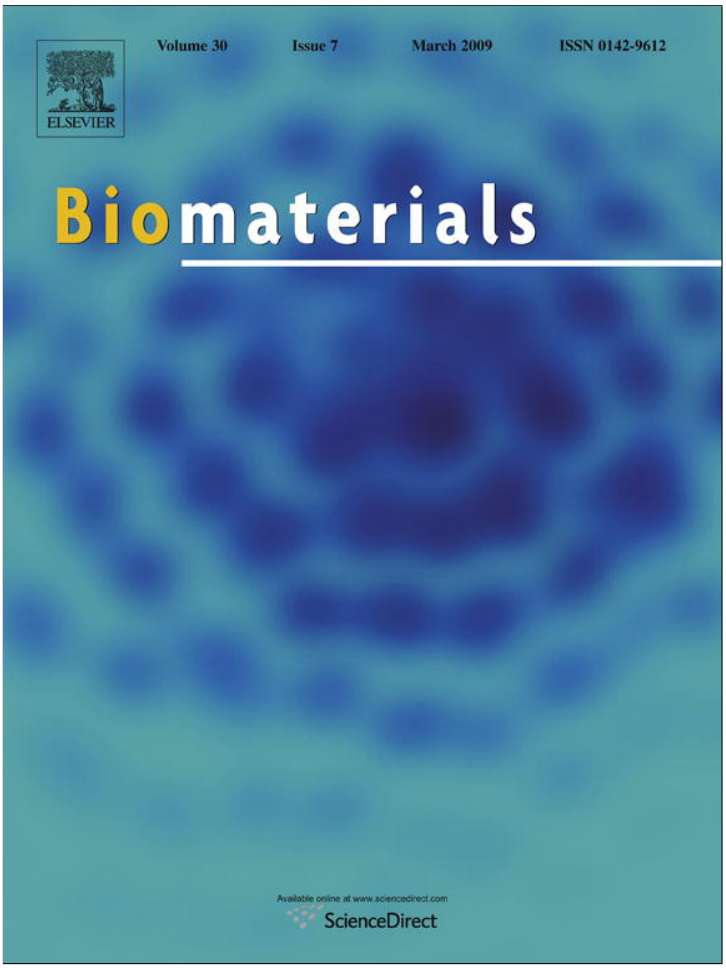

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Molecular basis of improved immunogenicity in DNA vaccination mediated by a mannan based carrier

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ABSTRACT

Receptor mediated gene delivery is an attractive non-viral method for targeting genetic material to specific cell types. We have previously utilized oxidized (OMPLL) and reduced mannan poly-L-lysine (RMPLL) to target DNA vaccines to antigen presenting cells and demonstrated that it could induce far stronger immune responses in mice compared to naked DNA immunization. In this study, we describe the immune enhancing attributes of mannan-PLL mediated DNA vaccination at the molecular level. Several attributes observed in similar gene delivery conjugates, such as entry via the endocytic pathway, low toxicity, protection from nucleases and compaction of particle size, were also evident here. In addition, OMPLL and RMPLL conjugates had profound effects on the antigen presentation functions of dendritic cells and macrophages, through the stimulation of cytokine production and maturation of dendritic cells. Interestingly, we demonstrate that OMPLL–DNA and RMPLL–DNA are able to mediate dendritic cell activation via toll-like receptor 2 as opposed to mannan alone which mediates via toll-like receptor 4. Overall, this report leads to greater understanding of how oxidized and reduced mannan mediated gene delivery could augment immune responses to DNA vaccination and provide insights into ways of further improving its immunogenicity.

1. Introduction

DNA vaccination has been proven effective in small animal models for many years. Its efficacy in humans however does not mirror these successes, hence the need to investigate novel methods to improve its immunogenicity. Understanding the process of efficient gene delivery allows us to identify problems and devise methods to overcome them. Many of these efforts are focused on using viral or non-viral carriers to act as vehicles to transport genetic material into cells. Although viral vectors are highly efficient in transducing cells, they lack in targeting specificity. This presents several disadvantages in in-vivo applications such as eliciting undesired effects on bystander cells, the need for larger doses and restriction to local administrations. Amongst the non-viral strategy is receptor mediated gene delivery, which allows targeting to specific cell types. It involves the use of a targeting moiety linked to a polycation that is capable of interacting electrostatically with the negatively charged backbone of DNA [1]. Many have utilized ligands of various C-type lectins, such as the asialoglycoprotein receptor and mannose receptor, to target DNA into cells via their endocytic properties. Thus far, there have been several reports of successful transfection of foreign DNA into antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages in vitro [2–6]. Similarly, to target DNA vaccines to APCs in vivo, we have performed immunization studies in mice using DNA complexed to mannan in its oxidized (OM) or reduced (RM) forms.

In our earlier studies, we have shown that OM conjugated to MUC1 fusion protein (MUC1FP) was able to induce strong cytotoxic T lymphocyte (CTL) responses against the tumor associated antigen and conferred tumor protection to immunized mice [7,8]. Instead of T-cell responses, RM-MUC1FP induced strong antibody responses and could not protect mice from tumor challenge. It was determined that cytokines secreted by splenic T cells from OM-MUC1FP immunized mice when stimulated with MUC1FP induced a classical Th1 cytokine profile IL-12, TNF-α and IFN-γ, whilst T cells from RM-MUC1FP immunized mice secreted IL-4 and IL-10 [9]. Further, both OM and RM were determined to interact with and stimulate DCs via toll-like receptor 4 (TLR4) inducing DC maturation, and

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increasing both antigen presentation capability and expression of Th1 and Th2 cytokines respectively [10]. More recently, we have demonstrated using ovalbumin (OVA) DNA [11] and mucin 1 (MUC1) DNA [12] that OMPLL–DNA and RMPLL–DNA immunization induced superior T-cell responses compared to naked DNA. OMPLL–DNA immunized mice induced specific IFN-γ secreting T-cell responses and weak antibody responses while RMPLL–DNA immunized mice induced IFN-γ, IL-4 and strong antibody responses. In both cases, better therapeutic and prophylactic tumor protection in mice were induced compared to DNA alone immunizations.

In the present study, we investigate the factors important to gene delivery and DNA vaccination that could contribute to the improved immunogenicity of OMPLL–DNA and RMPLL–DNA immunization. We show that OMPLL and RMPLL were able to complex with DNA to form particles that are compacted into sizes optimal for endocytic uptake. The particles formed were able to protect DNA from DNase I digestion, thus raising the possibility of delivery of DNA that is stable in the presence of nucleases in the extracellular environment. More importantly, OMPLL and RMPLL had a direct effect on the antigen presentation of DCs. In-vitro and in-vivo DCs were stimulated by OMPLL–DNA or RMPLL–DNA to induce a mature phenotype, which is more efficient in antigen presentation. In addition, pro-inflammatory cytokines were secreted by DCs when stimulated with the mannan DNA complexes. Surprisingly, unlike OM and RM which stimulate DCs via Toll-like receptor 4 (TLR4), both OMPLL–DNA and RMPLL–DNA stimulates via TLR2.

2. Materials and methods

2.1. Animals

C57BL/6, MyD88 knockout, C3H/He and C3H/HeJ mice, aged 6–10 weeks, used throughout this study were purchased from the animal facilities of the Walter and Eliza Hall Institute (Victoria, Australia) and maintained in the animal house facilities of Burnet Institute (Victoria, Australia).

2.2. Materials

Complete RPMI-1640 media were prepared by supplementing with 2% HEPES, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% (v/v) fetal calf serum. Recombinant GM-CSF used to culture DCs was purchased from BD Pharmingen (San Diego, USA) and was reconstituted in PBS. CpG (GeneWorks, Adelaide, Australia), LPS (L3137, Sigma, Castle Hill Australia), Poly(I:C) (P9582, Sigma, Castle Hill, Australia) were reconstituted in sterile distilled water. Anti-CD11c-APC, anti-DEC205–biotin and streptavidin–PE-Cy7 antibody were purchased from BD Pharmingen (San Diego, CA). Anti-CD40, CD80 and CD86 antibodies were prepared in-house. Cytochalasin D from *Zygosporium mansonii* (Sigma, Missouri, USA) and Filipin III from *Streptomyces filipinensis* (Sigma, Missouri, USA) were reconstituted in DMSO.

