile PBS was used in the primary emulsion formation to prepare PLA-PEG-PBS nanoparticles to serve as a negative control.

PLA-PEG-PBS nanoparticles were conjugated to FITC as reported [7]. Briefly equimolar amout of nanoparticles and FITC were mixed in borated buffer (50 mM, pH 8.5) and stirred at RT for 2 hr in dark. The resulting PLA-PEG-FITC nanoparticles were collected by centifugation, washed and lyophilized.

2.2 Scanning electron microscopy (SEM)

The morphology of PLA-PEG-FITC was investigated using SEM (Zeiss EVO 50 VPSEM) [3, 4]. The particles were mounted on metal pegs using conductive double-sided tape, and sputter coated with a gold layer prior to SEM analysis.

2.3 Stimulation of APCs with Nanoparticles

Mouse J774 macrophages [3, 4] and human THP1 monocytes [8] were used as APCs. Cells were stimulated with concentrations of PLA-PEG-M278 ranging from 1-40 µg/mL in 24-well plates and incubated at 37°C in a humidified atmosphere with 5% CO_2 from 24 to 72 hr. At the end of each incubation, cultures were centrifuged at 400*g* (4°C) to collect cell-free supernatants. PLA-PEG-PBS-exposed cells served as the negative control.

Cytokine ELISAs paired antibodies and kits (BD-Pharmingen, San Jose, CA) were employed to detect IL-6 and IL-12 secretion in cell-free supernatants [3, 8].

2.4 Immunofluorescence staining (IFA)

We conducted IFA to confirm uptake of nanoparticles by mouse J774 macrophages. Macrophages (2.5×10^4 cells/well) seeded in 8-well chamber slides were exposed to PLA-PEG-PBS and PLA-PEG-FITC nanoparticles. After 24 hr the cells were washed, fixed with 2% paraformaldehyde and then blocked with 10% normal goat serum. Cells were stained with PE-conjugated macrophage surface marker ER-MP58 (1:100 dilution) for 1 hr, washed, and then counterstained with 4',6-diamidino-2-phenylindole (DAPI) combined with an anti-fade mounting solution (Life Technologies, Grand Island, NY). Nanoparticles were visualized in macrophages by using a Nikon Eclipse Ti Confocal Microscope (Nikon Instrument, Melville, NY).

2.5 Mouse Immunization

Female BALB/c mice 5-6 weeks old were purchased from Charles River Laboratory, Raleigh, NC. Animals were maintained according to Alabama State University IACUC guidelines, housed under standard pathogen free environmental condition at ambient temperature, and supplied with food and water. Four groups of mice (at least 4 mice/group) received three subcutaneous immunizations at 2-week intervals with PBS, bare M278 (50 µg), PLA-PEG-M278 (50 µg encapsulated M278) and PLA-PEG-PBS (equivalent weight of encapsulated-M278). Immunogens were administered in 100 µL of sterile PBS [4]. Vaginal wash samples were collected two week after the last immunization.

2.6 Vaginal Wash Antibody Determination

ELISA as described previously [4] was used to determine antibody levels in vaginal wash samples. Brefily 100 µL of M278 protein at 1 µg/mL were coated in ELISA plates followed by added serial dilutions (1:25, 1:50, and 1:100) of vaginal wash samples made in dilution buffer (2% Bovine Albumin Serum (in PBS) with 0.05% Tween 20). Goat anti-mouse HRP-conjugated secondary antibodies to IgG and IgG2b were diluted to 1:4000 and 100 µL of each was added to the appropriate wells. The reaction was then developed, and absorbance was read at 450 nm.

3.0 RESULTS AND DISCUSSIONS

3.1 SEM Analysis

Scanning electron microscopy (SEM) analysis showed PLA-PEG-PBS (data not shown) and PLA-PEG-FITC nanoparticles are spherical in shape with diameters between 150-250 nm which are within agreement with our previous findings for PLA-PEG-M278 and PLA-PEG-PBS [4].

