Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 914–923
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

# Pharmaceutical analysis of synthetic lipid A-based vaccine adjuvants in poly (D,L-lactic-*co*-glycolic acid) nanoparticle formulations

Samar Hamdy *, Azita Haddadi, Vishwa Somayaji, David Ruan, John Samuel

*Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada*

Received 6 December 2006; received in revised form 9 March 2007; accepted 12 March 2007
Available online 19 March 2007

**Abstract**

The present study had two main objectives. First, was to compare the immune stimulatory effect of two synthetic lipid A analogues (7-acyl lipid A and pentaerythritol-based lipid A (PET lipid A)) on maturation/stimulation of bone marrow derived dendritic cells (DCs). Our second objective was to develop a liquid chromatography/mass spectrometry (LC-MS) method for the quantitative analysis of lipid A-based vaccine adjuvants. Treatment of immature DCs with 7-acyl lipid A and PET lipid A up regulated the surface expression of CD86 and CD40 molecules, and also induced similar profile of pro-inflammatory cytokine secretion. LC-MS analyses were performed using a Waters Micromass ZQ 4000 spectrometer, coupled to a Waters 2795 separations module with an autosampler. Calibration curves with $R^2 > 0.999$ were constructed over the concentration range of 1.25–20 μg/ml for the solution of 7-acyl lipid A and PET lipid A. The method was tested in a 3 day validation protocol. The accuracy of the assay at different concentrations tested ranged from 89 to 108% and from 92 to 107% for 7-acyl lipid A and PET lipid A, respectively. The limit of quantification for both 7-acyl lipid A and PET lipid A was 1.25 μg/ml (signal/noise (S/N)) ratio >15:1. The sensitivity of the method (the limit of detection) was 0.35 and 0.15 ng for 7-acyl lipid A and PET lipid A, respectively (S/N ratio between 4:1 or 3:1). As a preliminary application, this method has been successfully applied to the determination of 7-acyl lipid A and PET lipid A content in poly (D,L-lactic-*co*-glycolic acid) nanoparticles (PLGA-NP).


*Keywords:* Immunostimulatory adjuvant; Dendritic cells; Nanoparticles; Lipid A; Liquid chromatography/mass spectrometry

## 1. Introduction

Adjuvants are typically immune stimulants, which contain characteristic molecular patterns derived from the pathogens to which the immune system is pre-exposed. In designing a vaccine, an adjuvant serve as powerful companion to the antigen, to which the immune system is expected to mount response [1]. The most important issue in adjuvant development is safety, which has restricted the development of adjuvants since alum was first introduced more than 50 years ago [2]. Many experimental adjuvants have advanced to clinical trials and some have demonstrated high potency, but most have proved too toxic for routine clinical use [3].

Discovery of Toll-like receptors (TLR)s and their essential role in controlling innate and adoptive immune response has lead to better understanding of adjuvant actions. Recent identification of a broad spectrum of specific TLR binding structural features of pathogenic origin has expanded the repertoire of adjuvants and their structural definition. Lipopolysaccharide (LPS) preparations from Gram-negative bacteria are effective in activating innate immunity of the host following bacterial infection. Lipid A, the lipid anchor of LPS, has been shown to be responsible for the biological activities of LPS through its binding to TLR4 complex [4]. Different lipid A analogues have been used as stand-alone immunomodulators because of their ability to significantly enhance both innate and adaptive immune responses. Among the most thoroughly studied lipid A analogues, monophosphoryl lipid A (MPLA) appears to be the most promising candidate.

MPLA is a chemically modified derivative of LPS that exhibits potent adjuvant activity but is 100–10,000 fold less toxic than LPS [5]. A series of preclinical safety investigations in various animal models was performed to support clinical use of MPLA as an adjuvant and comprised cardiovascular/respiratory assessment in dog, rat and rabbits. MPLA was

* Corresponding author at: Faculty of Pharmacy and Pharmaceutical Sciences, 3118 Dentistry-Pharmacy Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2N8. Tel.: +1 780 492 9337; fax: +1 780 492 1217.
*E-mail address:* shamdy@pharmacy.ualberta.ca (S. Hamdy).

0731-7085/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.03.010

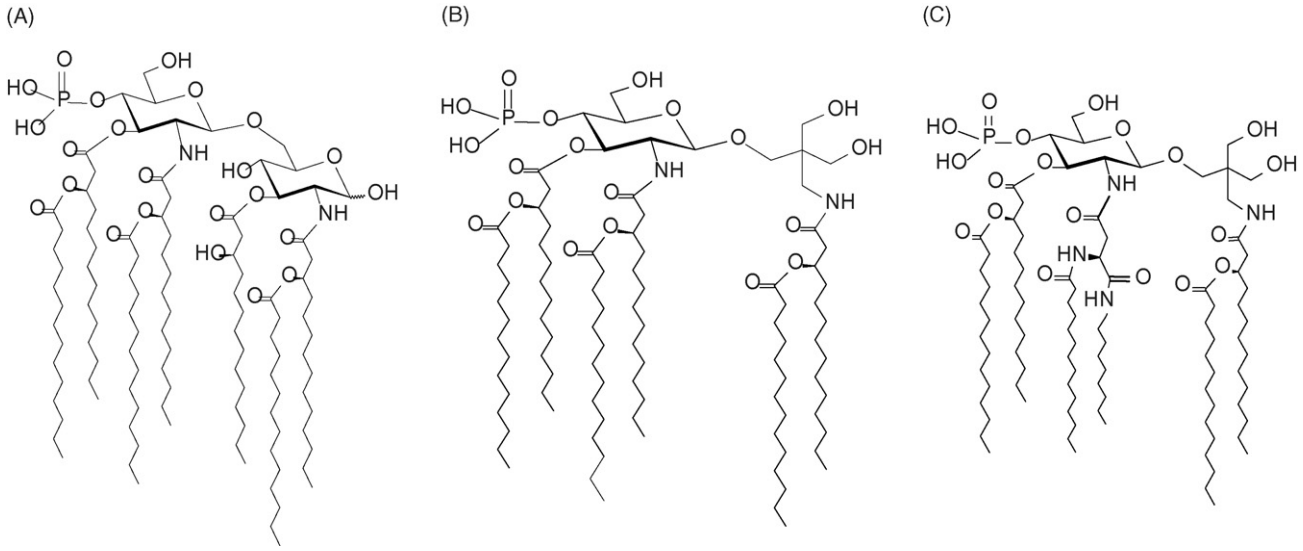


Fig. 1. Chemical structure of (A) 7-acyl lipid A (BC1-005), (B) pentaerythritol based lipid A (PET lipid A or BC1-042) and (C) aspartic (Asp)-PET lipid A (BC1-079) (internal standard).

shown to have no adverse effects on cardiovascular/respiratory function, reproduction, and genotoxicity [6]. MPLA has been evaluated extensively in human clinical trials with infectious diseases (Hepatitis B, Malaria, herpes simplex virus), cancer (metastatic melanoma, glioma, breast cancer, colorectal cancer and ovarian cancer) and allergy vaccines (for prevention or relief of symptoms that are caused by pollens from certain grasses, trees, and household dust mites). Through these trials, more than 33,000 doses of MPLA adjuvant have been administered to over 12,000 patients, establishing both the safety and efficacy of MPLA adjuvant for use in human vaccine, reviewed in [7–9].