2.3. OVA DNA plasmid preparation

Plasmid sOVA-C1 was kindly provided by Dr. Peter Smooker, RMIT University, Australia [13,14]. The plasmid was constructed by subcloning an EcoRI–XbaI fragment of whole chicken ovalbumin sequence into the mammalian expression plasmid pCI (Promega, Madison, WI), which is under the control of the CMV promoter. Expression of the plasmid yields the secretion of soluble OVA. DNA plasmids were purified using the Qiagen Endofree Plasmid Maxi Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions.

2.4. Ethidium bromide quenching assay

The degree of complexation between carrier and OVA DNA with varied R values ($R = 0.1, 0.4, 1, 3$ and 5) was analyzed by the ethidium bromide quenching assay. The R value is defined as the molar charge ratio of DNA (PO_4^-) to PLL (NH_3^+). Ethidium bromide (1 μg/ml) was incubated with DNA complexes containing 2 μg of OVA DNA for 30 min at RT before being transferred to FluoroNunc plates (NUNC, Roskilde, Denmark). The fluorescence intensity was detected by the Fluostar Optima microplate reader (BMG Labtech, Offenburg, Germany) at spectra of excitation wavelength of 540 nm and emission wavelength of 590 nm. Gain adjustment for each assay was set on uncomplexed OVA DNA with ethidium bromide.

2.5. Evaluation of particle size and charge

The particle size of the complexes was determined by measuring the mean hydrodynamic diameter by dynamic light scattering using the Zetasizer Nano ZS (Malvern Instruments, UK) at the fixed angle of 173° at 25 °C. Complexes used in the measurement were of 5 μg DNA content and formed in 800 μl of MilliQ water that was pre-filtered with a 0.22 μm filter. Measurements were made in automatic mode. The Z average value of at least ten measurements was reported as the mean diameter of the particles. Polydispersity index (PDI) values represent the width of the particle size distributions and are an indicator of the homogenicity of the particles present in the sample. PDI = 1 indicates a highly heterogenous sample and PDI = 0 highly homogenous.

The surface charge of particles was quantified as zeta potential by laser Doppler velocimetry. Samples used for light scattering experiments were transferred into a folded capillary cell (Malvern Instruments, UK) before analysis on the Zetasizer Nano ZS. Zeta-potential values (mV) of at least ten measurements were computed automatically from the mean electrophoretic mobility by applying the Smoluchowski equation.

2.6. DNAse I digestion of DNA complexes

OVA DNA of 2 μg was complexed with OMPLL at $R = 0.1$. Complexes were treated with either (1) DNase I alone (4 units), (2) DNase I and heparin (1 mg/ml) simultaneously, (3) DNase I then heparin or (4) heparin alone. For DNase I treatment, 2 μg DNA was incubated with 4 units of DNase I in a 100 μl reaction mix (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 50 μg/ml BSA) at 37 °C for 30 min or 1 h and finally with 5 μl of 0.5 M EDTA pH 7 to stop the reaction followed by heparin or buffer. The DNA complexes were analyzed with 0.6% agarose gel electrophoresis.

2.7. Generation of bone marrow-derived DCs

Murine DCs were generated as described previously [15]. Briefly, bone marrow cells were extracted from the lumen of femurs and tibias. Bone marrow cells were then treated with sterile 0.73% (w/v) NH_4Cl for 10 min at 37 °C to lyse erythrocytes. Cells were washed and resuspended in complete media (2×10^6 cells/3 ml) supplemented with 10 ng/ml of GM-CSF. These cells were cultured for 4 days in a 24-well plate (1 ml/well). Cells were harvested by gentle pippeting of the culture media. GM-CSF cultured bone marrow cells yield large numbers of MHC class II expressing DCs that are potent mixed lymphocyte reaction (MLR) stimulator cells [15].

2.8. In-vitro DC maturation studies

C57BL/6, MyD88 knockout, C3H/He and C3H/HeJ mice derived DCs were used in maturation studies. DCs were removed from culture plates and 1×10^5 DCs were resuspended in 150 μl of complete RPMI supplemented with 10 ng/ml GM-CSF and seeded into 48 well plates. 50 μl OVA DNA alone, RMPLL–OVA DNA or OMPLL–OVA DNA complexes were added to wells (14.3 μg/ml DNA and 200 μg/ml mannan content). CpG (10 μg/ml), LPS (1 μg/ml) and poly(I:C) (50 μg/ml) were used as positive controls and negative control (diluent: 5 mM NaCl) was also added into respective wells and incubated at 37 °C for 18 h. Cells were harvested and stained with anti-CD11c-APC together with anti-CD86 that was conjugated with FITC. $CD11c^{high}$ cells were gated and intensity of FITC was determined by histogram analysis to determine DC maturation states.

2.9. DC maturation studies using anti-TLR2 blocking antibodies on C57BL/6 DCs

Day 4 DCs (2×10^5) were resuspended in 50 μg/ml TLR2 blocking antibody clone T2.5 (eBioscience, San Diego, USA) for 30 min at 37 °C. OMPLL–OVA DNA or RMPLL–OVA DNA (14.3 μg/ml DNA and 200 μg/ml mannan content) were added to DCs which were pre-incubated with or without anti-TLR2 blocking antibody, at a final volume of 100 μl. LPS (ligand of TLR4) (1 μg/ml) and zymosan (ligand of TLR2) (500 μg/ml) were used as controls. Treated DCs were incubated at 37 °C for 18 h before being harvested and stained with anti-CD11c-APC and anti-CD86-FITC. $CD11c^{high}$ cells were analyzed for CD86 expression.

2.10. In-vivo DC maturation studies

C57BL/6 mice were injected intradermally (ID) on both hind footpads with 50 μl of OVA DNA, RMPLL–OVA DNA, OMPLL–OVA DNA and LPS (positive control). 50 μl of RMPLL–OVA DNA and OMPLL–OVA DNA contained 231.5 μg of mannan and 15 μg of DNA. OVA DNA groups received 15 μg of DNA while positive controls received 10 μg LPS per footpad. Popliteal lymph nodes were removed from injected mice 18 h later and cell suspensions were prepared. Cells were then resuspended in BSS buffer (2% v/v FCS, 0.1% NaN_3 and 2 mM EDTA in PBS) and incubated with CD11c microbeads (10 μl per 10^7 cells) (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4 °C. Cells were washed with BSS buffer. The labelled $CD11c^+$ cells were sorted by AutoMacs (Miltenyi Biotec, Bergisch Gladbach, Germany). To differentiate DC subpopulations, cells were stained with anti-DEC205–biotin and streptavidin–PE-Cy7 and anti-CD8-PE together with anti-CD11c-APC. The maturation states of DC subpopulations were determined by anti-CD86-FITC.

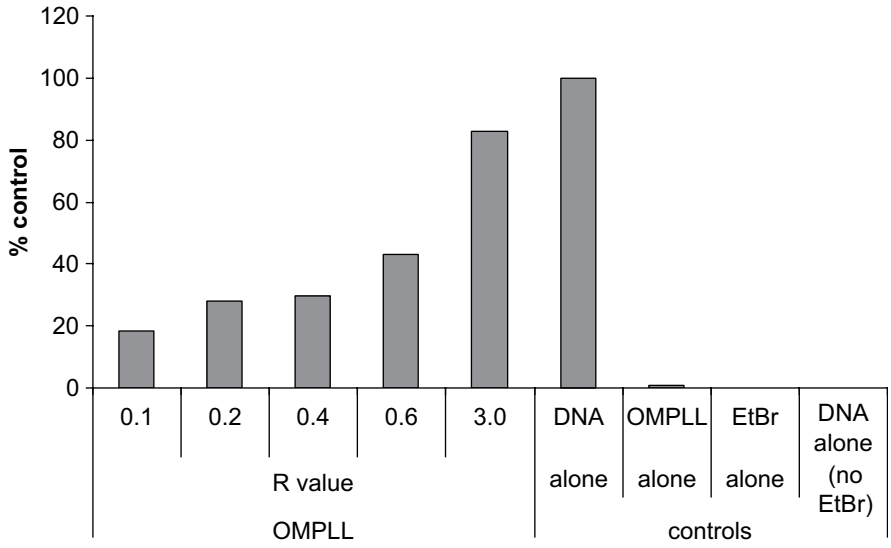

Fig. 1. OMPLL complexes with OVA DNA. DNA complexation efficiency was assessed by ethidium bromide quenching assay. The degree of interaction between PLL, OM and OMPLL with DNA was assessed by the amount of fluorescence induced by binding of ethidium bromide to exposed DNA binding sites. 1 μg/ml of ethidium bromide was reacted with 2 μg of complexed DNA for 15 min before detection of fluorescence (at Ex 540 and Em 590). Data are presented as the percentage of fluorescence relative to OVA DNA alone; representative of 3 separate experiments with variations less than 5% between experiments.

