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2017

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ORIGINAL ARTICLE

A nano particle vector comprised of poly lactic-co-glycolic acid and monophosphoryl lipid A and recombinant *Mycobacterium avium* subsp *paratuberculosis* peptides stimulate a pro-immune profile in bovine macrophages

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Keywords

Anaplasma marginale, monophosphoryl lipid A, *Mycobacterium avium* subsp. *paratuberculosis*, peptide vaccine, PLGA.

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2017/0386: received 27 February 2017, revised 19 April 2017 and accepted 9 May 2017

doi:10.1111/jam.13491

Abstract

Aims: We evaluated the potential of a nanoparticle (NP) delivery system to improve methods of delivery of candidate peptide-based vaccines for Paratuberculosis in cattle.

Methods and Results: Peptides derived from *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), and the pro-inflammatory monophosphoryl lipid A (MPLA) were incorporated in polymeric NPs based on poly (D,L-lactide-co-glycolide) (PLGA). The PLGA/MPLA NPs carriers were incubated with macrophages to examine their effects on survival and function. PLGA/MPLA NPs, with and without *Map* antigens, are efficiently phagocytized by macrophages with no evidence of toxicity. PLGA/MPLA NP formulations did not alter the level of expression of MHC I or II molecules. Expression of TNF α and IL12p40 was increased in *Map*-loaded NPs. T-cell proliferation studies using a model peptide from *Anaplasma marginale* demonstrated that a CD4 T-cell recall response could be elicited with macrophages pulsed with the peptide encapsulated in the PLGA/MPLA NP.

Conclusions: These findings indicate PLGA/MPLA NPs can be used as a vehicle for delivery and testing of candidate peptide-based vaccines.

Significance and Impact of the Study: These results will assist on more in depth studies on PLGA NP delivery systems that may lead to the development of a peptide-based vaccine for cattle.

Introduction

Paratuberculosis is a costly, chronic, progressive granulomatous disease of the small intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*), with high prevalence in US dairy herds (Harris and Barletta 2001). Control programmes for paratuberculosis have been impeded by an incomplete understanding of the immune response to *Map* and the lack of a vaccine

that induces protective immunity (Harris and Barletta 2001; Chen *et al.* 2012; Settles *et al.* 2014; Bannantine and Talaat 2015). Attempts to develop more efficient vaccines against *Map* have not been successful (Bannantine and Talaat 2015; Bannantine *et al.* 2014). Commercial vaccines available for ruminants reduce production losses, mortality, histopathological lesions (Wentink *et al.* 1994; Griffin *et al.* 2009), bacterial shedding (Kormendy 1994) and extend the productive life of vaccinated animals, but

do not provide complete protection from infection (Reddacliff *et al.* 2006; Bannantine and Talaat 2015). Moreover, the use of a killed or live attenuated vaccine is limited mainly to sheep due to the cross-reaction it produces with the immunological diagnosis of bovine tuberculosis in cattle (Stringer *et al.* 2011). The research highlighted above emphasizes the need to explore development and testing of new candidate vaccines for efficacy.

Recent studies with a *Map* relA deletion mutant suggest it might be possible to develop a peptide-based vaccine (Park *et al.* 2014). Deletion of relA, a global gene regulator, disrupts the capacity of *Map* to establish a persistent infection indicating that the response directed towards one or more *Map* proteins is sufficient to block the establishment of a persistent infection. Identification of which proteins account for blocking development of persistent infection could lead to the development of a peptide-based vaccine. Such a vaccine would clearly have advantages over killed and live attenuated mutant vaccines in terms of feasibility for large-scale commercial production, and human safety. As mentioned, one of the impediments in developing peptide-based vaccines has been identifying a delivery system that protects and optimizes delivery of peptides to antigen presenting cells (APC) through pattern recognition receptors (PRRs) that modulate the expression of cytokines that drive differentiation of T cells with different effector activity. The objective of the present study was to evaluate a novel delivery system for its potential use in next-generation vaccines for Paratuberculosis, and other pathogens affecting the livestock industry.

Poly lactic-co-glycolic acid (PLGA) is a safety polymer with diverse formulations available commercially and approved by health regulatory agencies (Danhier *et al.* 2012). PLGA NPs have been shown to boost immune responses in several animal models (Elamanchili *et al.* 2007; Klippstein and Pozo 2010; Hamdy *et al.* 2011; Danhier *et al.* 2012). For example, enhanced immune responses to entrapped antigens were observed in mice following oral administration of PLGA NPs. These findings were considered to be a consequence of their uptake into M cells, and their ability to stimulate APC in Peyer's patches in the intestines (Eldridge *et al.* 1992). PLGA NPs polarize immune responses from predominantly antibody- and Th2-type responses to the more protective Th1-type responses (Conway *et al.* 2001), and also facilitate the induction of CD8 cytotoxic T lymphocytes in several disease models (Conway *et al.* 2001; Chong *et al.* 2005; Elamanchili *et al.* 2007). Depending on the goal, an extensive variety of particle sizes with unique physicochemical characteristics, antigen load and release profiles can be formulated using PLGA. Studies have shown that 300 nm to 2 μ m NPs are readily phagocytized by

macrophages (Elamanchili *et al.* 2007; Hamdy *et al.* 2011; Danhier *et al.* 2012). Importantly, as biochemically distinct molecules can be encapsulated in PLGA NPs, a wide variety of adjuvants can be incorporated in the same PLGA NP formulation, thereby increasing the effectiveness of the vaccine (Chong *et al.* 2005; Elamanchili *et al.* 2007; Klippstein and Pozo 2010; Hamdy *et al.* 2011).

Monophosphoryl lipid A (MPLA) is a commercially available endotoxin derivative that has been used as a vaccine adjuvant (Ulrich and Myers 1995; Kathaperumal *et al.* 2008; Fichter *et al.* 2015). MPLA is produced by the hydrolysis of native diphosphoryl lipid A, the component of LPS that is recognized specifically by the pro-inflammatory receptor TLR4 (Wang 2013). The structural alterations that occur during this process (i.e. removal of all but a single phosphate group; deacylation) decrease systemic toxicity by >99% when compared to native bacterial LPS. This results in an immunomodulatory agent with greater potential for clinical use (Wang 2013; Cui *et al.* 2014; Fichter *et al.* 2015). The attenuated toxicity of MPLA is associated with reduced induction of pro-inflammatory cytokines such as TNF α , IL1 β and IFN γ during initial exposure. Despite this, MPLA retains significant immunomodulatory activity and prior treatment with MPLA increases survival after otherwise lethal exposure to LPS in animal models (Wang 2013; Cui *et al.* 2014; Fichter *et al.* 2015).