Following clinical success of MPLA containing vaccine, several other lipid A analogues are now in development. Natural Lipid A is produced from bacterial cultures and is a mixture of several undefined structures, where as synthetic adjuvants are highly pure, stable, well-defined single structures. Biomira Inc. has developed several synthetic lipid A analogues. The two compounds that are described in this paper are BC1-005 and BC1-042. BC1-005 (Fig. 1A) is a 7-acyl lipid A comprised of β-(1 → 6) diglucosamine moiety bearing three identical (*R*)-tetradecanoyl-oxytetradecanoyl residues on the 2, 2′ and 3′ positions and one (*R*)-3-hydroxytetradecanoyl group on 3 position. Structurally this analogue is closer to the natural MPLA but differs from it in having all acyl chains of uniform length and acylated at 3 position. Where as in BC1-042 (Fig. 1B), also called PET lipid A, a penta erythritol moiety mimics the first sugar and comprised of three identical (*R*)-tetradecanoyloxy-tetradecanoyl residues similar to that of 7-acyl lipid A. Both these analogues have shown promising activity both in soluble form (present study) as well as in liposomal [4] or nanoparticle formulations [10].

Despite of the successful clinical development of many lipid A-based adjuvants, there is a poor understanding of their pharmacokinetics, partially because of lack of sensitive and reliable methods for their analysis. Since lipid A is not a good chromophore, spectrophotometric detection sensitivity have been improved in earlier studies by derivatisation with dinitrobenzyloxyamine [11,12] or dansyl hydrazine (a fluorescent tag) prior to analysis [13], such tedious derivatization process was accomplished in 3 h at 60 °C [11,12]. In the present study, we present a sensitive, reliable and fast method for the analysis of lipid A-based adjuvants by liquid chromatography coupled with mass spectrometry (LC-MS). This method overcame all the potential problems associated with the poor UV-absorbance of lipid A derivatives, and pre-column derivatization. We have used this method for analysis of both 7-acyl lipid A and PET lipid A. The compound that was used as an internal standard is also Biomira's another synthetic lipid A derivative (BC1-079) or (Aspartic (Asp)-PET lipid A, Fig. 1C), so this method may be of general application to various lipid A analogues.

The formulation of potent adjuvants in particulate vaccine delivery systems ensure that both antigen and adjuvant are delivered into the same population of antigen-presenting cells. In addition, delivery systems can focus the effect of the adjuvants onto the key cells of the immune system and limit the systemic distribution of the adjuvant, to minimize its potential to induce adverse effects [14]. Lipid A analogues have been successfully encapsulated in various particulate vaccine formulations including, emulsions, liposomes [15,16], and biodegradable poly (D,L-lactic-*co*-glycolic acid) nanoparticles (PLGA-NP) [12,17–19]. Inclusion of antigens in PLGA-NP offers a vaccine delivery approach that can target the DCs both in vitro and in vivo, in order to activate potent cellular immune responses. We have recently shown that particulate delivery of ovalbumin (OVA) and 7-acyl lipid A to the DCs lead to dramatically increase in antigen specific $CD8^+$ T and $CD4^+$ T cell immune responses, as evidenced by 3000-fold increase in in vitro $CD8^+$ T cell proliferative responses and >13 folds increase in in vivo clonal expanded $CD4^+$ T cells [10]. However, difficulty in extracting and quantifying lipid A analogues in PLGA-NP has lead to lack of understanding of the ways to optimize their loading, release

profile or stability inside PLGA-NP. In the current study, extraction and quantification of 7-acyl lipid A content in the PLGA-NP using the newly developed LC-MS method are described. The same method has been applicable to PET lipid A encapsulated in PLGA-NP. In conclusion, this new method can be a very useful tool to quantify lipid A analogues in vaccine formulations.

## 2. Materials and methods

### 2.1. Mice

C57Bl/6 mice were bred and maintained at the University of Alberta's Health Science's Laboratory Animal Facility. All experiments were performed in accordance to the University of Alberta guidelines for the care and use of laboratory animals. All experiments were performed using 6–12 week old male mice.

### 2.2. Materials

7-Acyl lipid A (BC1-005), PET lipid A (BC1-042), and Asp PET lipid A (BC1-079) (molecular weights: 1956.5, 1685.70 and 1699.2 Da, respectively) were kindly provided by Biomira Inc. (Edmonton, Alberta, Canada). Polyvinyl alcohol (PVA), m.w. 21–50,000 Da. PLGA co-polymer, monomer ratio 50:50, m.w. 7000 Da, was purchased from Absorbable Polymers International (Pelham, AL, USA). Recombinant murine Granulocyte Monocyte Colony Stimulating Factor (GM-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). Murine IL-6 and TNF-α ELISA kits were purchased from E-Bioscience (San Diego, CA, USA). Murine IL-12 ELISA kit was purchased from BD Biosciences (Mississauga, ON, Canada). RPMI-1640, L-glutamine, and gentamycin were purchased from Gibco-BRL (Burlington, Ont., Canada). Fetal Calf Serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA). Anti mouse CD16/CD32 mAb (2.4G2), CD40, and CD86 antibodies, and their respective isotype controls were purchased from BD Biosciences (Mississauga, Ont., Canada). Chloroform, methanol, acetonitrile, water (all HPLC grades) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

### 2.3. Preparation of murine bone marrow derived DCs

DC primary cultures were generated from murine bone marrow precursors from femurs of C57Bl/6 mice in complete media in presence of GM-CSF as described earlier [20]. Cell culture medium was RPMI-1640 supplemented with gentamycin (80 μg/ml), L-glutamine (2 mM), 10% heat-inactivated FCS and 20 ng/ml GM-CSF. At day 7 cells can be used already. The purity of the DC population on day 7 was found to be between 70 and 75% based on the expression of CD11c on the semi-adherent and non-adherent cell populations.

### 2.4. Dendritic cells activation/maturation studies

#### 2.4.1. Cytokine secretion

On day 7 of the primary culture, the semi-adherent and non-adherent cell populations were harvested and re-suspended at $4 \times 10^5$ cells/ml in complete media containing 20 ng/ml of GM-CSF. Stock solutions of 7-acyl lipid A and PET lipid A were prepared at 1 mg/ml in tertiary butanol, then diluted with plain RPMI media to give the corresponding concentration (0.05, 0.1, 0.5, 1, 5 and 10 μg/ml of culture media) before adding to the cells. DCs treated with tertiary butanol (diluted similarly with plain RPMI media) were used as vehicle control. After an overnight incubation, culture supernatants were analyzed for IL-6, IL-12p70, and TNF-α secretion by ELISA kits in a 96 well microplate using a microplate reader (Powerwave with KC Junior software; Bio-Tek, Winooski, VT) at OD of 450 nm with reference set at 570 nm according to the manufacturer's directions. All samples were analyzed in duplicates. The minimum detection levels of the cytokines were: IL-12p70, 5 pg/ml; IL-6, 10 pg/ml; TNF-α, 15 pg/ml.