2.11. YOYO-1 complexation with DNA

DNA intercalating dimeric cyanine fluorescent dye, YOYO-1 (Molecular Probes, Leiden, The Netherlands), was used to label OVA DNA for uptake studies in RAW cells (macrophage like cell line). To make labelled DNA complexes, OVA DNA was incubated with the appropriate amount of YOYO-1 for 15 min at 37 °C before the addition of RMPLL/OMPLL and incubated for a further 30 min at 37 °C. The amount of YOYO-1 used was 1 dye molecule to 300 DNA base pairs which is equivalent to 5 μl of 1 μM Dye to 1 μg OVA DNA (5201 bp). The efficiency of complexation between RMPLL and OMPLL with OVA DNA was not affected by the presence of DNA dye as determined by agarose gel electrophoresis (data not shown).

2.12. Uptake pathway

Raw cells (3×10^5) were seeded into 24 well plates to adhere overnight at 37 °C. Various inhibitors of cellular uptake processes including sodium azide (10 mM), dextran sulphate (20 μg/ml) and cytochalasin D (1 μg/ml) were added to DC cultures and incubated for 1 h at 37 °C. Thereafter, YOYO-1 labelled OVA DNA complexes were added to the treated cells and incubated for a further 4 h at 37 °C. Cells were subsequently harvested by scraping, followed by staining with propidium iodide (1 μg/ml) and analyzed on a flow cytometer (BD Bioscience, California, USA).

2.13. Cytokine analysis of supernatant from DCs stimulated with OM/RMPLL–OVA

Day 4 GM-CSF grown C57BL/6 DCs (3×10^5) were incubated with OVA DNA (14.3 μg/ml), OM/RMPLL–OVA DNA (14.3 μg/ml DNA and 200 μg/ml mannan content) and LPS (1 μg/ml) in 200 μl culture media in a 48-well plate for 18 h before the supernatants were collected and analyzed for the presence of cytokines using a multiplex assay, Bioplex kit (Bio-Rad, California, USA).

2.14. Statistical analysis

All data are represented as means ± standard error of mean. One-way analysis of variance (ANOVA) with Bonferroni's post-test was used to compare significant difference between 3 or more groups. $p \leq 0.05$ indicates a statistically significant difference.

3. Results

3.1. Physical characterization of DNA complexation

The degree of complexation with respect to *R* values was analyzed by the ethidium bromide quenching assay, which indicates the relative amount of interaction between DNA and carrier by comparing level of fluorescence emitted by intercalation of ethidium bromide into DNA. OVA DNA was used in this study. From the results, it was clear that OMPLL interacted with OVA DNA as there was inhibition of ethidium bromide quenching in OMPLL–OVA DNA complexes of $R = 0.1$ and $R = 0.4$ (Fig. 1). Such inhibition was increased when *R* value decreased. OM alone did not inhibit binding of ethidium bromide to DNA while PLL alone showed stronger inhibition compared to OMPLL (data not shown).

Particle size and surface charge of DNA complexes were determined by dynamic light scattering and laser Doppler velocimetry respectively (Fig. 2A). It was observed that the size of OVA DNA was considerably reduced in the presence of OMPLL or PLL. Furthermore, it appeared that *R* value not only affected the degree of complexation between carrier and DNA but also the particle size of the complex. Decreasing *R* values decreases the particle size. It was noted that the size of the complex was smaller than the carrier alone at lower *R* values, indicating condensation and complex formation, hence supporting ethidium bromide quenching data. In addition, polydispersity index (PDI), which reflects the range of the size distribution of particles present in the sample, indicated that OMPLL–OVA DNA complexes were more heterogenous than PLL–DNA complexes (data not shown). This could reflect the heterogeneity in size of mannan, which contains carbohydrate that ranges from 50 kDa to 1000 kDa. The surface charge of the particles is presented in Fig. 2B. The charge of OVA DNA and PLL was −39.4 mV and 34.8 mV respectively, while OMPLL was 1.94 mV. PLL–OVA complexes were negatively charged at $R = 2$ and $R = 1$ and positively charged at $R = 0.4$ and below. OMPLL–OVA DNA, however, did not form complexes of positive charge even at low *R* value, which was to be expected since its carrier has a close to neutral charge compared to PLL. Results here indicate that OMPLL does interact and complex with DNA to form particles that are compacted in size and less negatively charged than DNA alone.

3.2. DNase I protection studies

DNA complexed to various polyamines had been shown to be protected from nuclease degradation due to the formation of compacted DNA particles [16–18]. To investigate whether DNA complexed to OMPLL could be protected, OMPLL–OVA DNA was treated with DNase I followed by incubation with heparin to release any non-degraded DNA bound to PLL (Fig. 3). Gel electrophoresis