One question that had to be addressed at the outset of this study was which *Map* protein should be encapsulated into the NPs. We restricted our selection to lipoproteins because they have been shown to interact with the TLR2 pathway (Noss *et al.* 2001; Bowdish *et al.* 2009). By measuring cytokine proliferation in response to 42 *Map* lipoproteins, we identified two protein candidates (MAP2322c and MAP0981c) that induced high levels of the immune-modulatory cytokine IL12 and the pro-inflammatory cytokine TNF α , while inducing low levels of the anti-inflammatory protein IL10 (Bannantine *et al.* 2015). These two proteins were incorporated into NPs for this study.

As described herein, examination of the methods of construction and functional activity of MPLA/PLGA NP as vectors for delivery of peptide-based vaccines have shown PLGA NPs are a good choice as a delivery system for peptide-based vaccines for Paratuberculosis, Anaplasmosis and other pathogens of ruminants.

Materials and methods

Bovine monocyte isolation and *in vitro* macrophage generation

The study was carried out in accordance with the recommendations, institutional guidelines and approved Animal

Care and Use Committee (IACUC) protocols (#04543-003) at Washington State University. Peripheral blood used for monocyte isolation were collected from healthy adult Holstein dairy cows that tested negative for paratuberculosis as determined by faecal culture and polymerase chain reaction (PCR) assays of blood and faecal samples. Isolation and generation of bovine macrophages was carried out as previously described (Souza 2015). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Percoll (Sigma-Aldrich, St. Louis, MO, USA) density gradient. Briefly, blood was layered onto 50-ml conical tubes containing Histopaque 1077 (Sigma-Aldrich), and following density gradient centrifugation (500 g for 20 min) at room temperature, PBMC were collected. The PBMC were washed twice with sterile phosphate-buffered saline (PBS; Invitrogen™; Thermo-Fisher Scientific, Waltham, MA, USA) before being resuspended in PBS. Monocytes were then isolated using microbeads conjugated to a cross-reactive mouse anti-human CD14 Mab (Miltenyi Biotec Ltd, San Diego, CA). The isolation was performed according to the manufacturers' instructions. The identity and purity of monocytes was >95% as determined by flow cytometry using an anti-CD14 fluorescein-labelled antibody as described (Souza 2015). Purified monocytes were seeded at 1×10^6 per well on standard 24-well tissue culture plates containing RPMI 1640 medium (Invitrogen, Life Technologies Corporation) containing 10% heat-inactivated FBS (Sigma-Aldrich), gentamicin (5 g l^{-1} ; Sigma-Aldrich), $100 \mu\text{g l}^{-1}$ of bovine-specific GM-CSF (Kingfisher, St. Paul, MN, USA) and 1 mol l^{-1} β -mercaptoethanol. Subsequently, cells were incubated at 37°C and 5% CO_2 . On Day 7, confluent monolayers of MoM Φ displayed the characteristic macrophage morphology as confirmed by light microscopy and expected immunophenotype. Thereafter, MoM Φ were used for the *in vitro* challenge experiments with PLGA NPs formulations.

Production and purification of recombinant *Map* proteins

Recent evidence suggests that *Map* proteins play key roles in the host-immune responses to *Map* (Souza *et al.* 2013), and many vaccine trial studies using *Map* proteins as immunogens have shown promise (Rigden *et al.* 2006; Kathaperumal *et al.* 2008, 2009; Roupie *et al.* 2008). We therefore screened a large number of *Map* proteins annotated as lipoproteins to test in our *in vitro* macrophage model. These proteins were recombinantly expressed in *Escherichia coli* and purified by affinity chromatography (Bannantine *et al.* 2015). Briefly, *Map* genes annotated as lipoproteins (MAP2322c and MAP0981c), along with several control proteins were cloned into the pMAL-c2x

expression vector and transformed into *E. coli* DH5. All clones were confirmed to be correct and in-frame with the maltose-binding protein by DNA sequencing. Confirmed transformants were cultured, induced with isopropyl β -D-1-thiogalactopyranoside (IPTG), and purified as described previously (Bannantine *et al.* 2010). The only modification was that proteins eluted off the amylose resin column were collected and loaded onto a second amylose column to maximize removal of potential LPS contamination. These proteins were expected to be non-lipidated and were used for the PLGA NP synthesis.

Synthesis of PLGA NP formulations

PLGA NPs were formulated to internally carry one of five different proteins at 10% w/v: MAP0981c, MAP2322c, OVA, OVA-FITC or *Anaplasma marginale* outer membrane (OM)-derived peptides, VirB9-2 P5, or VirB9-1 P8 (which were gifts from Dr Brown, Washington State University). Externally PLGA NPs were loaded with MPLA at 1% w/v. PLGA NPs were generated using the double emulsion water-in-oil-in-water (w/o/w) technique as previously described by our group (Guldner *et al.* 2016a). Briefly, 9 g l^{-1} of PLGA 75:25 (PolySciTech®, IN) at a molecular weight of 25–35 kDa were dissolved in $450 \mu\text{l}$ of chloroform; $50 \mu\text{l}$ of PBS was then added to create a water-in-oil (w/o) emulsion. The resultant solution was emulsified by sonication twice for 30 s each on ice using a Branson Ultrasonics Sonifier™ Cup Horn sonicator at an amplitude of 20%. This first emulsion was then added dropwise into 1 ml of 1% poly vinyl alcohol (PVA) (Sigma Aldrich, St. Louis, MO, USA) at a molecular weight of 31–50 kDa, while the mixture was being continuously vortexed. Initially, 2.5 ml of a PLGA chloroform solution (60 g l^{-1}) was mixed with 0.1 ml of a MPLA from S. Minnesota R595-TLR-based adjuvant vaccine grade™ (InvivoGen, San Diego, CA) solution (20 g l^{-1}) in methanol: chloroform (1 : 4 v/v). Emulsion (w/o) was carry out by adding 0.5 ml of 20 g l^{-1} of the several peptides used in this study adjusted for the required concentration into the PLGA/MPLA recipe. A bath sonicator (Vibra Cell VC-505) at a 40% amplitude for 40 s was used for the emulsification step. Then, the emulsion (w/o) was added into a 12 ml , 1% w/v PVA solution. The recipe was then sonicated at a 40% amplitude for 2 min. The resulting emulsion was vortex overnight for proper solvent evaporation. The PLGA NPs were purified by means of four successive centrifugations at 5000 g and 4°C for 10-min dispersion cycles, and were subsequently lyophilized. Empty and PLGA/MPLA NPs served as controls. PLGA NPs were then dispersed in a 9% (w/v) sucrose solution (10 g l^{-1} NP per ml sucrose solution) followed by sterilization in a UV incubator for 10 min to 20 min.