#### 2.4.2. Flow cytometry analysis

DCs pulsed with 0.1 μg/ml of either 7-acyl lipid A or PET lipid A were harvested (after overnight incubation), and tested for up-regulation of maturation surface markers (CD40 and CD86). Briefly, $2.5 \times 10^5$ cells were suspended in FACS buffer (PBS with 5% FCS, and 0.09% sodium azide) and incubated with anti mouse CD16/CD32 mAb to block Fc receptors and then stained appropriate fluorescence conjugated antibodies. DCs pulsed was 1 μg/ml LPS was used as a positive control. All samples were finally acquired on a Becton–Dickinson Facsort and analyzed by Cell-Quest software.

### 2.5. Establishment of liquid chromatography/mass spectrometry (LC-MS) method for quantification of lipid A-based adjuvants

#### 2.5.1. LC-MS method parameters

LC-MS analyses were performed using a Waters Micromass ZQ 4000 spectrometer, coupled to a Waters 2795 separations module with an autosampler (Milford, MA, USA). The mass spectrometer was operated in negative ionization mode with selected ion recording (SIR) acquisition. The nebulizer gas was obtained from an in house high purity nitrogen source. The temperature of source was set at 150 °C, and the voltages of the capillary and cone were 3.11 kV and 60 V, respectively. The gas flow of desolvation and the cone were set at 550 and 90 l/h, respectively. Chromatographic separation was achieved using an Agilent Technologies (Palo Alto, CA, USA) Zorbax eclipse XDB C8 3.5 μm (2.1 mm × 50 mm) as the stationary phase. The mobile phase was consisting of 2 solutions; *Solution A* is 0.1% glacial acetic acid, 0.1% triethylamine in methanol and *Solution B* is 0.1% glacial acetic acid, 0.1% triethylamine, 10% methanol in tetrahydrofuran (THF). The mobile phases were delivered at a constant flow rate of 0.2 ml/min. The gradient conditions are shown in Table 1. Asp-PET lipid A (another synthetic lipid A derivative) was used as an internal standard (IS). SIR at *m/z* 1955.5, 1684.7 and 1698.2, related to M-H were selected for quantification of 7-acyl lipid A, PET lipid A and the internal standard, respectively.

Table 1
Chromatographic gradient program over LC-MS analysis time (15 min)

| Time (min) | Mobile phase A[a] (%) | Mobile phase B[b] (%) | Flow rate (ml/min) | Gradient curve |
|---|---|---|---|---|
| 0 | 100 | 0 | 0.2 | 1 |
| 10 | 50 | 50 | 0.2 | 6 |
| 10.2 | 100 | 0 | 0.2 | 1 |

[a] Mobile phase A is methanol containing 0.1% glacial acetic acid and 0.1% triethylamine.
[b] Mobile phase B is 10% methanol in tetrahydrofuran containing 0.1% glacial acetic acid and 0.1% triethylamine.

#### 2.5.2. Standard and stock solutions

The stock solutions were prepared by dissolving 2 mg of 7-acyl lipid A or PET lipid A in 1 ml of chloroform:methanol mixture (4:1, v/v). The stock solutions were stored at −20 °C between experiments. The working solutions of 7-acyl lipid A and PET lipid A were prepared fresh each day by making a 50-fold dilution of the stock solution in chloroform:methanol mixture (4:1, v/v), and (1:4, v/v), respectively. The calibration standards were then prepared by serial dilution of the working solution. The stock solution of the internal standard was prepared by dissolving 2 mg of Asp-PET lipid A in 1 ml chloroform:methanol mixture (4:1, v/v), followed by the preparation of a working solution of 20 μg/ml by a further 100-fold dilution of the stock solution. The stock solution of the internal standard was stored at −20 °C between experiments, and the working solution was prepared fresh at each experiment.

#### 2.5.3. Accuracy and precision of the LC-MS method

Each sample was prepared in triplicates on three different days. The accuracy (the nearness of a measured value to the true value) was expressed as the mean percentage error, [mean measured concentration/expected concentration × 100]. The precision (agreement between replicate measurements) was evaluated as inter and intra-day coefficient of variation (CV), which was calculated as: [%CV = (standard deviation/mean) × 100]. Least-squared regression method was used to determine the regression coefficient and the equation for the best fitting line.

### 2.6. Preparation and characterization of PLGA nanoparticles containing either 7-acyl lipid A or PET lipid A

#### 2.6.1. Preparation of nanoparticles

PLGA nanoparticles containing 7-acyl lipid A or PET lipid A were prepared as oil/water single emulsion formulation by the solvent evaporation method. Briefly, 200 μg 7-acyl lipid A or 600 μg PET lipid A in 100 μl of 1:4 methanol–chloroform mixture (v/v) was added to the polymer–chloroform solution (100 mg polymer in 300 μl chloroform). The resulting solution was then was emulsified in 2 ml of PVA solution (9%, w/v PVA in PBS) by sonication for 45 s at level 4, using a microtip sonicator (Heat systems Inc., Farmingdale, NY, USA). The emulsion was added drop-wise into 8 ml of stirring PVA solution. Nanoparticles were collected after 3 h of stirring by centrifugation of the emulsion at 40,000 × *g* for 10 min at 4 °C. The nanoparticles were washed twice with cold deionized water and lyophilized. The mean size diameter of the nanoparticles was determined by dynamic light scattering technique using a Zetasizer 3000 (Malvern, UK).

#### 2.6.2. Analysis of 7-acyl lipid A and PET lipid A content

We have recently described the method for extraction of 7-acyl lipid A from PLGA-NP [10]. Since 7-acyl lipid A is not soluble in acetonitrile unlike PLGA, extraction was done by first dispersing 10 mg of nanoparticles in 400 μl of acetonitrile, followed by centrifugation at 15,000 × *g* for 15 min. The supernatant was removed. The residue was then extracted by adding 500 μl of 1:4 methanol–chloroform mixture. The sample was centrifuged at 15,000 × *g* for 15 min and the supernatant was assayed for 7-acyl lipid A by LC/MS. In contrast to 7-acyl lipid A, PET lipid A is soluble in acteonitrile, so the extraction of PET lipid A from PLGA-NP was done by slightly different method. Briefly, 10 mg of nanoparticles were dispersed in 100 μl of acetonitrile. After brief sonication (2–3 s), both the polymer and PET lipid A were dissolved. PET lipid A was then extracted by adding 1400 μl ethanol followed by centrifugation at 15,000 × *g* for 15 min. After centrifugation, polymer was precipitated and the supernatant was analyzed by LC/MS. The supernatant containing extracted 7-acyl lipid A or PET lipid A, were then mixed in (1:1, v/v ratio) with working solution (20 μg/ml) of the internal standard. An aliquot of 10 μl of this mixture was then injected into the LC/MS system. Based on the measured concentration, the encapsulation efficiency was calculated as the amount of encapsulated 7-acyl lipid A or PET lipid A relative to the total amount of 7-acyl lipid A or PET lipid A used during nanoparticle preparation, respectively. The loading was calculated from the weight of the nanoparticles and the amount of 7-acyl lipid A or PET lipid A incorporated.

### 2.7. Statistical analysis

The data obtained in the current studies was subjected to statistical analysis wherever appropriate. Student's *t*-test was used to compare data sets for statistical significance.