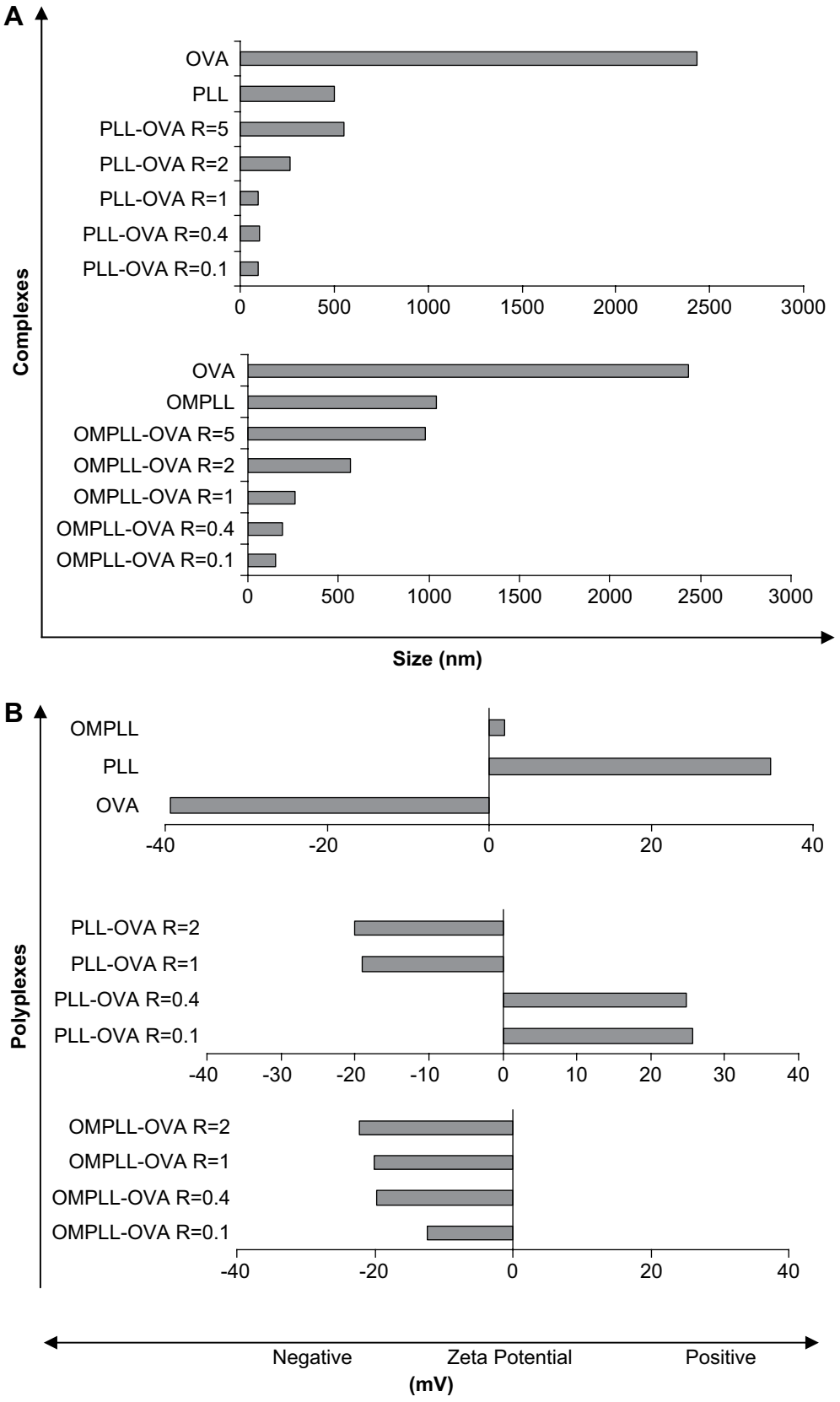

Fig. 2. Particle size and zeta-potential analysis of DNA complexes. (A) The size of OVA, PLL, OMPLL and OMPLL–OVA DNA complexes of various *R* values was assessed. Measurements were made at the fixed angle of 173° at 25 °C. *Z* average value of at least ten measurements was reported as the mean diameter of the particles. (B) The charge of OVA DNA, PLL, OMPLL and OMPLL–OVA DNA complexes at various *R* values. Zeta-potential values (mV) of at least ten measurements were computed automatically from the mean electrophoretic mobility by applying the Smoluchowski equation; representative of 3 separate experiments.

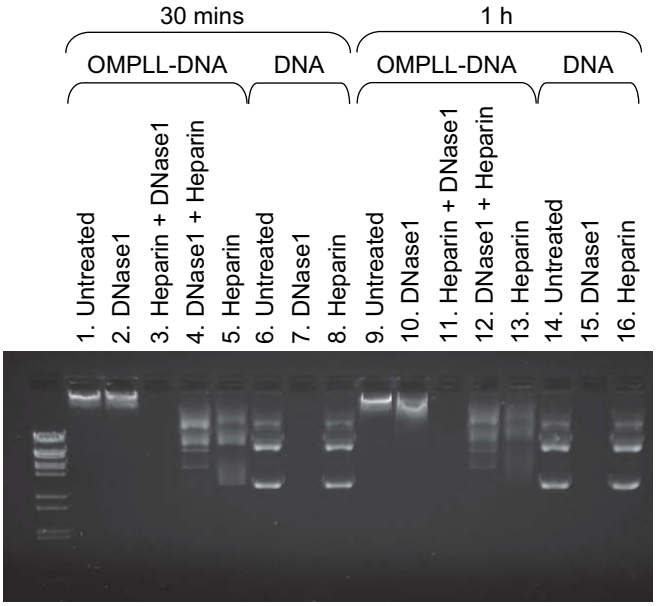

Fig. 3. OMPLL protects DNA from DNase I digestion. Protection of DNA from degradation was assessed by treatment with DNase I, DNase I and heparin, DNase I then heparin and heparin alone. DNase I treatment for 30 min and 1 h is shown. OMPLL–DNA complexes were formed at $R = 0.1$ with 2 μg of OVA DNA. DNA complexes after treatment were analyzed with 0.6% agarose gel electrophoresis.

revealed that OVA DNA alone was completely degraded by DNase I but not affected by heparin treatment as expected (lanes 6, 8, 14 and 16). Conversely, treatment of OMPLL–OVA DNA complexes with DNase I (lanes 2 and 10) did not result in the digestion of the DNA as the same intensity band as untreated OVA DNA (lanes 1 and 9) was evident. OMPLL–OVA DNA complexes treated with heparin to release bound DNA followed by treatment with DNase I (lanes 3 and 11) showed that all DNA were digested. While OMPLL–DNA complexes that were treated with DNase I and subsequently heparin (lanes 4 and 12) showed bands with intensity similar to OMPLL–OVA DNA treated with heparin only (lanes 5 and 13), therefore proving that OMPLL did protect bound OVA DNA from nucleases' digestion. Results here demonstrate that OMPLL does protect DNA from nuclease digestion.

3.3. OMPLL–DNA and RMPLL–DNA uptake kinetics and pathway

In order to visualize and quantitate the rate of uptake of OMPLL–DNA and RMPLL–DNA by APCs, OVA DNA was labelled with a fluorescent DNA intercalator, YOYO-1 [19], before complexation with OMPLL and RMPLL. The pathway of OMPLL–DNA, RMPLL–OVA DNA, PLL–OVA DNA and OVA DNA uptake by RAW cells was investigated by pre-treatment with biochemical inhibitors (Fig. 4). Firstly, it was demonstrated that the uptake of all labelled complexes was predominantly energy dependent, as depletion of cellular ATP pool by pre-incubation with sodium azide resulted in extensive (60–70%) inhibition of uptake. From the competition assay with dextran sulphate, it was observed that there was a substantial reduction in the binding between the particles and cells via a charge interaction. Uptake of PLL–OVA DNA complexes was the most affected by dextran sulphate. Cytochalasin D, which disrupts the function of actin filament by inhibiting F-actin elongation [20], was used to determine if OMPLL–OVA DNA and RMPLL–OVA DNA are taken up by macropinocytosis or other endocytic processes. Indeed, OMPLL–OVA DNA, RMPLL–OVA DNA and OVA DNA uptakes were significantly inhibited by the cytochalasin D treatment, whilst PLL–OVA DNA was not affected. This demonstrates that unlike PLL–OVA DNA,

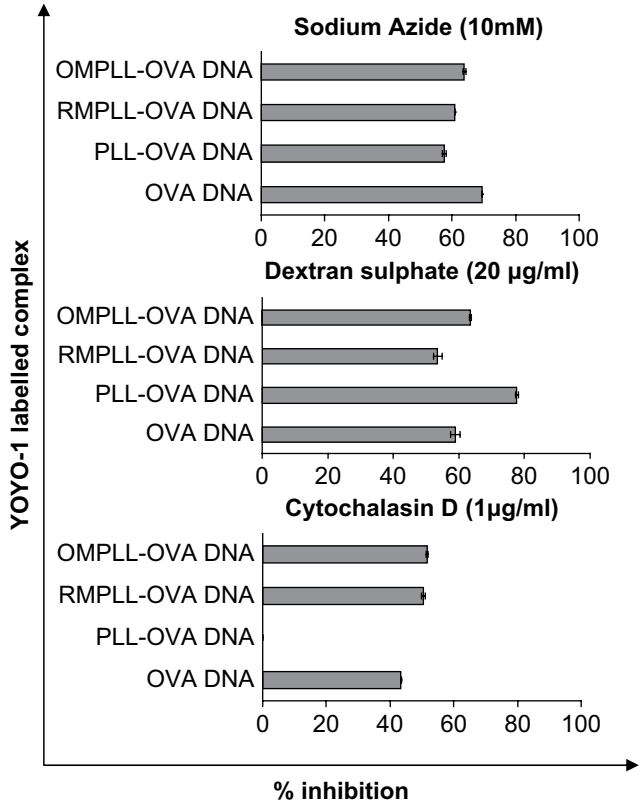

Fig. 4. Inhibition of uptake of complexes by treatment with inhibitors of various cellular uptake pathways. OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA were labeled with intercalating fluorescent DNA dye YOYO-1 and incubated with RAW cells for 4 h. The amount of uptake was compared between cells that were un-inhibited or inhibited with sodium azide (10 mM), ammonium chloride (50 mM), dextran sulphate (20 μg/ml), cytochalasin D (1 μg/ml) or filipin (5 μg/ml) by pre-treatment for 1 h before addition of the DNA complex. Percentage of inhibition is reflected. Triplicate samples ± standard error of mean are shown; representative of 3 different experiments.

in addition to uptake via electrostatic interactions, there are a proportion of OMPLL–OVA DNA and RMPLL–OVA DNA particles that are taken up by RAW cells via endocytic mechanisms.