Determination of PLGA NP morphology

The morphology of the PLGA NPs was determined by scanning electron microscopy (SEM). Briefly, dried PLGA/MPLA preparations containing MAP2322c, MAP0981c or LPS-free OVA were applied onto a pin stub covered with double-sided carbon tape and then coated with 3.5 nm of platinum/palladium using a Cressington Sputter Coater. Images were taken using an FEI Quanta 200F SEM (Franceschi Microscopy and Imaging Center, WSU, Pullman, WA) at 5–10 kV accelerating voltage. The PLGA NP preparation sizes were then measured using ImageJ software (NIH, Bethesda, MD, USA, 1.48v).

Determination of PLGA NPs polydispersibility and zeta potential

Polydispersibility and zeta potential were performed using Zetasizer nano ZS instrument and DTS software (Malvern Instruments, Worcestershire, UK). Briefly, the lyophilized PLGA NP formulations contained in microcentrifuge tubes were suspended in PBS, pH 7.4 and introduced into the instrument according to manufacture guidelines.

Determination of MAP0981c, MAP2322c, OVA and MPLA encapsulation efficiency

A protein assay kit (Pierce Biotechnology, Rockford, IL) was used for the quantification of protein loading in NP preparations (wt%). Briefly, 2.5 g l⁻¹ of PLGA NPs were dissolved in 0.2 ml of 0.1 mol l⁻¹ NaOH solution. After overnight incubation at 4°C, the protein concentration was measured using a BCA protein assay kit according to the manufacturer's instructions. Sample absorbance was measured at 562 nm by means of a microplate reader (BioTek, Winooski, VT). Empty PLGA NPs served as the control. Protein encapsulation efficiency (EE) is determined as the ratio of the protein content in the PLGA NPs divided by the total mass of protein used. A limulus amoebocyte lysate (LAL) kit (ThermoFisher Scientific, Rockford, IL, USA) was used for the determination of the MPLA LC (wt%) in the NPs. Accordingly, aqueous MPLA solutions were analysed with LAL in a plate reader to set up a calibration curve. The calibration curve for the MPLA concentration ranged from 0.01 to 10 µg l⁻¹ with a calculated correlation coefficient of $R^2 = 0.9$. MPLA content in the PLGA NPs was calculated by subtracting the concentration of supernatant-containing MPLA from the starting concentration of MPLA in the formula. MPLA EE was determined by the ratio of the measured MPLA concentration in the PLGA NPs to the total concentration of MPLA in the formula.

Intracellular uptake of PLGA NP preparations by bovine macrophages

MoMΦ were incubated with 50 mg l⁻¹ PLGA/MPLA alone or PLGA/MPLA NP containing LPS-free OVA-FITC (EndoFit™; InvivoGen) for 24 h. The cells were detached from the 12-well plates after a 10 min-incubation at 37°C with a prewarmed solution of 10 mol l⁻¹ EDTA containing 5% FBS. After two washes, the cells were fixed with 2% paraformaldehyde containing 2% glutaraldehyde in 0.1 mol l⁻¹ PBS overnight at 4°C. MoMΦ were dehydrated with ethanol: acetone solution and embedded in resin. Sections were realized via a microtome (Leica Reichert Ultracut R Microtome, Franceschi Microscopy and Imaging Center, WSU). The sections were then placed on a nickel grid, followed by staining with 2% aqueous uranyl acetate. Analysis of the uptake of PLGA/MPLA OVA-FITC NPs by MoMΦ was performed by transmission electron microscopy (TEM) (FEI Tecnai G2 20 Twin, Franceschi Microscopy and Imaging Center) at 200 kV.

Cellular uptake of PLGA/MPLA OVA-FITC NP was subsequently evaluated by confocal microscopy (Zeiss 510 META Confocal Laser Scanning Microscope, Franceschi Microscopy and Imaging Center). Briefly, MoMΦ were cultured on coverslips in a six-well culture plastic plate and then incubated for 2 h with 50 mg l⁻¹ of PLGA NP formulations as described above. After cell culture medium removal, the cells were fixed with 2% paraformaldehyde for 10 min at 37°C. The coverslips were rinsed with PBS and stained with a drop of ProLong® Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) and evaluated by confocal microscopy. DAPI was excited with a diode laser (405 nm) and the emission was collected with a BP420-480 IR filter. FITC was excited with an argon laser at 488 nm and the emission was collected with BP505-570 IR filter. Multitracking was used to decrease interchannel cross-talk and confocal scanning parameters were set up for bright field background.

Further evaluation of phagocytosis was performed by flow cytometric analysis. Briefly, after 7 days of culture, 1×10^6 MoMΦ per ml were treated with either PBS, 50 mg l⁻¹ OVA-FITC or PLGA/MPLA OVA-FITC NP for 24 h. Four wells of a standard 12-well plastic culture plate were assigned to each of these treatment groups. The cells were harvested using a sterile cell scraper, and thoroughly washed using PBS at 37°C to remove non-phagocytized PLGA NPs. Samples were analysed on a BD FACSCalibur and analysed using FCS Express 4 (DeNovo Software, Los Angeles, CA). Results were presented as histograms, with the macrophages incubated with PBS and an isotype Mab as a control for nonspecific binding.

Viability of bovine macrophage exposed to PLGA NP preparations

MoM Φ viability was evaluated by trypan blue exclusion assay and morphology by light microscopy. MoM Φ were incubated with 50 mg l⁻¹ of PLGA/MPLA NPs, 50 mg l⁻¹ of PLGA/MPLA/MAP0981c or 50 mg l⁻¹ of PLGA/MPLA/MAP2322c NPs for 2 or 24 h. The adherent cells were harvested after incubation at 37°C with pre-warmed solution of 10 mol l⁻¹ EDTA in 5% PBS and mixed with trypan blue (1 : 10). The percentage of viable MoM Φ (cells that did not take up trypan blue), was assessed in a haemocytometer chamber using light microscopy. Aliquots of 1 × 10⁶ MoM Φ were further cytocentrifuged at 4000 g for 5 min (Cytospin™; ThermoFisher, Waltham, MA), and stained with a Wright's-based stain. Thereafter, macrophage morphology was evaluated by light microscopy for evidence of apoptosis or cell necrosis by a Board Certified Clinical Pathologist.