## 3. Results

### 3.1. Phenotypic/functional maturation of DCs following 7-acyl lipid A or PET lipid A stimulation

The induction of DCs maturation is critical for the stimulation of antigen specific T lymphocyte responses and may be essential for the development of human vaccines relying on T cell immunity [21]. The maturation process is characterized by IL-12 production and the up-regulation of costimulatory molecules. In this study, we have investigated the effects of soluble form of two synthetic lipid A analogues (7-acyl lipid A and PET lipid A) on their ability to mature bone marrow derived DCs both phenotypically (up-regulation of key markers, CD40 and CD86) and functionally (secretion of pro-inflammatory cytokines; IL-12p70, TNF-α and IL-6).

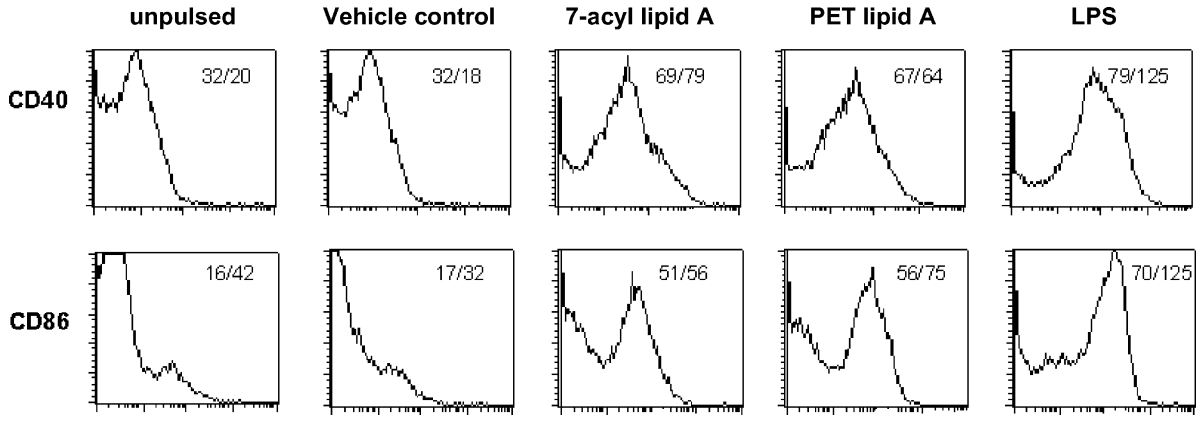


Fig. 2. Effect of 7-acyl lipid A and PET lipid A on up-regulation of CD40 and CD86 on murine bone marrow derived dendritic cells (DCs). Day 7 bone marrow derived DC cultures were incubated in complete media containing GM-CSF and left untreated (unpulsed) or treated with 0.1 μg/ml of 7-acyl lipid A or PET lipid A, or 1 μg/ml LPS. After overnight incubation, non-adherent cells were harvested and stained with the antibodies and analyzed by FACS. Stock solutions of 7-acyl lipid A and PET lipid A were prepared at 1 mg/ml in tertiary butanol, then, diluted with plain RPMI media to give the corresponding concentration. DCs treated with tertiary butanol (diluted with RPMI media) were used as vehicle control. Numbers at the right corner of histograms represent percentages of positive cells/mean florescence intensity (MFI). One representative out of three individual experiments is shown.

Stimulation of DCs with either 0.1 μg/ml of 7-acyl lipid A or PET lipid A enhanced the expression of CD40 and CD86 to a similar extent (Fig. 2). Numbers at the right corner of histograms in Fig. 2 represent percentages of positive cells/mean florescence intensity (MFI). Tertiary butanol alone (vehicle control) did not have any effect on either percentage of cells or MFI. The expression of CD40 was highly up-regulated following treatment with either 7-acyl lipid A or PET lipid A, as evidenced by more than 2 folds increase in the percentage of positive cells (relative to the unpulsed DCs), and also 3.2 and 3.9 folds increase in the MFI, respectively of CD40, respectively. Both adjuvants resulted also in up-regulation of CD86 expression, as more than 3 folds increase in the percentage of positive cells for CD86 have been observed relative to the unpulsed DCs.

In parallel to the phenotypic up-regulation of costimulatory molecules, fully mature DCs also secrete pro-inflammatory cytokines in response to a danger signal [22]. For this purpose, the production of IL-12p70, IL-6 and TNF-α upon exposure to 7-acyl lipid A or PET lipid A was assessed by quantitative ELISA kits. Immature DCs from day 7 of in vitro propagation were cultured overnight in the presence of titrating concentration of 7-acyl lipid A or PET lipid A (0.05–10 μg/ml of culture medium). Vehicle control was DCs treated with tertiary butanol (diluted similarly with culture medium). After overnight incubation supernatant was analyzed for cytokine secretion. Results are shown in Fig. 3. As observed in the phenotype maturation studies, both 7-acyl lipid A and PET lipid A have very similar dose response effect on secretion of pro-inflammatory cytokines. However, at the lowest concentration (0.05 μg/ml), PET lipid A produced significantly higher amount of IL-12p70, TNF-α, and IL-6 ($p < 0.05$) than 7-acyl lipid A. As the concentration of adjuvants increased, both 7-acyl lipid A and PET lipid A showed similar cytokine secretion profile. All three cytokine have reached their highest peak concentration when DCs were stimulated with 1 μg/ml of either 7-acyl lipid A or PET lipid A. When the concentration of adjuvants was further increased, secretions of the cytokines have started to decrease. However, more sudden and sharp decrease have been observed by 7-acyl lipid A at 5 μg/ml, especially in the secretion of IL-12p70 (Fig. 3A) and TNF-α (Fig. 3B), whereas PET lipid A was still resulting in significantly higher secretion of those cytokines ($p < 0.05$). At 10 μg/ml, 7-acyl lipid A resulted in slight increase in TNF-α secretion, however this increase was not significantly different than what was observed at 5 μg/ml ($p > 0.05$). Minimum level of cytokine secretion has been observed at the highest concentration (10 μg/ml). Further studies are being undertaken in our laboratory to assess the toxic effect of titrating doses of 7-acyl lipid A versus PET lipid A on murine bone marrow derived DCs. Taken together these data (Figs. 2 and 3) support our hypothesis that PET lipid A is as effective as 7-acyl lipid A at least in terms of stimulation of bone marrow derived DCs cells and secretion of pro-inflammatory cytokines that induce T helper 1 (Th1)-biased immune response.

### 3.2. Validation of LC-MS method for quantification of lipid A-based adjuvants

The mass spectra of 7-acyl lipid A, PET lipid A and Asp-PET lipid A (internal standard) dissolved in 50:50 (v/v) solution A:solution B is shown in Fig. 4. The molecular ions at *m/z* of 1955.5, 1684.7 and 1698.2 corresponding to [M−H] were selected for quantification of 7-acyl lipid A, PET lipid A, and the internal standard (IS), respectively. Fig. 5 shows the single ion recording (SIR) chromatograms of either 7-acyl lipid A and IS (Fig. 5A) or PET lipid A and IS (Fig. 5B). The peaks of both 7-acyl lipid A and PET lipid A were well separated from that of the IS in the established chromatographic condition. The retention times of 7-acyl lipid A, PET lipid A and IS were approximately 10.2, 9.2 and 8 min, respectively. The analytical run time was 15 min.