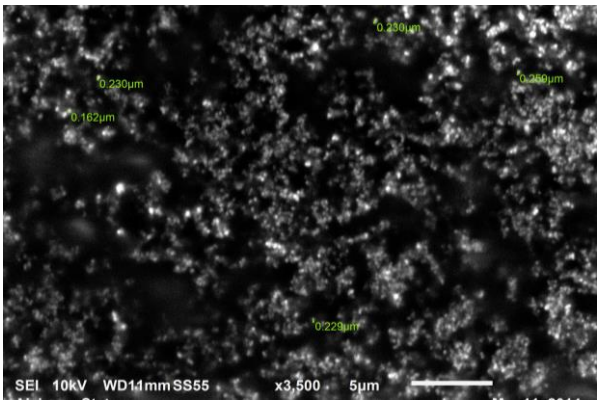
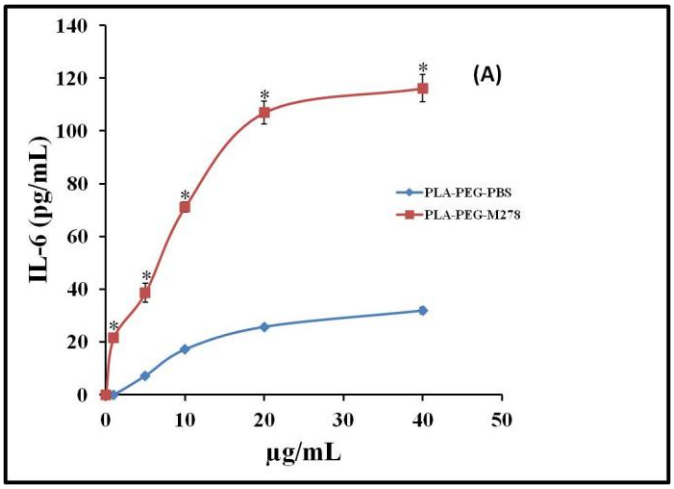
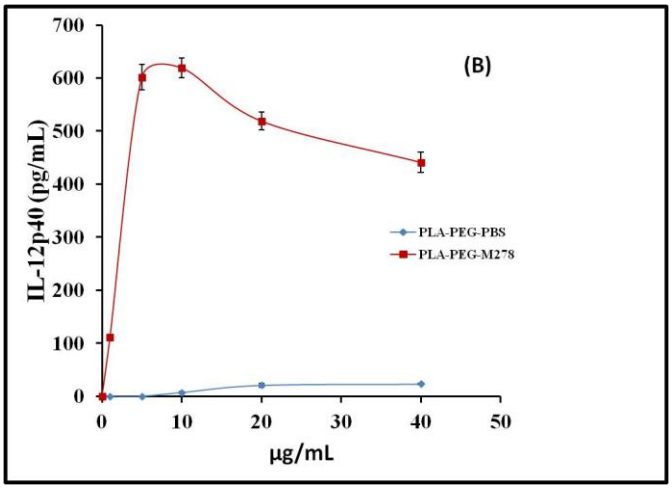


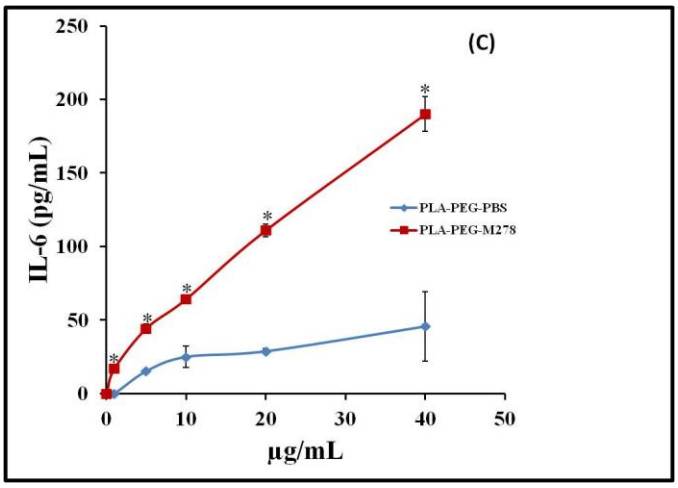
Figure 1. (A) SEM photograph of PLA-PEG-FITC. The nanoparticles were mounted on aluminum pegs using conductive double-sided tape, and sputter coated with a gold layer prior to SEM analyses.