Determination of TNF α , IL12p40 and IL10 expression by qRT-PCR

Seven-day-old bovine MoM Φ were incubated with 50 mg l⁻¹ of PLGA/MPLA NPs, 50 mg l⁻¹ of PLGA/MPLA/MAP0981c, 50 mg l⁻¹ of PLGA/MPLA/MAP2322c NPs or 1 mg l⁻¹ of LPS for 0, 24, 36 or 72 h. Total RNA was extracted from the treated cells using the Aurum™ Total RNA Mini Kit (BioRad, Hercules, CA) following the manufacturer's recommendations for cultured cells. The amount of total RNA was determined spectrophotometrically (NanoDrop™ 1000 Spectrophotometer; Thermo Scientific, Waltham, MA). Reverse transcription of mRNA into cDNA was processed in accordance with the manufacturer's recommendations (iScript™ Reverse Transcription Supermix for RT-qPCR; BioRad, Hercules, CA) to obtain a cDNA final volume of 20 μ l from 100 ng of total RNA using a thermal cycler (Axygen® MaxyGene™ II Thermal Cycler, Corning, NY). The cDNA product was amplified by PCR using validated primers designed by Qiagen RT2 qPCR Primer Assay as described (Souza 2015). Expression levels of GAPDH gene were used to normalize the input of cDNA. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's recommendations. Briefly, each well contained a 20- μ l reaction mixture consisting of 10 μ l of the SYBR Green qPCR Master Mix, 4 μ l of water, 2 μ l of each specific primer set and 4 μ l of 1/20 diluted cDNA template. The thermal profile settings consisted of a 10-min denaturation at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C and a dissociation stage at the end of the run from 60 to 95°C with 0.3°C

increments, and were carried out in a StepOnePlus™ Real-Time PCR system (Applied Biosystems). At least two replicates of each product to verify for accuracy and a nontemplate control to test for contamination of assay reagents were included in each experiment. The relative quantity of target, normalized to an endogenous control and relative to a reference sample, was calculated based on the threshold cycle (Ct) value of 2^{- $\Delta\Delta$ Ct} using StepOne™ software (ver. 2.3).

Immunophenotypic analysis of bovine macrophages incubated with PLGA NP preparations

For a 24-h period prior to collection, 1 × 10⁶ MoM Φ in 1 ml were incubated with PBS, 50 mg l⁻¹ of PLGA/MPLA NPs and 50 μ g ml⁻¹ PLGA/MPLA/MAP2322c NP in individual standard wells of a 12-well plastic culture plate. Three to four wells were assigned to each of the aforementioned study groups. The macrophages treated with PBS were utilized as a control. The cells were collected using a sterile cell scraper, and gently washed using PBS at 37°C. For phenotypic maturation studies, the MoM Φ of each group were separately incubated with 15 mg l⁻¹ of COLIS69A (Mab isotype control with specificity for *E. coli* J5), major histocompatibility complex (MHC) I (clone H6A) and MHC II (clone CAT82A), followed by incubation with a fluorescein-conjugated IgG/IgM antibody. All Mabs were obtained from the Washington State University Monoclonal Antibody Center, Pullman, WA. Samples were acquired on a Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ) by gating on the live cell populations. All non-CD14⁺ populations were excluded. The data were further evaluated using FCS Express 4 (DeNovo Software) and presented as dot plots.

CD4T Cell proliferation assay

Blood was collected from calf 48432 (DRB3*1101/*1401) that was previously immunized four times subcutaneously at 3-week intervals with 60 μ g of purified *A. marginale* St. Maries strain OMs (Lopez *et al.* 2005) emulsified in 6 mg of saponin diluted in 1.3 ml PBS. PBMC were purified from blood samples by Histopaque gradient centrifugation, repeatedly washed with Hanks' buffered salt solution (ThermoFisher Scientific, Waltham, MA), suspended in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100 mmol l⁻¹ L-glutamine, 50 mol l⁻¹ 2-ME, 24 mmol l⁻¹ HEPES buffer, and 50 mg l⁻¹ gentamycin sulphate (complete RPMI; cRPMI). To establish 2-week T-cell lines, 4 × 10⁶ PBMC were cultured in 1.5 ml cRPMI with 1 mg l⁻¹ *A. marginale* OM in 24-well plates and incubated in 5% CO₂ at

37°C for 1 week allowing an enrichment of OM-specific T cells. T cells were collected, washed and resuspended in cRPMI, and cultured in 24-well plates at 7×10^5 cells per well with 2×10^6 cells per well of irradiated (3000 rads) autologous PBMC as a source of APC without antigen (rested) for 1 week in 5% CO₂ at 37°C. Cells were then centrifuged, washed, and resuspended in cRPMI. 3×10^4 T cells and 2×10^5 irradiated PBMC were cultured in a total volume of 100 μ l in 96-well round-bottomed plates. *Anaplasma marginale* OM-derived peptides VirB9-1 P8 (negative control) and VirB9-2 P5 were tested at 1 mg l⁻¹ and 10 mg l⁻¹. NP formulations PLGA/MPLA (control), PLGA/MPLA/VirB9-1 P8, and PLGA/MPLA/VirB9-2 P5 were tested at 5 and 25 mg l⁻¹ NP. For all assays, cells with medium only served as a negative control and cells with 10% T-cell growth factor (TCGF) served as a positive control. Cells were incubated in 5% CO₂ at 37°C for 3 days, radiolabelled with 0.25 μ Ci 3H-thymidine per well and incubated an additional 18 h before harvesting with a Tomtec Harvester96 and quantifying with a PerkinElmer 1450 TriLux MicroBeta liquid scintillation counter (Waltham, MA, USA). Results are presented as the Stimulation Index, calculated as the mean CPM of cells cultured with antigen/mean CPM of cells cultured with medium.

Statistical analysis

The experiments were run in biological triplicates. A two-tailed paired *t*-test was used to compare two data. Values of *P* < 0.05 were considered statistically significant.