#### 3.2.1. Linearity range

The regression analysis was constructed by plotting the peak-area ratio of either 7-acyl lipid A or PET lipid A to IS (response

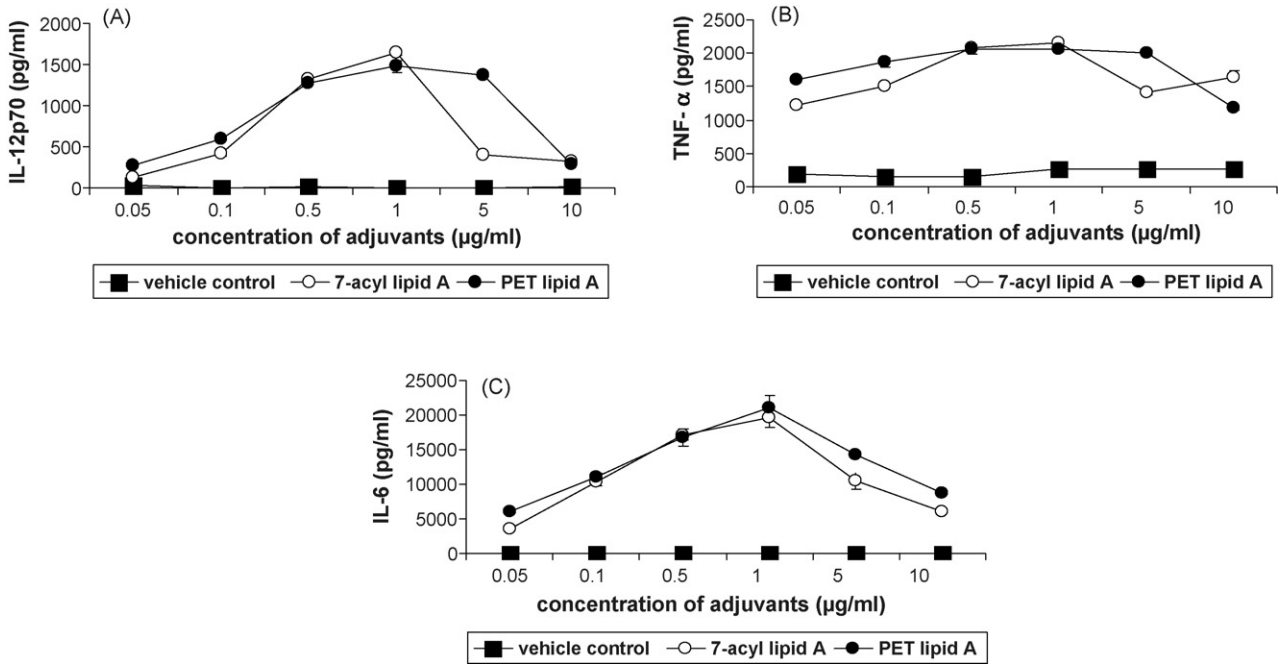


Fig. 3. Effect of titrating doses of 7-acyl lipid A and PET lipid A on the secretion of pro-inflammatory cytokines by bone marrow derived dendritic cells (DCs). On day 7 of the primary culture, the semi-adherent and non-adherent cell populations were harvested and re-suspended at $4 \times 10^5$ cells/ml in complete media containing 20 ng/ml of GM-CSF. Stock solutions of 7-acyl lipid A and PET lipid A were prepared at 1 mg/ml in tertiary butanol, then diluted with plain RPMI media to give the corresponding concentration (0.05, 0.1, 0.5, 1, 5 and 10 μg/ml of culture media) before adding to the cells. DCs treated with tertiary butanol were used as vehicle control. After an over night incubation, culture supernatants were analyzed for cytokine secretion, IL-12p70 (A), TNF-α (B) and IL-6 (C).

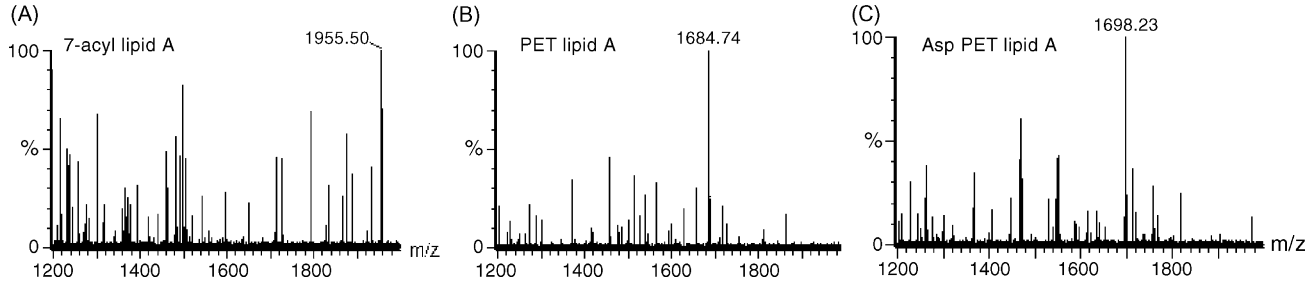


Fig. 4. Mass spectra of (A) 7-acyl lipid A, (B) pentaerythritol based lipid A (PET lipid A) and (C) aspartic (Asp)-PET lipid A (internal standard). 7-Acyl lipid A, PET lipid A and BC1-079 were dissolved in 50:50 (v/v) solution A:solution B. *Solution A* is methanol containing 0.1% glacial acetic acid and 0.1% triethylamine, *solution B* is 10% methanol in tetrahydrofuran containing 0.1% glacial acetic acid and 0.1% triethylamine.

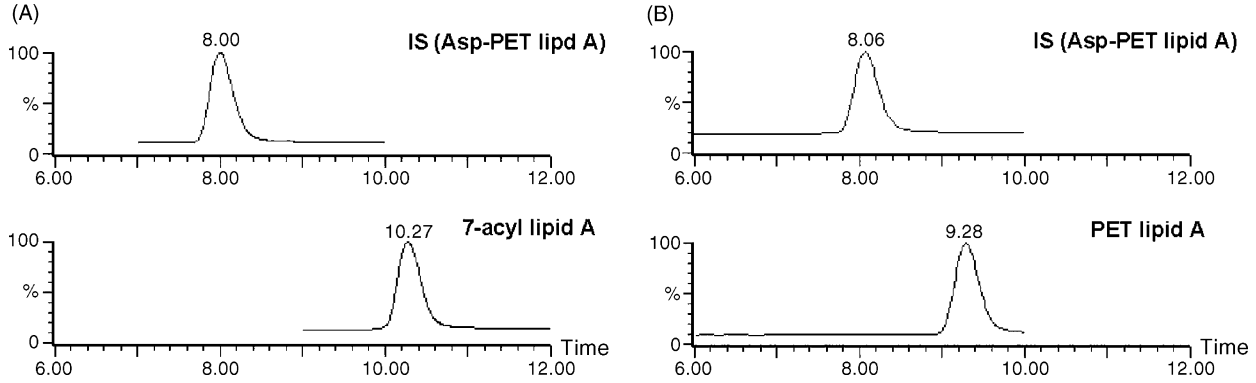


Fig. 5. Single ion recorder (SIR) chromatograms of Asp-PET lipid A (internal standard, IS), with either 7-acyl lipid A (A) or PET lipid A (B). Chromatographic gradient conditions are described in Table 1.