3.4. Induction of cytokine secretions by OMPLL–DNA and RMPLL–DNA stimulated DCs

To detect the amount of IL-2, IL-12, IFN-γ and TNF-α present in the supernatant of DCs incubated with OMPLL–DNA and RMPLL–DNA (14.3 μg/ml DNA and 200 μg/ml mannan content) or an equivalent amount of DNA alone for 18 h, the Bioplex kit from Bio-Rad was used (Fig. 5). Supernatant from untreated and LPS (1 μg/ml) stimulated DCs were negative and positive controls respectively. None of the tested cytokines were detected in the supernatant of un-stimulated DCs, whilst LPS stimulated DCs showed detectable levels of all cytokine tested. The secretion of each cytokine relative to DNA stimulated DCs was different between RMPLL–DNA and OMPLL–DNA. RMPLL–DNA showed significant increase in IL-2 secretion compared to DCs stimulated with DNA alone, other cytokine levels are comparable to DNA alone. OMPLL–DNA induced the highest level of secretion across all cytokines tested. OMPLL–DNA was a more potent stimulator of cytokine production in DCs compared to RMPLL–DNA. In addition, OMPLL–DNA has over 2-fold increase in IL-12, a Th1 cytokine, compared to RMPLL–DNA. Results here demonstrate that RMPLL–DNA and OMPLL–DNA induce a different profile of cytokine secretion levels by DCs and are strong stimulants of cytokine release compared to DNA alone.

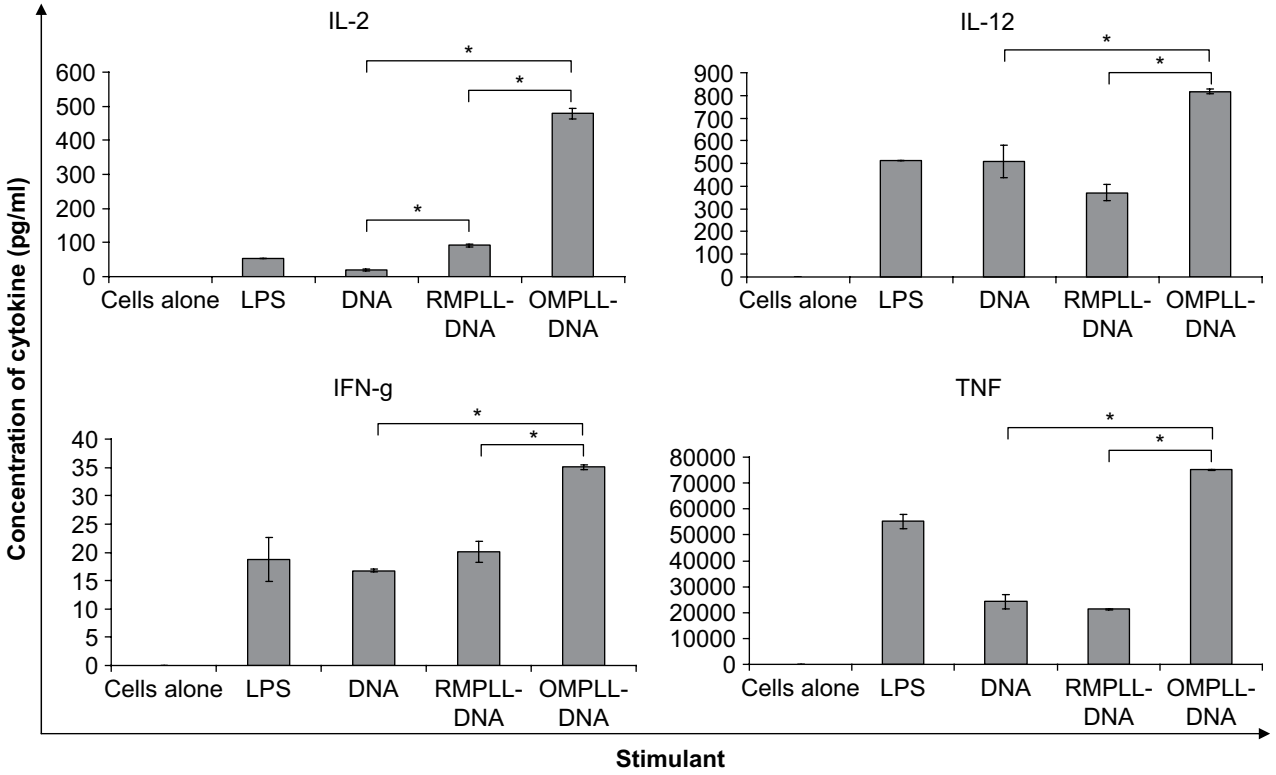

Fig. 5. Bioplex analysis of cytokine secretion in GM-CSF cultured bone marrow cells stimulated with RM/OMPLL–OVA DNA. Day 4 GM-CSF cultured C57BL/6 DCs were incubated with OVA DNA, RMPLL–OVA DNA or OMPLL–OVA DNA (at 14.3 μg/ml DNA and 200 μg/ml mannan content) for 18 h. Supernatants were collected and IL-2, IL-12, IFN-γ and TNF-α secretion were assessed by Bioplex. Duplicate samples ± standard error of mean are shown. $^{*}p \leq 0.05$ indicates a statistically significant difference. Results are presented as pg/ml and representative of two separate experiments is shown.

3.5. Maturation of in-vitro and in-vivo grown DCs by RMPLL–OVA DNA and OMPLL–OVA DNA

Maturation of DCs is an essential process to prevent energy and generate an effective immune response against the target antigen in immunization strategies. It has been shown that mannan and mannose based conjugates mature DCs [10,21]. To determine if OMPLL–DNA and RMPLL–DNA could induce maturation in DCs, bone marrow derived DCs from C57BL/6 mice were incubated with OMPLL–OVA DNA and RMPLL–OVA DNA for 18 h (Fig. 6A). Diluent (5 mM NaCl) and LPS (1 μg/ml) were used as negative and positive controls respectively. High CD11c expressing cells were assessed for their expression of CD86. OVA DNA was not a strong inducer of DC maturation when uncomplexed. In contrast, OVA DNA complexed with RMPLL and OMPLL (at 200 μg/ml mannan concentration of the complex) stimulated a level of maturation that was comparable to LPS stimulation at 1 μg/ml. A stronger upregulation was noted in OMPLL–OVA DNA compared to RMPLL–OVA DNA. PLL–OVA, at a lower non-toxic dose, was unable to mature DCs (data not shown). Hence, results here indicate that RMPLL–DNA and OMPLL–DNA are able to induce stronger DC maturation compared to DNA alone.

We further investigated the maturation effect of OMPLL–DNA and RMPLL–DNA in in-vivo DC subsets (Fig. 6B). C57BL/6 mice were injected intradermally on the footpads and 18 h later popliteal lymph nodes were removed and enriched for CD11c expressing cells using AutoMacs. Based on their expression for CD8 and DEC205, lymph node DCs were generally categorized into $CD8^{+}DEC205^{-}$, $CD8^{int}Dec205^{+}$ and $CD8^{-}DEC205^{-}$. Using LPS (10 μg/footpad) as a positive control, the maturation effect could be observed in all three populations of DCs (Fig. 5B). This maturation effect was most profound in the $CD8^{+}DEC205^{-}$ subset. Parallel to the above in-vitro DC maturation study, uncomplexed OVA DNA did not mature DCs in vivo. The maturation effect of RMPLL–DNA and OMPLL–DNA was observed only in the $CD8^{+}DEC205^{-}$ subset. There was no significant upregulation of CD86 in the $CD8^{int}DEC205^{+}$ and $CD8^{-}DEC205^{-}$ populations. Similarly, LPS stimulation was also not able to induce strong CD86 upregulation in these two populations as seen in $CD8^{+}DEC205^{-}$ DCs. Overall, these results indicate that RMPLL–OVA DNA immunization and OMPLL–OVA DNA immunization mature DCs in vivo, and more specifically the $CD8^{+}DEC205^{-}$ DC subset.