Results

Characterization of PLGA NP formulations

Physical–chemical characterization, including hydrodynamic diameter, zeta potential, Polydispersity Index, mobility, conductivity and encapsulating efficiency (EE) of peptides and MPLA, of the several PLGA NPs

constructs used in this study are summarized in Table 1. The mean diameters of PLGA varied from 431 to 1218 nm. Zeta potential showed negative values for all NP preparations (−8.58 to −14.8 mV). PLGA/MPLA NP showed reduced overall surface negativity except for PLGA/MPLA/MAP2322c. The results described herein suggest negligible differences among the PLGA NPs preparations used in this study. The EE for the PLGA NPs preparation varied from approximately 50–100%. Higher EE were also obtained for preparation containing MPLA with exception of the PLGA/MPLA/MAP2322c. SEM of the PLGA/MPLA/Map peptide and Ova NPs showed that the NPs were spherical, presenting with smooth surface with absence of superficial irregularities containing an electron dense core when peptides were present. A representative example is shown in Fig. 1a,b. The *in vitro* release of PLGA/MPLA/MAP0981c and PLGA/MPLA/Ova NP in PBS at 37°C was evaluated over 4 weeks. Results indicated that ~30% of MAP0981c is released from PLGA/MPLA NPs during the first week reaching at maximum a released profile of ~60% after 4 weeks (data not shown). The obtained results were in agreement with those previously reported in the literature for the same PLGA NPs under similar conditions (Sarti *et al.* 2011; Danhier *et al.* 2012; Guldner *et al.* 2016a).

PLGA NP uptake by bovine macrophages

Uptake of the PLGA NP preparations by bovine MoM Φ was evaluated by flow cytometry, confocal microscopy and TEM. Only PLGA/MPLA preparations containing OVA-FITC were used for the confocal and flow cytometry experiments. Bovine MoM Φ were incubated with PLGA/MPLA/OVA-FITC for 24 h and mean fluorescent intensity (MFI) of FITC was measured by flow cytometry against macrophages incubated with PBS or OVA-FITC (i.e. positive control). Results showed that PLGA/MPLA NPs were efficiently internalized by MoM Φ (Fig. 2g,h). These observations were consistent with previous studies performed on the ability of APCs to efficiently

Table 1 Properties of PLGA NPs constructs used in this study

Formulation	Hydrodynamic diameter (nm)	Zeta Potential (mV)	Polydispersity Index	Mobility (μ m-cm Vs ⁻¹)	Conductivity (mS cm ⁻¹)	Protein EE (%)	MPLA EE (%)
PLGA	431 SD \pm 162	−14.8	0.141	−1.159	18	–	–
PLGA/OVA	658.1 SD \pm 273.7	−11.3	0.518	−0.885	19	100	–
PLGA/MPLA	400 SD \pm 125	−8.58	0.947	−0.672	18	–	98.41
PLGA/MPLA/OVA	566.7 SD \pm 339.3	−9.41	0.358	−0.737	18	90.8	97.34
PLGA/MPLA/0981c	1218 SD \pm 118	−8.58	0.947	−0.672	18	108.5	68.9
PLGA/MPLA/2322c	764.7 SD \pm 628.5	−11.4	0.675	−0.896	18	50.13	37.2

EE, encapsulation efficiency; PLGA, poly (lactic-co-glycolic acid); MPLA, monophosphoryl lipid A; OVA, ovalbumin; 0981c and 2322c, MAP recombinant peptides.

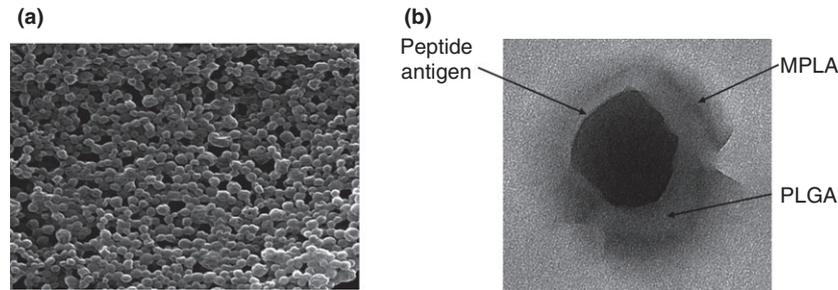


Figure 1 Morphological characterization of PLGA/MPLA NP. (a) Morphological analysis of PLGA/MPLA NP by scanning electron microscopy (SEM). (b) Transmission electron microscopy (TEM) of a single PLGA/MPLA/peptide NP. Scale bar = 1 μm (a) and 100 nm (b). PLGA, poly lactic-co-glycolic acid; MPLA, monophosphoryl lipid A.