Table 2
Assay validation data for 7-acyl lipid A

| Expected concentration (μg/ml) | Measured concentration ± S.D. (μg/ml) | CV (%) | Accuracy (%) |
|---|---|---|---|
| (A) Intra-day precision (coefficient of variation, CV) and accuracy (mean percent error) ($n = 3$) | | | |
| 1.25 | 1.1 ± 0.069 | 6.2 | 89 |
| 2.5 | 2.3 ± 0.200 | 8.6 | 92 |
| 5 | 5.0 ± 0.646 | 12.9 | 100 |
| 10 | 9.6 ± 0.880 | 9.1 | 96 |
| 20 | 18.8 ± 1.678 | 8.9 | 94 |
| (B) Inter-day precision (coefficient of variation, CV) and accuracy (mean percent error) ($n = 3$) | | | |
| 1.25 | 1.1 ± 0.061 | 5.3 | 91 |
| 2.5 | 2.5 ± 0.223 | 8.7 | 102 |
| 5 | 5.4 ± 0.385 | 7.1 | 108 |
| 10 | 10.5 ± 0.828 | 7.8 | 105 |
| 20 | 19.4 ± 1.350 | 6.9 | 97 |

factor) versus analyte concentration (μg/ml). The calibration curve was linear within the range of 1.25 μg to 20 μg/ml. The correlation coefficient ($R^2$) was always greater than 0.999, indicating a good linearity.

### 3.2.2. Sensitivity

The limit of detection (LOD) was assessed based on signal-to-noise (S/N) ratio. Determination of the S/N ratio was performed by comparing measured signals from samples with known low concentrations of 7-acyl lipid A or PET lipid A with those of blank samples and establishing the minimum concentration at which the signal can be reliably detected. A S/N ratio between 4:1 or 3:1 is generally acceptable for estimating the detection limit. The LOD for 7-acyl lipid A and PET lipid A was found to be 0.035, 0.015 μg/ml, respectively. Based on 10 μl injection volume, that corresponds to an amount of 0.35 and 0.15 ng, respectively. The limit of quantification (LOQ) or the minimum level at which 7-acyl lipid A or PET lipid A could be quantified with acceptable accuracy and precision have been found to be 1.25 μg/ml for both of them. Based on 10 μl injection volume, that corresponds to an amount of 12.5 ng.

### 3.2.3. Accuracy and precision

Intra- and inter-day precision were determined using different concentrations of 7-acyl lipid A or PET lipid A standards (1.25, 2.5, 5, 10 and 20 μg/ml). These concentrations were assayed in triplicates on 3 different days. The assay coefficient of variation at all of the intra- and inter-day assessment was less than 13%. The accuracy of the assay at different concentrations tested ranged from 89 to 108% and from 92 to 107% for 7-acyl lipid A and PET lipid A, respectively, and the measured amounts were not significantly different than expected, based on Student's $t$-test ($\alpha = 0.05$). All validation results are reported in Tables 2 and 3 for 7-acyl lipid A and PET lipid A, respectively.

### 3.2.4. Selectivity

Injection of reagent blanks showed no interfering peaks in the 7-acyl lipid A or PET lipid A in the region of the chromatograms. Furthermore, a structure analogue used as an internal standard (Asp-PET lipid A) was well separated from both test compounds.

## 3.3. Application of the LC-MS method: analysis of 7-acyl lipid A and PET lipid A content in PLGA nanoparticles

The mean hydrodynamic diameter of the nanoparticles ranged between 350 and 450 nm, and the polydispersity was below 0.1. We have used the LC-MS method to analyze 7-acyl lipid A and PET lipid A content in PLGA-NP. The results of the analysis are summarized in Table 4. The efficiency of the extraction procedure was measured by a spiking study, where

Table 3
Assay validation data for PET lipid A

| Expected concentration (μg/ml) | Measured concentration ± S.D. (μg/ml) | CV (%) | Accuracy (%) |
|---|---|---|---|
| (A) Intra-day precision (coefficient of variation, CV) and accuracy (mean percent error) ($n = 3$) | | | |
| 1.25 | 1.3 ± 0.123 | 9.4 | 105 |
| 2.5 | 2.7 ± 0.101 | 3.6 | 110 |
| 5 | 5.3 ± 0.491 | 9.2 | 106 |
| 10 | 10.2 ± 0.28 | 2.7 | 102 |
| 20 | 19.7 ± 1.15 | 5.8 | 98 |
| (B) Inter-day precision (coefficient of variation, CV) and accuracy (mean percent error) ($n = 3$) | | | |
| 1.25 | 1.15 ± 0.13 | 11.7 | 92 |
| 2.5 | 2.5 ± 0.29 | 11.3 | 102 |
| 5 | 5.3 ± 0.4 | 7.4 | 107 |
| 10 | 10.6 ± 0.55 | 5.2 | 106 |
| 20 | 19.0 ± 0.7 | 3.6 | 95 |

Table 4
Analysis of 7-acyl lipid A and PET lipid A content in PLGA nanoparticles

| Adjuvant | Total amount used in preparation | Extraction recovery | Loading[a] | Encapsulation[b] efficiency |
|---|---|---|---|---|
| 7-Acyl lipid A | 200 μg | ∼100% | 1.79 μg ± 0.18 | 67.3 ± 6.9% |
| PET lipid A | 600 μg | ∼80% | 1.76 μg ± 0.10 | 22.0 ± 1.2% |

[a] The amount of 7-acyl lipid A or PET lipid A incorporated per 1 mg of the nanoparticles.
[b] Based on 75% polymer recovery, encapsulation efficiency was calculated as the amount of encapsulated 7-acyl lipid A or PET lipid A relative to the total amount of 7-acyl lipid A or PET lipid A used during nanoparticle preparation (200 and 600 μg, respectively).

known amounts of 7-acyl lipid A or PET lipid A were added to empty PLGA-NP. Following extraction, the supernatant was injected to the LC-MS system and the extraction recovery was calculated as the amount detected/amount used in spiking × 100. The procedures of extraction of 7-acyl lipid A and PET lipid A was easy and was performed in less than 1 h, the recoveries were ∼100 and ∼80%, respectively. PET lipid A is smaller in size (6 fatty acid chains versus 7 in 7-acyl lipid A), thus it is less hydrophobic than 7-acyl lipid A. Accordingly, the encapsulation efficiency inside PLGA-NP was less than that of 7-acyl lipid A (22.0% versus 67.3%, respectively). During the preparation of nanoparticles, 600 μg of PET lipid A was used (versus 200 μg of 7-acyl lipid A), so that the loading of both 7-acyl lipid A and PET lipid A was almost the same; 1.79 μg and 1.76 μg per 1 mg dry weight of nanoparticles, respectively (Table 4).

## 4. Discussion

The development of safe and efficient vaccines for cancer and infectious diseases remains a major goal in global public health [23]. Key elements of effective vaccines include the antigen against which the immune responses are elicited, an adjuvant which triggers early immune responses and aid in the generation of robust and long-lasting adoptive immune responses and finally, delivery system that target the vaccine to the appropriate cells of the immune system [24].