3.6. Maturation of C3H/He and C3H/HeJ DCs by RMPLL–OVA DNA and OMPLL–OVA DNA

TLR are the sentinels of the immune system which are able to recognize a wide array of pathogen-associated molecular patterns from bacterial DNA, viral double stranded RNA, microbial lipids and LPS. Once activated, it would induce a wide array of pathogen clearing immune responses such as cytokine and chemokine secretion, co-stimulatory and adhesion molecule upregulation in immune cells. It was previously shown that RM and OM were able to stimulate DC maturation via TLR4 activation [10]. To determine if DC maturation by RMPLL–OVA DNA and OMPLL–OVA DNA is mediated by TLR4, maturation studies were performed on DCs cultured from C3H/HeJ mice bone marrow cells. C3H/HeJ inbred mice are defective in TLR4 signalling due to the mutation in the gene expressing Ran/TC4 GTPase and are resistant to LPS stimulation [22] (Fig. 7). Wild-type C3H/He DCs which have normal TLR4 signalling were used as negative controls. RM and OM at 800 μg/ml were included as controls [10]. LPS (1 μg/ml) and CpG (10 μg/ml) which are TLR4 and TLR9 agonists respectively were used as positive controls. Expression levels of CD86 on pulsed DCs were analyzed. Similar to C57BL/6 DCs, RMPLL–DNA and OMPLL–DNA were strong inducers of maturation in the wild-type C3H/He DCs.

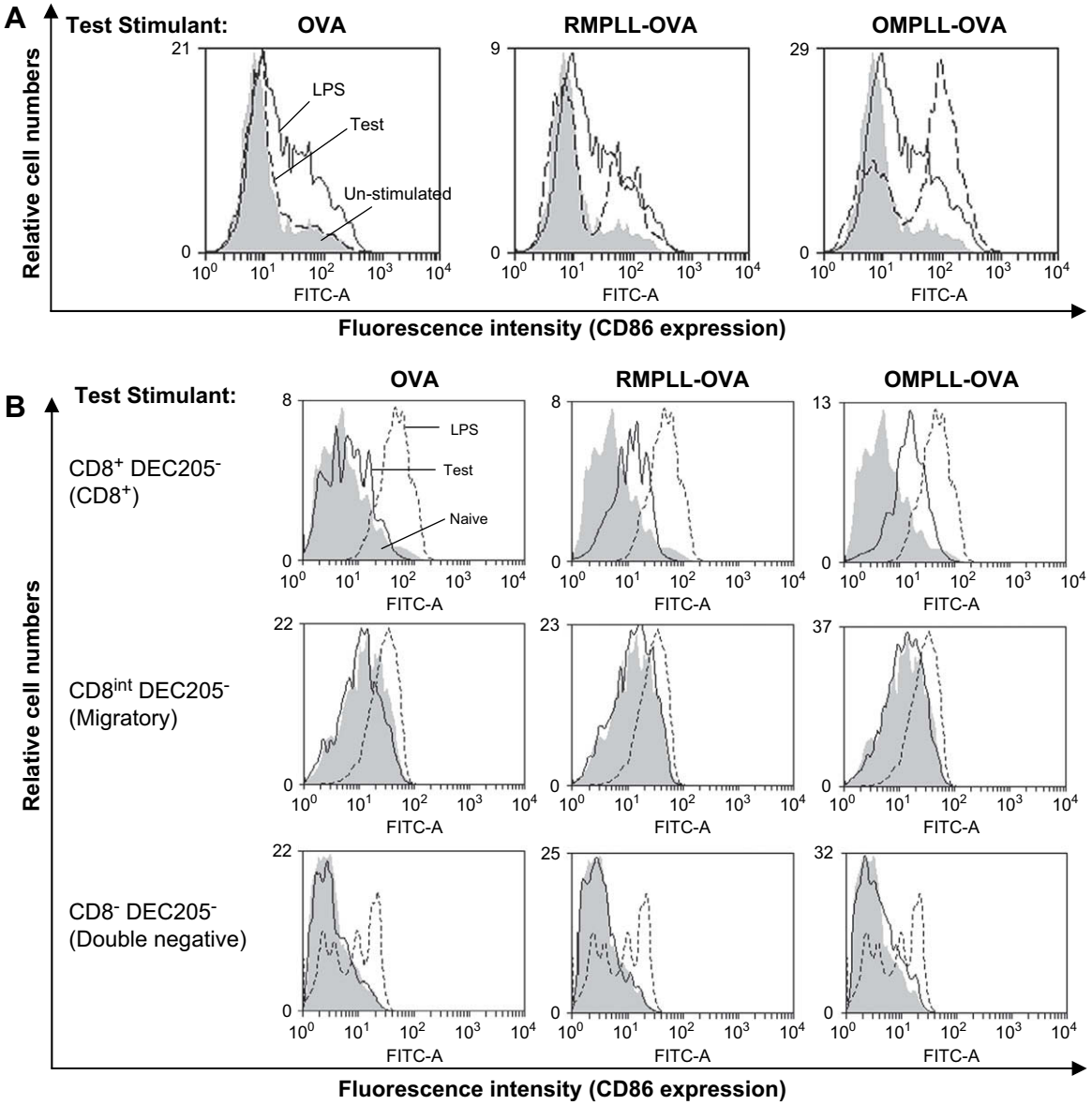

Fig. 6. DC maturation effect of OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA. (A) In-vitro grown C57BL/6 DCs. Day 4 GM-CSF grown C57BL/6 DCs were incubated with OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA (at 14.3 μg/ml DNA and 200 μg/ml mannan content) for 18 h before the detection of DC maturation marker CD86 using flow cytometry. LPS (1 μg/ml) stimulated DCs were used as positive controls. Analysis was performed on live cells (from SSC vs FSC plots) and CD11c positive cells. (B) In-vivo C57BL/6 popliteal DCs. C57BL/6 mice were injected with OVA DNA, RMPLL–OVA DNA or OMPLL–OVA DNA (at 235.5 μg/ml mannan content) intradermally at the footpads before popliteal lymph nodes were removed and isolated for DCs using AutoMacs. LPS (10 μg/footpad) immunized mice were used as positive controls. DCs were separated into 3 different populations based on their expression for DEC205 and CD8 (not shown). DC maturation marker CD86 levels were detected on these populations. Analysis was performed on live cells (from SSC vs FSC plots) and CD11c positive cells (from the already enriched sample). Data are presented as histograms; representative of two separate experiments.

OVA DNA alone was not able to induce appreciable upregulation of CD86 levels. LPS matured C3H/He DCs whilst CpG induced maturation in both C3H/He and C3H/HeJ DCs. In accordance with previous findings, RM and OM were able to mature C3H/He DCs but could not induce maturation in C3H/HeJ DCs [10]. Surprisingly, both RMPLL–OVA DNA and OMPLL–OVA DNA complexes induced strong maturation in wild-type and TLR4 defective DCs, indicating TLR4 is not required for DC stimulation by OMPLL–DNA and RMPLL–DNA. These findings suggest that RMPLL–OVA DNA and OMPLL–OVA DNA interact with DCs differently from RM and OM.