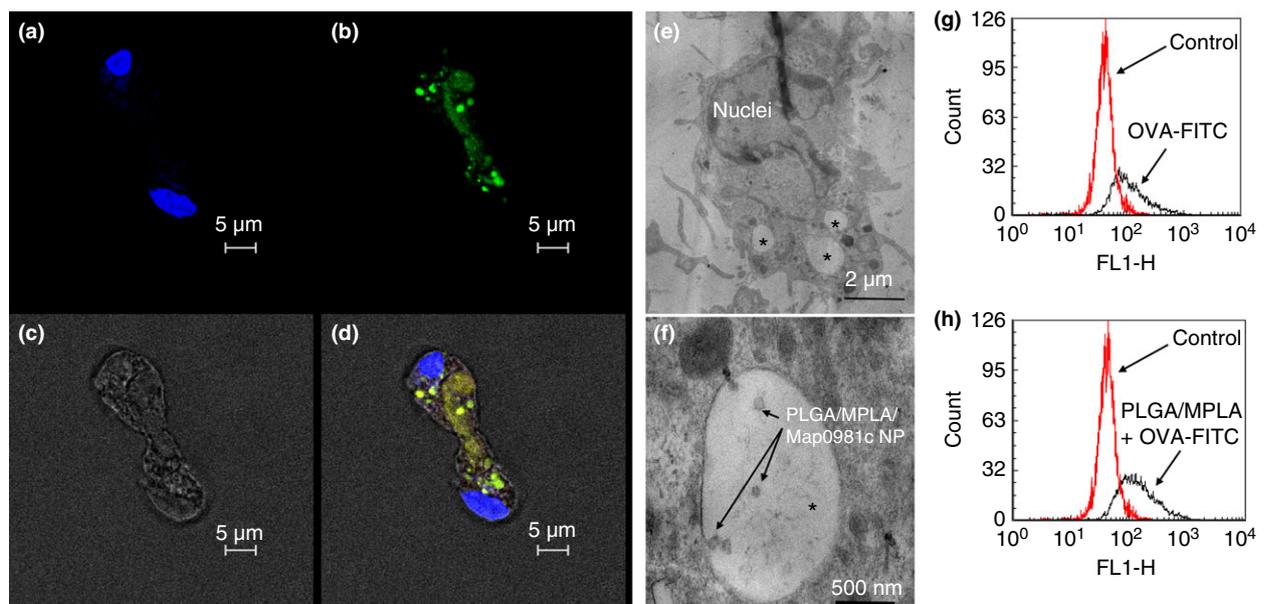


Figure 2 Cellular localization of PLGA/MPLA/OVA-FITC NPs uptake by macrophages and evaluated by confocal laser scanning microscopy after 2 h of incubation. Day 7 bovine MoM Φ cultures (1×10^6) were incubated with 50 mg l^{-1} of PLGA/MPLA/OVA-FITC NPs. After 24 h, MoM Φ were harvested and analysed by confocal microscopy. One representative experiment is shown. (a) Nucleus of macrophages stain blue by DAPI. (b) PLGA/MPLA/OVA-FITC NPs stain green. (c) Bovine MoM Φ observed by phase contrast microscopy. (d) Merge of (a) and (b) demonstrating internalization of NPs. Scale bar = 5 μm . DAPI = 4',6-diamidino-2-phenylindole, dihydrochloride; FITC, fluorescein isothiocyanate. Transmission electron microscopy (TEM) evaluation of cellular uptake of PLGA/MPLA NP preparations by bovine macrophages. Day 7 bovine MoM Φ cultures (1×10^6) were incubated with PBS or 50 mg l^{-1} of PLGA/MPLA/Map0981c NPs. After 24 h, nonadherent cells were harvested and analysed by TEM. (e, f) MoM Φ incubated with PLGA/MPLA/Map0981c NPs for 24 h showing PLGA/MPLA NPs within phagocytic vacuoles (*). Flow cytometric evaluation of cellular uptake of PLGA NP preparations by bovine MoM Φ . Flow cytometric analysis of MoM Φ treated with PLGA/MPLA/OVA-FITC NPs. MoM Φ cultures (1×10^6) were incubated with 50 mg l^{-1} of PLGA/MPLA/OVA-FITC NP or OVA-FITC. After 24 h, nonadherent cells were harvested and analysed by flow cytometry. One representative experiment is shown. (g) Mean fluorescence intensity (MFI) of MoM Φ incubated with 50 mg l^{-1} OVA-FIT for 24 h. (h) MFI of MoM Φ incubated with 50 mg l^{-1} PLGA/MPLA/OVA-FITC for 24 h. [Colour figure can be viewed at wileyonlinelibrary.com]

phagocytize PLGA NPs (Keijzer *et al.* 2011, 2013; Danhier *et al.* 2012). TEM studies demonstrated that MoM Φ incubated with PLGA/MPLA NPs containing OVA or MAP0981c for 24 h displayed several electron dense intracytoplasmic structures compared to the control (i.e. PBS alone, data not shown), consistent with

internalization of the PLGA NPs (Fig. 2e,f). In addition to flow cytometry and TEM, the cellular localization of PLGA NPs was assessed by confocal microscopy and showed efficient uptake of NPs (Fig. 2a–d). Comparable results were obtained when PLGA without MPLA NPs were used (data not shown), suggesting that MPLA had

no effect on the ability of bovine MoM Φ to phagocytize the PLGA NPs formulations used in this study.

Macrophage viability studies

MoM Φ viability was evaluated by incubating 7-day-old bovine MoM Φ with PLGA/MPLA, PLGA/MPLA/MAP0981c and PLGA/MPLA/MAP2322c NPs for 2 or 24 h. The results showed that there were no significant differences between the control groups and the several PLGA NPs tested at 2 or 24 h postincubation (Fig. 3a). In addition, cultures of MoM Φ incubated with different PLGA NPs constructs for 24 h and evaluated by light microscopy by a Board Certified Clinical Pathologist demonstrated absence of abnormal cytomorphological features consistent with cell death (i.e. apoptosis and necrosis; Fig. 3b). Lack of toxicity of PLGA NPs with or without MPLA to APCs was expected and corroborate with studies performed previously (Silva *et al.* 2015; Guldner *et al.* 2016a). Daily visual evaluation of the

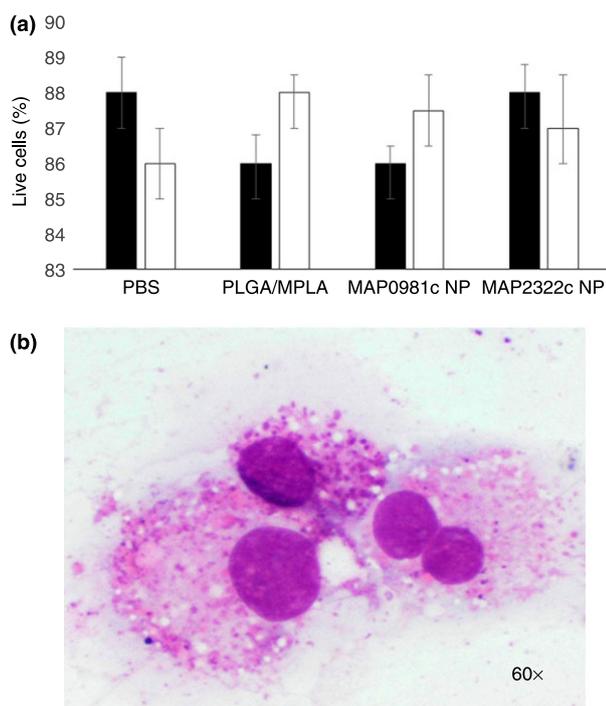


Figure 3 Evaluation of bovine macrophage viability incubated with PLGA NP formulations. Day 7 bovine MoM Φ cultures (1×10^6) were incubated with 50 mg l^{-1} of different PLGA NP formulations for 2 h or 24 h at 37°C . MoM Φ were then stained with Trypan Blue and evaluated microscopically. (a) Cells that take the stain have impaired membranes and are considered dead cells. (b) A total of Day 7 (1×10^6) MoM Φ were cytocentrifuged and evaluated under light microscopy for evidence of pyknosis or cellular necrosis. Similar data were obtained in three independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

MoM Φ in culture for up to 5 days postviability analysis did not demonstrate cytomorphological changes that could be consistent with cell death. In addition, the RNA concentration and its quality also did not change throughout the set points we used for the experiments used on this study (data not shown).