Numerous publications have described the potential use of MPLA as a vaccine adjuvant [7–9,15,16]. MPLA has excellent immunostimulatory activity through its diverse effects on the cellular elements of the immune system including macrophage activation and T- and B cell interaction with concomitant cytokine release. With regard to specific T-helper-type1 or 2 (Th1 or Th2)-derived cell-mediated immunity, studies clearly show that MPLA has greater Th1 than Th2 stimulating potential. One consequence of Th1 activation is a selective IgG production, which has been demonstrated with MPLA. The induction of IgG may prolong protective immunity which is a further benefit of use of MPLA as an adjuvant [8]. MPLA adjuvant has now been produced on an industrial scale and injected into thousands of individuals as an essential component of several next-generation vaccines. Following the successes of MPLA, development of other lipid A analogues can lead to the development of new generation of vaccine adjuvants that could be as promising as MPLA in terms of safety and immunstimulatory effect. In the current study we have examined two newly designed synthetic lipid A mimetic compounds (7-acyl lipid A and PET lipid A). Synthetic lipid A analogues have many advantages over the natural lipid A in terms of reproducibility, feasible large scale production, better control over purity of the final products, and minimal variations from batch to batch. We compared both synthetic 7-acyl lipid A and PET lipid A in their ability to induce bone marrow derived DC maturation. Our results here showed that both 7-acyl lipid A and PET lipid A have similar effect on the stimulation of murine DCs, as evidenced by the increase in the expression of DC key activation/maturation markers, and the dose-dependant secretion of pro-inflammatory cytokines (IL-12, IL-6 and TNF-α). These results are consistent with earlier studies, in which natural lipid A was able to mature DCs from human donors, causing the up-regulation of human leukocyte antigen-DR, CD80, CD86, CD40, and an activation marker, CD83, in vitro [21].

The successful clinical development of MPLA or any lipid A analogue requires the establishment and validation of a satisfactory analytical method for the conduction of pharmaceutical and pharmacokinetic studies. Natural MPLA is a heterogenous product composed of several MLA species which differ primarily with respect to the degree and type of fatty acid acylation and to a lesser extent with respect to intrinsic microheterogeneity in fatty acid chain length [14]. Past attempts to analyze MPLA with reversed-phase HPLC (RP-HPLC) have used either UV–vis detection at 210 nm, where MPLA is weakly absorbing, or radiodetection utilizing metabolically-labeled preparations [25,26]. Beside the low sensitivity of MPLA, UV–vis detection of MPLA has been unattractive because of the different species present in MPLA, which may have different molar extinction coefficients. The use of radiolabelled material is also unattractive for safety reasons and is obviously inappropriate for clinical material [11]. An additional complication in the use of RP-HPLC to analyze MPLA is that the phosphomonoester group in MPLA interferes with elution from RP columns. In the present study, we present a quick, sensitive, accurate and reliable method for the analysis of lipid A analogues by liquid chromatography coupled with mass spectrometry (LC-MS). This method can overcome all the potential problems associated with the poor UV-absorbance, use of radiolabeling, difficulty in elution from RP columns, and pre-column derivatization of lipid A compounds. In addition, this method can detect 4 samples/h (analysis time is only 15 min), which is much faster than the conventional HPLC method in which the analysis time was 45 min/one run plus 3 h pre-column derivatization. This method is also much more sensitive than HPLC, as evidenced by LOD (0.35 and

0.15 ng for 7-acyl lipid A and PET lipid A, respectively, as compared to 540 ng in previously reported HPLC method for MPLA analysis) [11]. One additional advantage to the LC/MS method is that, it is easily applicable to various lipid A analogues (7-acyl lipid A, PET lipid A and Asp-PET lipid A without changing any parameters. Lipid A analogues have been used in various vaccine formulations either alone or combined with other adjuvants [27,28]. In fact, AS04 adjuvant system (a combination of 3-*O*-desacyl-4′ MPLA and aluminium salt) have induced an immune response of higher magnitude and persistence compared to a vaccine formulated with aluminium salt only [28]. Therefore, in the future, if more than one lipid A analogue were combined in the same vaccine formulation, using the LC-MS method that we have developed could easily enable simultaneous quantification of both analogues in a considerable short time.

Encapsulation of lipid A-based adjuvants within PLGA-NP offers a significant advantage immunologically [10,29]. However, the difficulty in extracting and quantifying lipid A analogues in PLGA-NP have lead to lack of understanding of the ways to optimize their loading, release profile or stability inside PLGA-NP. As pre-column derivatization process required at least 1 mg of MPLA [11], a major problem of HPLC analysis for quantifying MPLA content in PLGA-NP is how to get this 1 mg of from the nanoparticle formulation to start the derivatization reaction. For example, in a preparation of PLGA-NP containing 7-acyl lipid A, a total of 200 μg 7-acyl lipid A is added to 100 mg polymer. That means, in order to analyze the 7-acyl lipid A content from PLGA-NP, we have to use at least 500 mg PLGA-NP to be able to extract 1 mg of 7-acyl lipid A to start the derivatization process (assuming 100% encapsulation and 100% polymer recovery, which is not practically true). However, our method enabled us to extract and quantify both 7-acyl lipid A and PET lipid A from only 10 mg PLGA-NP and in less than 1 h.

In earlier studies, the encapsulation efficiency of MPLA in PLGA-NP has been estimated to be 100%, as it is very hydrophobic and lipid soluble molecule [30]. However, assuming that all lipid soluble molecules could be formulated in PLGA-NP with 100% encapsulation efficiency could be quite misleading. For example, in the present study we have formulated both 7-acyl lipid A and PET lipid A in PLGA-NP. Although both compounds are lipid soluble and very similar in size and structure, but the encapsulation efficiency of PET lipid A was significantly lower than 7-acyl lipid A inside PLGA-NP (Table 4) ($p < 0.0005$). The amount of PET lipid A that was used in the preparation (600 μg) was three fold higher than the amount of 7-acyl lipid A (200 μg), however both formulations had the same loading (almost 1.7 μg of 7-acyl lipid A or PET lipid A per 1 mg dry weight nanoparticles). The reason of the low encapsulation of PET lipid A could be because of it is more hydrophilic molecule than 7-acyl lipid A. Hydrophlic molecules have lower affinity to the polymer, and have a tendency to move from the organic phase to the outer aqueous phase and not in the precipitating nanoparticles. We are currently investigating the effect of various formulation parameters to improve the encapsulation efficiency of PET lipid A inside PLGA-NP.

## 5. Conclusion

All together, our results support the potential use of lipid A-based adjuvant in clinic and also introduce two promising candidates from lipid A family (7-acyl lipid A and PET lipid A), as candidates for further studies in future vaccine trials. We have also developed a sensitive, quick and reliable LC-MS based method for quantification of 7-acyl lipid A and PET lipid A, that can be easily applied for other lipid A analogues. This method has overcome all the potential problems associated with previous methods for lipid A analysis. Such method will lay the foundation for further optimization of the pharmaceutical preparations and characterization of pharmacokinetics studies of lipid A mimetics, and will lead to better understanding and improvement of the immune stimulant properties associated with this entire class of adjuvants.

## Acknowledgments

This project was supported by research grants from Canadian Health Institute of Research (MOP 42407) and Natural Sciences and Engineering Council of Canada (STPGP 336987). Authors thank Biomira Inc., Edmonton, our industrial partner for their incessant co-operation and providing the synthetic lipid A analogues used in this study. Thanks are extended to Dr. Damayanthi Yalamati and Dr. Rao Koganty (Biomira Inc., Edmonton) for providing outstanding assistance and advise during the project.