3.7. Maturation of MyD88 knockout DCs by RMPLL–OVA DNA and OMPLL–OVA DNA

To investigate if RMPLL–DNA and OMPLL–DNA require TLR stimulation to induce DC maturation, we performed DC maturation studies on myeloid differentiation factor 88 (MyD88) knockout DCs. MyD88 is an adaptor molecule that associates with the cytoplasmic Toll/interleukin-1 receptor (TIR) domain of TLRs. The majority of the members of the TLR family utilizes MyD88 as their exclusive adaptor molecule for downstream signalling events except for TLR3 and TLR4 [23]. Hence, MyD88 knockout DCs would be unresponsive to stimulation from all TLR agonists except TLR3 and TLR4. In this study, C57BL/6 DCs were used as a control because MyD88 knockout mice were derived from C57BL/6 mice and have normal MyD88 function. CpG (10 μg/ml), LPS (1 μg/ml) and poly(I:C) (50 μg/ml), which are agonists of TLR9, TLR4 and TLR3 respectively, were used to demonstrate the unresponsiveness of MyD88 knockout DCs to TLR9 stimulation but strongly activated by TLR3 and TLR4 agonists (Fig. 8A). Indeed, with the exception of CpG on MyD88 knockout DCs, poly(I:C) and LPS were able to mature C57BL/6 and MyD88 knockout DCs. Similar to results demonstrated in

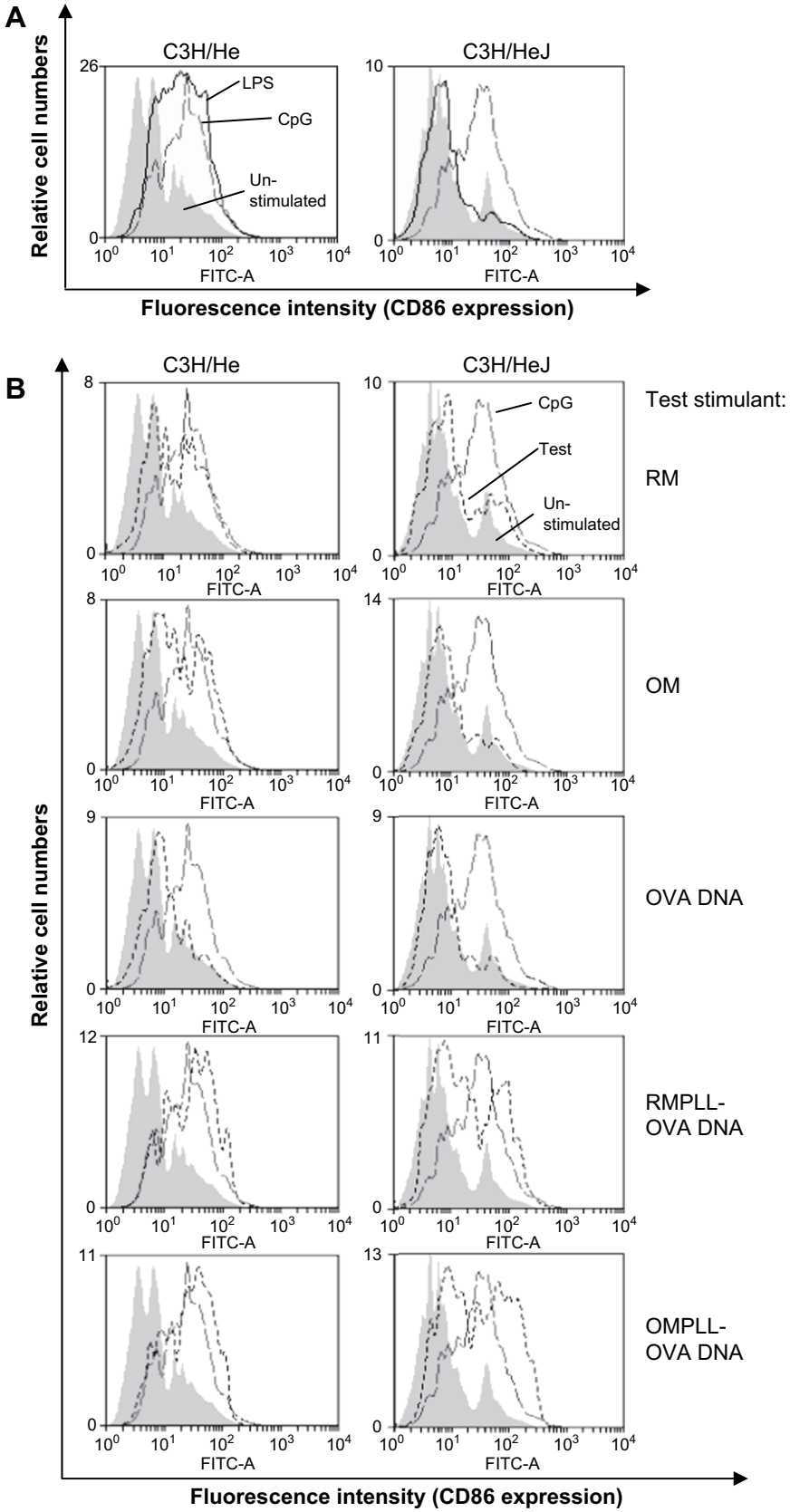

Fig. 7. DC maturation effect of OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA on in-vitro grown C3H/He and C3H/HeJ DCs. Day 4 GM-CSF grown C3H/He and C3H/HeJ DCs were incubated with OVA DNA, OM, RM, RMPLL–OVA DNA or OMPLL–OVA DNA (at 14.3 μg/ml DNA and 200 μg/ml mannan content) for 18 h before the detection of DC maturation marker CD86 using flow cytometry. LPS (1 μg/ml) stimulated DCs were used as positive controls. Analysis was performed on live cells (from SSC vs FSC plots) and CD11c positive cells. Data are presented as histograms; representative of two separate experiments.

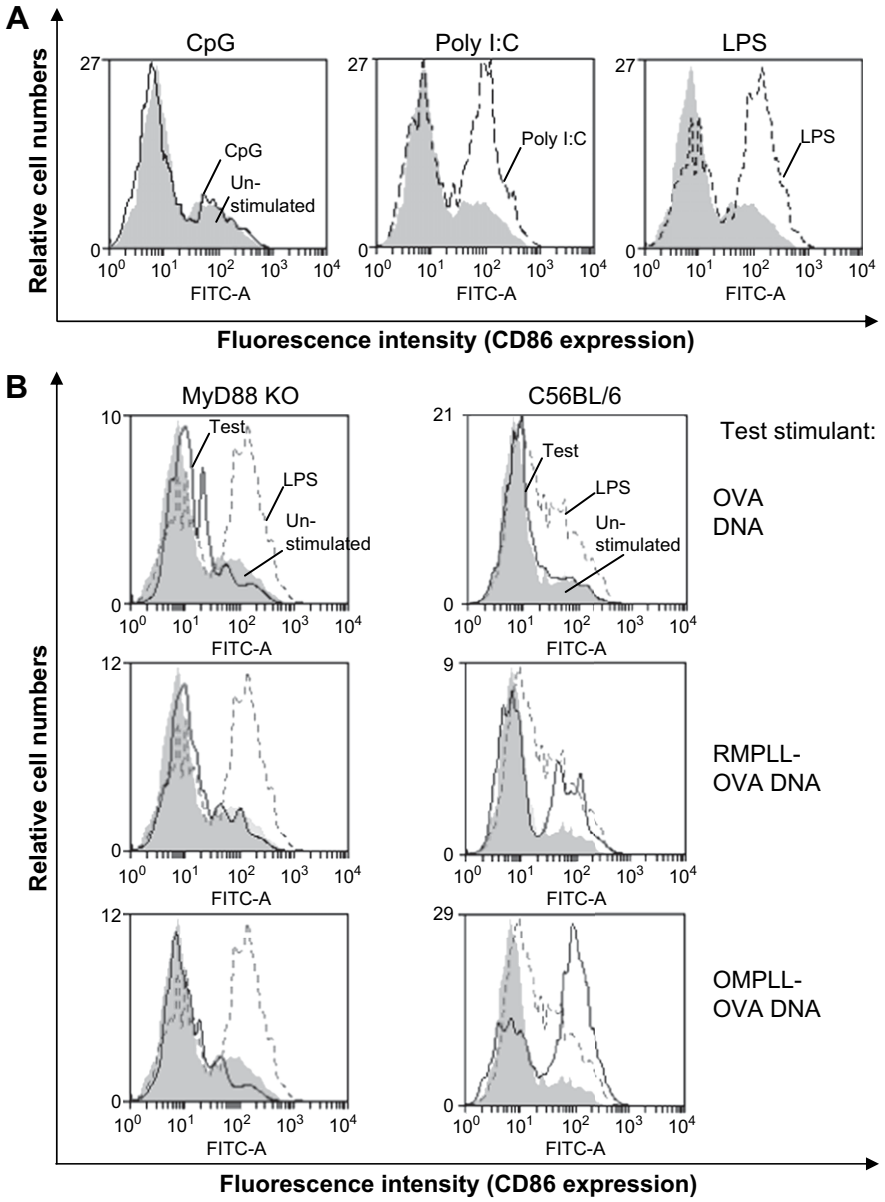

Fig. 8. DC maturation effect of OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA on in-vitro grown MyD88 knockout DCs. (A) MyD88 knockout DCs were stimulated with CpG (TLR 9 agonist), poly(I:C) (TLR 3 agonist) and LPS (TLR 4 agonist). (B) MyD88 knockout and C57BL/6 DCs were incubated with OVA DNA, RMPLL–OVA DNA or OMPLL–OVA DNA (at 14.3 μg/ml DNA and 200 μg/ml mannan content) for 18 h before the detection of DC maturation marker CD86 using flow cytometry. Analysis was performed on live cells (from SSC vs FSC plots) and CD11c positive cells. Data are presented as histograms; representative of two separate experiments.