3.2 PLA-PEG-M278 stimulates APCs

Our results show that PLA-PEG-M278 induced significant production of IL-6 and IL-12 by APCs in a dose- dependent manner in comparison to PLA-PEG-PBS. Both IL-6 and IL-12 are essential for resolving primary infections and/or resisting infections. Both play important roles in the host defense against chlamydial infection as well as other infections. Stimulation of production of cytokines further confirms the encapsulation of protein, its release and functionality.







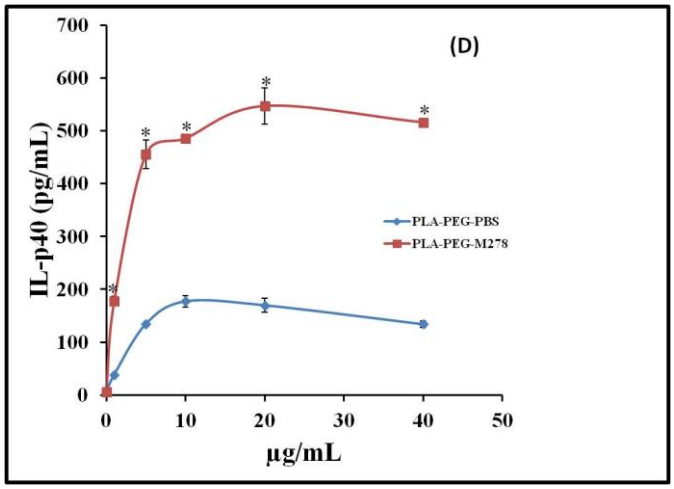
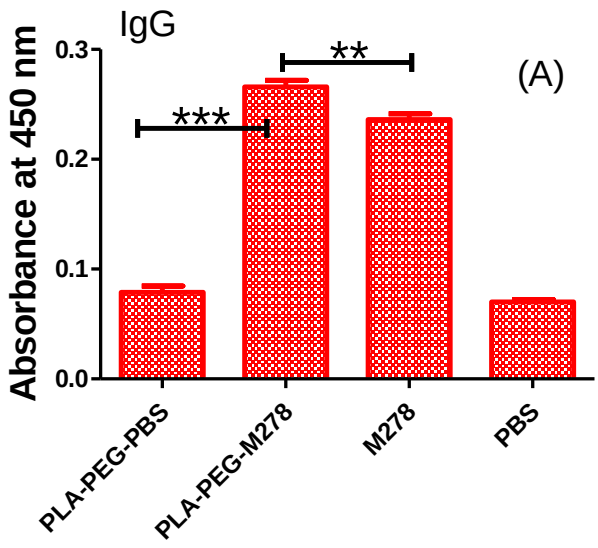


Figure. 2: IL-6 and IL-12p40 production by mouse J774 macrophages (A, B) and THP-1 cells (C,D) in response to increasing concentrations of PLA-PEG-PBS and PLA-PEG-M278 nanoparticles after 48 hr. * signifcance at $P < 0.05$

3.3 Mucosal Antibody Level

To study the mucosal immune response of encapsulated – M278 groups of BALB/c mice were immunized subcutaneously with PLA-PEG-PBS, PLA-PEG-M278, bare M278 and PBS. Vaginal wash samples were collected from mice on days 42 post-immunizations and analyzed for the presence of M278 specific antibodies by ELISA. Immunization with PLA-PEG-M278 elicited significantly ($P < 0.001$) higher IgG as well IgG2b antibodies compared to bare M278 immunized mice. Moreover, the antibody response was predominantly Th2-driven similar to our recent findings for systemic antibodies in immunized mice [4]. This enhanced antibody response may be attributed to the slow release of M278 from nanoparticles, an important attribute for a vaccine candidate.