Cytokine expression

Pro-inflammatory and pro-immune cytokine production by primed APCs are considered important components of an effective immune response and thus, essential in designing effective vaccines. In this study, pro-inflammatory (i.e. TNF α), pro-immune (i.e. IL12p40) and anti-inflammatory (i.e. IL10) cytokine expression were measured to evaluate the potential of PLGA NP preparations to induce early, protective, immune responses initiated by MoM Φ . Bovine MoM Φ were incubated with different PLGA NP constructs and the expression of the above cytokines was determined at different time points (Fig. 4). As expected, incubation of MoM Φ with PLGA NPs containing MPLA (i.e. LPS derivative), upregulated TNF α expression at all time points in this study. Increases of more than 10-fold compared to control were observed. The results also showed that PLGA/MPLA with or without addition of MAP0981c or MAP2322c upregulated TNF α at comparative levels as bacterial LPS at early time points. Surprisingly, this increase was significantly higher than the one elicited by LPS at later time points, particularly when *Map* antigens were included in the formulations (Fig. 4a). Similar increases were seen with IL12p40 expression at all time points studied when macrophages were incubated with LPS or PLGA NPs preparations with or without addition of MAP0981c or MAP2322c (Fig. 4b). Importantly, expression of IL10 did not appear to be significantly induced at later time points by incubation of MoM Φ with any of the PLGA NP formulations used in this study (Fig. 4c).

Analysis of MHC I and MHC II expression

Seven-day-old MoM Φ were stimulated with empty PLGA/MPLA NP or PLGA/MPLA/MAP0981c NPs for 24 h and thereafter cells were evaluated by flow cytometry. Results showed no difference in the expression of MHC I or II by bovine MoM Φ stimulated with the above PLGA NPs (data not shown).

T-cell stimulation assay

As a proof of concept, to show that the PLGA/MPLA NPs loaded with antigen could stimulate CD4 T cells, we used T cells from a calf that had been previously

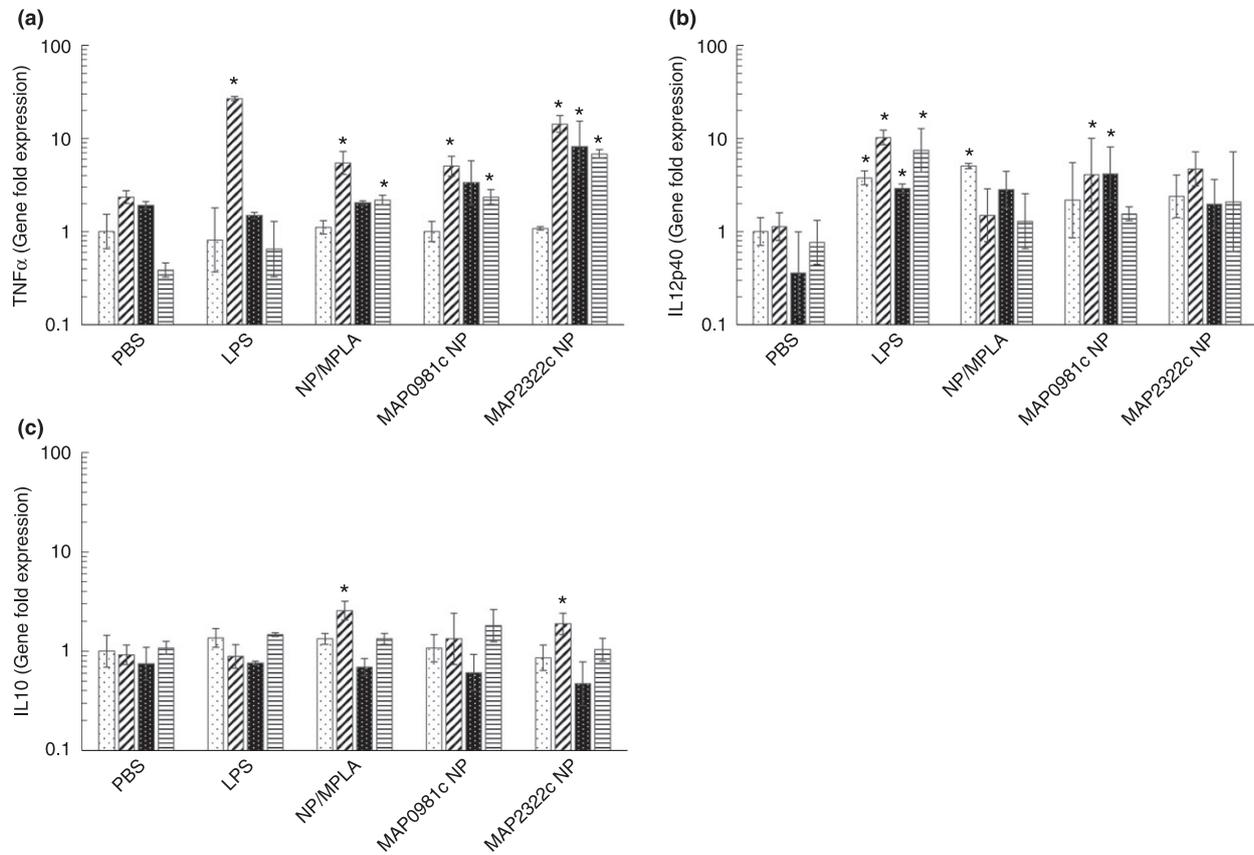


Figure 4 Effects of incubation of PLGA NP preparations on TNF α (a), IL12p40 (b) and IL10 (c), gene expression by bovine MoM Φ . Day 7 MoM Φ (1×10^6 per mL) were pretreated with 50 mg l^{-1} of PLGA/MPLA NP, PLGA/MPLA/MAP0981c NP, PLGA/MPLA/MAP2322c NP or LPS (1 mg l^{-1}) for 0, 24, 48 or 72 h. GAPDH was used to normalize the results. *Statistically significant difference when compared to PBS and PLGA/MPLA NP-treated group ($P < 0.05$). Similar data were obtained in three independent experiments. □ 0 h; ▨ 1 day; ■ 3 day; ▤ 5 day.

immunized with *A. marginale* OM expressing MHC II DRB3 haplotype *1101, which was shown to respond to peptide 5 of VirB9-2, but not peptide 8 of VirB9-1 (Morse *et al.* 2012). PLGA/MPLA NP were loaded with each peptide (as described above) and tested with free peptide in a proliferation assay using a short-term T-cell line, started from PBMC stimulated with OM for 1 week and rested with APC without antigen for 1 week. The cell line responded specifically and significantly to VirB9-2 P5, but not VirB9-1 P8 as predicted (Fig. 5). Importantly, the T cells also responded specifically and significantly to PLGA NPs loaded with VirB9-2 P5, but not with VirB9-1 P8 or empty PLGA/MPLA NPs. The response to VirB9-2 P5-loaded PLGA/MPLA NP was lower than the response to free peptide VirB9-2 P5 as there is an estimated 10-fold less peptide present in the PLGA NPs formulation.