## References

[1] Z.H. Jiang, R.R. Koganty, Synthetic vaccines: The role of adjuvants in immune targeting, Curr. Med. Chem. 10 (2003) 1423–1439.
[2] R. Edelman, New Generation Vaccines, Marcel Dekker Inc., New York, 1997.
[3] M. Singh, D. O'Hagan, Advances in vaccine adjuvants, Nat. Biotechnol. 17 (1999) 1075–1081.
[4] Z.H. Jiang, W.A. Budzynski, L.N. Skeels, M.J. Krantz, R.R. Koganty, Novel lipid A mimetics derived from pentaerythritol: Synthesis and their potent agonistic activity, Tetrahedron 58 (2002) 8833–8842.
[5] K. Takayama, E. Ribi, J.L. Cantrell, Isolation of a nontoxic lipid A fraction containing tumor regression activity, Cancer Res. 41 (1981) 2654–2657.
[6] P. Baldrick, D. Richardson, G. Elliott, A.W. Wheeler, Safety evaluation of monophosphoryl lipid A (MPL): An immunostimulatory adjuvant, Regul. Toxicol. Pharmacol. 35 (2002) 398–413.
[7] J.T. Evans, C.W. Cluff, D.A. Johnson, M.J. Lacy, D.H. Persing, J.R. Baldridge, Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529, Expert Rev. Vaccines 2 (2003) 219–229.
[8] J.T. Ulrich, K.R. Myers, Monophosphoryl lipid A as an adjuvant. Past experiences and new directions, Pharm. Biotechnol. 6 (1995) 495–524.
[9] J.R. Baldridge, P. McGowan, J.T. Evans, C. Cluff, S. Mossman, D. Johnson, D. Persing, Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents, Expert Opin. Biol. Ther. 4 (2004) 1129–1138.
[10] P.E. Samar Hamdy, A. Alshamsan, O. Molavi, T. Satou, J. Samuel, Enhanced antigen-specific primary CD4+ and CD8+ responses by co-delivery of ovalbumin and Toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-*co*-glycolic acid) nanoparticles, J. Biomed. Mater. Res. Part A 81 (2007) 652–662.
[11] S.R. Hagen, J.D. Thompson, D.S. Snyder, K.R. Myers, Analysis of a monophosphoryl lipid A immunostimulant preparation from Salmonella minnesota R595 by high-performance liquid chromatography, J. Chromatogr. A 767 (1997) 53–61.

[12] C.S. Chong, M. Cao, W.W. Wong, K.P. Fischer, W.R. Addison, G.S. Kwon, D.L. Tyrrell, J. Samuel, Enhancement of T helper type 1 immune responses against hepatitis B virus core antigen by PLGA nanoparticle vaccine delivery, J. Control. Release 102 (2005) 85–99.
[13] M. Hopkins, B.G. Lees, D.G. Richardson, S.R. Woroniecki, A.W. Wheeler, Standardisation of glutaraldehyde-modified tyrosine-adsorbed tree pollen vaccines containing the Th1-inducing adjuvant, monophosphoryl lipid A (MPL), Allergol. Immunopathol. (Madr) 29 (2001) 245–254.
[14] D.T. O'Hagan, M. Singh, Microparticles as vaccine adjuvants and delivery systems, Expert Rev. Vaccines 2 (2003) 269–283.
[15] R.L. Richards, M. Rao, N.M. Wassef, G.M. Glenn, S.W. Rothwell, C.R. Alving, Liposomes containing lipid A serve as an adjuvant for induction of antibody and cytotoxic T-cell responses against RTS,S malaria antigen, Infect. Immun. 66 (1998) 2859–2865.
[16] J. Neidhart, K.O. Allen, D.L. Barlow, M. Carpenter, D.R. Shaw, P.L. Triozzi, R.M. Conry, Immunization of colorectal cancer patients with recombinant baculovirus-derived KSA (Ep-CAM) formulated with monophosphoryl lipid A in liposomal emulsion, with and without granulocyte-macrophage colony-stimulating factor, Vaccine 22 (2004) 773–780.
[17] M. Diwan, P. Elamanchili, H. Lane, A. Gainer, J. Samuel, Biodegradable nanoparticle mediated antigen delivery to human cord blood derived dendritic cells for induction of primary T cell responses, J. Drug Target. 11 (2003) 495–507.
[18] P. Elamanchili, M. Diwan, M. Cao, J. Samuel, Characterization of poly(D,L-lactic-co-glycolic acid) based nanoparticulate system for enhanced delivery of antigens to dendritic cells, Vaccine 22 (2004) 2406–2412.
[19] M.E. Lutsiak, G.S. Kwon, J. Samuel, Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses, J. Pharm. Pharmacol. 58 (2006) 739–747.
[20] M.B. Lutz, N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, G. Schuler, An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow, J. Immunol. Methods 223 (1999) 77–92.
[21] J. Ismaili, J. Rennesson, E. Aksoy, J. Vekemans, B. Vincart, Z. Amraoui, F. Van Laethem, M. Goldman, P.M. Dubois, Monophosphoryl lipid A activates both human dendritic cells and T cells, J. Immunol. 168 (2002) 926–932.
[22] T. De Smedt, B. Pajak, E. Muraille, L. Lespagnard, E. Heinen, P. De Baetselier, J. Urbain, O. Leo, M. Moser, Regulation of dendritic cell numbers and maturation by lipopolysaccharide in vivo, J. Exp. Med. 184 (1996) 1413–1424.
[23] R.M. Zinkernagel, On natural and artificial vaccinations, Annu. Rev. Immunol. 21 (2003) 515–546.
[24] A. Pashine, N.M. Valiante, J.B. Ulmer, Targeting the innate immune response with improved vaccine adjuvants, Nat. Med. 11 (2005) S63–S68.
[25] N. Qureshi, K. Takayama, E. Ribi, Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*, J. Biol. Chem. 257 (1982) 11808–11815.
[26] N. Qureshi, P. Mascagni, E. Ribi, K. Takayama, Monophosphoryl lipid A obtained from lipopolysaccharides of Salmonella minnesota R595. Purification of the dimethyl derivative by high performance liquid chromatography and complete structural determination, J. Biol. Chem. 260 (1985) 5271–5278.
[27] J.P. Valensi, J.R. Carlson, G.A. Van Nest, Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants, J. Immunol. 153 (1994) 4029–4039.
[28] S.L. Giannini, E. Hanon, P. Moris, M. Van Mechelen, S. Morel, F. Dessy, M.A. Fourneau, B. Colau, J. Suzich, G. Losonksy, M.T. Martin, G. Dubin, M.A. Wettendorff, Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only, Vaccine 24 (2006) 5937–5949.
[29] J. Kazzaz, M. Singh, M. Ugozzoli, J. Chesko, E. Soenawan, T.D. O'Hagan, Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency, J. Control. Release 110 (2006) 566–573.
[30] K.D. Newman, J. Samuel, G. Kwon, Ovalbumin peptide encapsulated in poly(d,l lactic-co-glycolic acid) microspheres is capable of inducing a T helper type 1 immune response, J. Control. Release 54 (1998) 49–59.