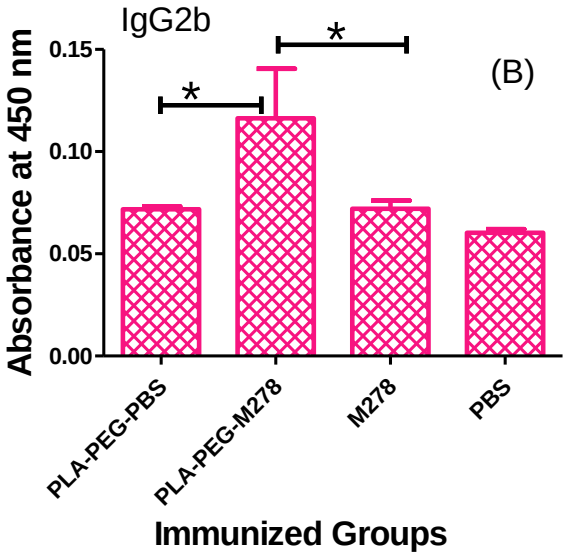


Figure. 3: (A) IgG and (B) IgG2b mucosal antibody profiles in vaginal wash samples collected from immunized groups of mice. * significnat at $P < 0.05$; ** signicance at $P < 0.01$ and *** signicance at $P < 0.001$

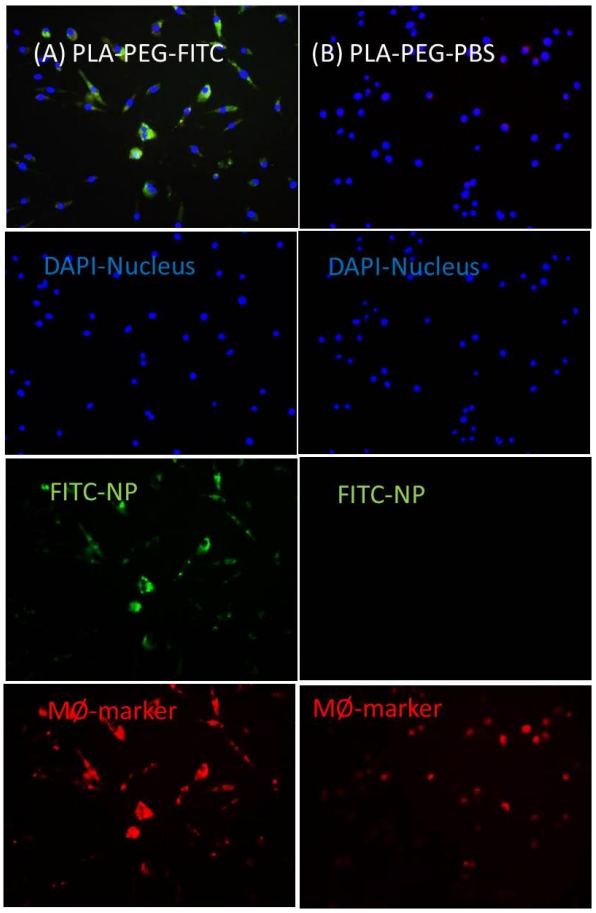


Figure 4. Macrophages were exposed to **(A)** PLA-PEG-FITC and **(B)** PLA-PEG-PBS for 24 hr. PLA-PEG-FITC nanoparticles. are visualized as green.

3.4 Nanoparticle Uptake

To determine subcellular localization and trafficking of nanoparticle, macrophages were exposed to PLA-PEG-PBS and PLA-PEG-FITC nanoparticles. Our results show, by immunoflourcence microscopy, the subcellular localization of nanoprticles in the cytoplasm of macrophages, thus confirming phagocytic internalization of nanoparticles. The phagocytic internalization of nanoparticles in macrophages is essential for activation of T-cell pathways that are invoved in adaptive immune responses. Assessments of the phagoctic internalization of nanoparticles by professional APCS like dendritic cells are currently being investigated.

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5.0 REFERENCES

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