Discussion

The objective of the present study was to evaluate the potential of PLGA/MPLA NPs, as a vector system, for

delivery of candidate peptide-based vaccines for Paratuberculosis. Initial studies showed a modified double emulsion solvent evaporation technique was the most efficient method to prepare PLGA/MPLA NPs (Danhier *et al.* 2012; Guldner *et al.* 2016a). Physicochemical analysis of the PLGA/MPLA NP constructs in solution demonstrated that NPs in solution were dispersed with limited aggregation of particles. Examination by SEM showed that PLGA NPs had a smooth regular surface in a size range optimal for uptake by MoM Φ (Table 1) similar to the size ranges observed in other studies (Elamanchili *et al.* 2007; Kanchan and Panda 2007; Guldner *et al.* 2016b). Examination by TEM showed the PLGA/MPLA NPs containing peptides were comprised of an outer ring of MPLA and an inner ring of PLGA with a dense peptide central core. Phagocytosis and viability studies showed the PLGA NPs and PLGA/MPLA NPs were taken up rapidly and efficiently with no evidence of cytotoxicity at the concentrations used in the studies. Similar to previous reports, phagocytosis of the PLGA/MPLA NPs increased the expression of TNF α and IL12p40. Flow

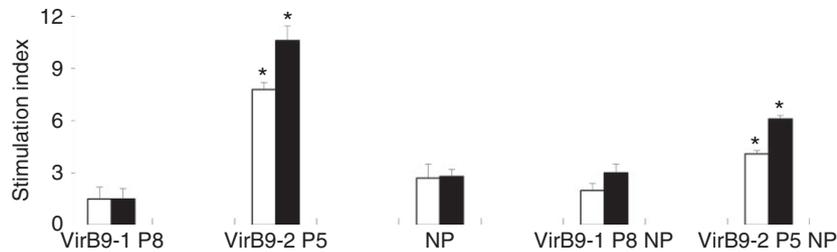


Figure 5 PLGA/MPLA NP loaded with *Anaplasma marginale* outer membrane antigen-stimulated CD4 T cells. *Anaplasma marginale*-immune T cells were washed, and cultured at 3×10^4 cells per well in triplicate with 2×10^5 irradiated, autologous PBMC as a source of APC. Free peptide (VirB9-1 P8 and VirB9-2 P5) were tested at 1 and 10 mg l⁻¹ and PLGA/MPLA NP formulations were tested at 5 and 25 mg l⁻¹ of PLGA NP. White bars and black bars represent low and high antigen concentration respectively. *VirB9-2 P5 and PLGA/MPLA/VirB9-2 P5 NP stimulated significant responses compared to VirB9-1 P8, or empty PLGA/MPLA NP or PLGA/MPLA/VirB9-1 P8 NP, respectively, at both antigen concentrations, where $P < 0.05$ using a Student's *t*-test.

cytometric analysis of the level of expression of MHC I and II molecules showed there was no clear change in the level of expression of MHC I or MHC II molecules following uptake of PLGA/MPLA NPs with and without inclusion of peptides (data not shown).

The last set of experiments were conducted for proof of concept to show whether antigenic peptides processed and presented by MoMΦ, pulsed with a peptide-containing PLGA/MPLA NPs can elicit a recall response *ex vivo*. Ongoing studies of the immune response to *A. marginale* with a steer (DRB3 haplotype *1101) had shown that a MHC II-restricted response could be elicited to peptide 5 of VirB9-2, encoding an epitope recognized by CD4 T cells. This afforded an unique opportunity to examine the functionality of MoMΦ in a genetically defined context with a source of autologous cells where experiments could be duplicated as needed (Morse *et al.* 2012). A control peptide, peptide 8 of the VirB9-1 protein had been identified which is not recognized by the MHC II allomorph expressed by the DRB3 haplotype *1101. Three repeats of the experiment with a CD4 T-cell line specific for peptide 5 of VirB9-2 demonstrated that MoMΦ pulsed with encapsulated peptide 5 consistently elicited a MHC II restricted immune response (Fig. 5).

The results obtained in the present study paves the way for use of PLGA/MPLA NP technology to investigate methods for modulating the functional activity of APC by targeting the PRRs that initiate signalling cascades that drive T-cell differentiation. The PLGA NPs have been shown to be safe for use *in vivo* (Chong *et al.* 2005; Hamdy *et al.* 2011; Silva *et al.* 2015; Guldner *et al.* 2016b). The present results support they can also be used *ex vivo*, expanding opportunities for further studies of Anaplasmosis and especially for ongoing studies focused on developing a peptide-based vaccine for Paratuberculosis. *Ex vivo* studies with monocyte-derived dendritic cells (MoDC) and MoMΦ have shown MoDC and MoMΦ pulsed with a *relA* deletion mutant elicit a MHC-restricted recall response with PBMC from a steer

vaccinated with the mutant (Park *et al.* 2016). Analysis of the response has shown both CD4 and CD8 T cells proliferate in response to antigens processed and presented to PBMC by MoDC and MoMΦ. Of considerable interest, part of the response is directed to another *Map* peptide under consideration for inclusion in a peptide-based vaccine, a major membrane protein (MMP) (Bannantine *et al.* 2003). Preliminary studies comparing the proliferative response to MMP alone and encapsulated in PLGA/MPLA have shown that MoDC pulsed with PLGA/MPLA/MMP NP elicit a more vigorous response than MoDC pulsed with MMP alone (C. Souza *et al.* unpublished).

The need for more safe and effective vaccines has stimulated the development of new vaccine technologies. These include delivery systems and adjuvants to target the innate immune system to provide a stimulus that potentiates the development of antigen-specific immune responses. Delivery systems based on PLGA containing the immunomodulatory molecule MPLA has been shown to be safe and able to elicit optimal immune responses. Moreover, they can be administered by several routes, which offer the possibility of developing both mucosal and systemic immune responses. Our preliminary studies in bovine set the stage for more in depth studies on NP delivery systems in a setting that may lead to the development of a peptide-based vaccine for economically important diseases of cattle. It will be possible to conduct studies to detail how the delivery of antigens to specific PRRs with PLGA/MPLA NPs modulate the signalling cascades in APC that drive T-cell differentiation. In addition, it will be possible to identify the peptide sequences presented by MHC I as well as MHC II molecules involved in T-cell activation and differentiation.

Acknowledgements

This research was supported in part by an intramural grant (#9830) from Washington State University College of Veterinary Medicine.

Conflict of Interest

The authors declare no conflict of interest on any of data published in this manuscript.

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