Fig. 6A, there was an upregulation of DC maturation in RMPLL–OVA DNA and OMPLL–OVA DNA pulsed wild-type DCs whilst OVA DNA did not. Interestingly, there was no appreciable level of CD86 upregulation in MyD88 knockout DCs when pulsed with RMPLL–OVA DNA or OMPLL–OVA DNA. Thus, these results suggest that RMPLL–OVA DNA and OMPLL–OVA DNA induce DC maturation via an MyD88 dependent pathway.

3.8. Maturation of TLR2 blocked C57BL/6 DCs by OMPLL–DNA and RMPLL–DNA

Similar to TLR4, TLR2 recognizes a variety of bacterial cell wall components such as lipoprotein from mycobacteria [24] and meningococcus [25], lipoteichoic acid [26], mycobacterial lipo-arabinomannan [27], zymosan [28] and peptidoglycan [26]. Since it has been determined that OMPLL maturation and RMPLL maturation are TLR4 independent but MyD88 dependent, TLR2 was investigated for its involvement in the complexes DC maturing properties. Immature C57BL/6 DCs were pre-incubated with TLR2 blocking antibodies before the addition of OMPLL–DNA or RMPLL–DNA complexes (Fig. 9). LPS (1 μg/ml) and zymosan (500 μg/ml), which mature DCs via TLR4 and TLR2, respectively were used as controls. LPS induced maturation was not affected by the pre-incubation of TLR2 blocking antibodies while zymosan treated DCs showed a decrease in upregulation of CD86 expression compared to unblocked DCs. This validates that TLR2 was blocked and could not be activated. In the presence of TLR2 blocking antibodies, OMPLL–OVA DNA and RMPLL–OVA DNA treated DCs appeared to have a reduced expression of CD86 molecules compared to the absence of blocking antibody. This level of reduction was similar to that observed in the zymosan treated DCs. DC maturation induced by OMPLL and RMPLL is also decreased in the presence of TLR2 blocking antibody (data not shown). In summary, these results indicate that OMPLL–DNA and RMPLL–DNA mature DCs via a TLR2 and not TLR4 dependent pathway.

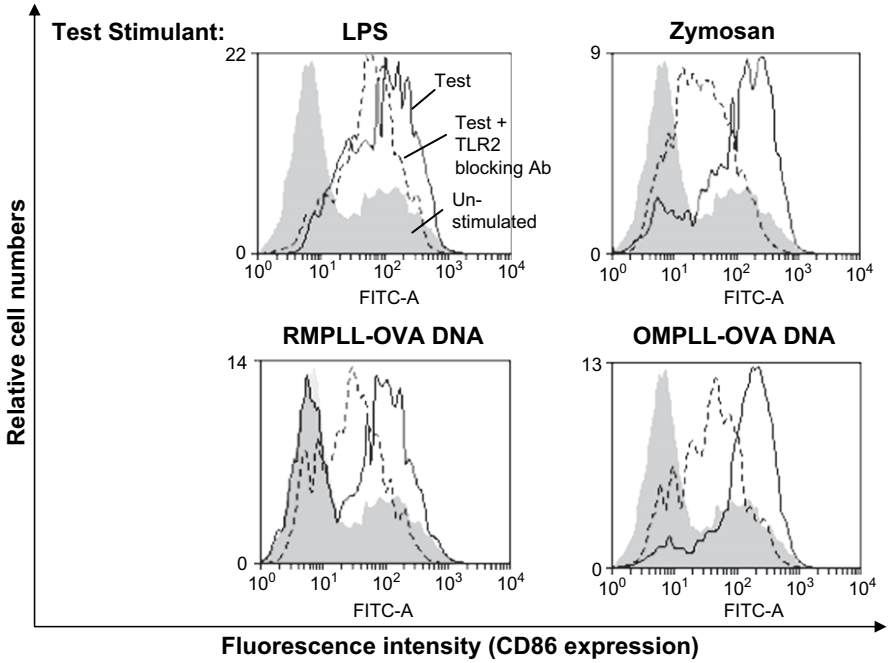

Fig. 9. DC maturation effect of OVA DNA, RMPLL–OVA DNA and OMPLL–OVA DNA on TLR2 blocked C57BL/6 DCs. Day 4 GM-CSF grown C57BL/6 DCs were pre-incubated for 1 h at 37 °C with TLR2 blocking antibody (T2.5 clone) before incubation with RMPLL–OVA DNA and OMPLL–OVA DNA (at 14.3 μg/ml DNA and 200 μg/ml mannan content) for 18 h before the detection of DC maturation marker CD86 using flow cytometry. LPS (TLR4 ligand) (1 μg/ml) and zymosan (TLR2 ligand) (500 μg/ml) were used as controls. Analysis was performed on live cells (from SSC vs FSC plots) and CD11c positive cells. Data are presented as histograms.

4. Discussion

Complexation of DNA to OMPLL and RMPLL was shown to augment immune responses to DNA vaccines in vivo [11]. Data presented here indicate that both carriers improve the immunogenicity of DNA vaccines in several ways. The pathway of uptake by cells may determine how the antigens are processed endogenously and the fate of the antigen. We showed that OMPLL–DNA and RMPLL–DNA particles were taken up by charge dependent binding and endocytic pathways. Furthermore, size analysis showed that the OMPLL–OVA DNA and RMPLL–OVA DNA particles were of the appropriate size for cellular uptake by receptor mediated internalisation processes such as clathrin dependent endocytosis [29]. For proper antigen presentation process to occur, two signals are required. First, the contacts between T-cell receptor and the antigen peptide that is loaded onto MHC molecules. The second signal is between co-stimulatory markers such as CD80 (B7.1) and CD86 (B7.2). Mature DCs that are primed for antigen presentation would have upregulated their expression for these co-stimulatory molecules. Thus, the expression levels of co-stimulatory molecules are related to how efficient the antigens are presented and ultimately affect the immune response generated [30]. In this study, it was demonstrated that both OMPLL–DNA and RMPLL–DNA were able to mature DCs in vitro and in vivo, unlike OVA DNA alone. This is supported by a previous finding which showed OM and RM induced phenotypic and functional maturation of mouse DCs [10]. Similar to that previous study, $CD8^{+}DEC205^{-}$ subset of lymph node DCs was the most responsive to OMPLL–DNA and RMPLL–DNA stimulation. This subset of DCs had been shown to be able to cross-present antigens in vivo [31]. PLL–DNA was not included in the study due to its cytotoxic effect on cells at the concentration used in the experiment. Furthermore, PLL had been shown not to induce DC maturation in a separate study using PLL coated microparticles [32]. One possible explanation for the ability of OMPLL to induce stronger CD8 responses compared to RMPLL could be due to OMPLL inducing higher co-stimulatory molecule expression (Fig. 6). It is suggested that CD8T cells require a stronger co-stimulatory signal than CD4T cells [33]. The ability of elevated co-stimulatory expression in APCs to augment immune response was also exploited in an MUC1 DNA immunization study, where the authors showed increased tumor protection in mice when immunized with plasmids that express MUC1 together with CD80 and CD86 compared to MUC1 alone [34].

DC maturation could occur in a variety of ways such as the high incidence of apoptotic cells [35], viral replication [36], heat shock protein release from necrotic cells [37], engagement of Fc receptors [38], Fas–FasL [39] and TLR activations. It was previously shown that OM and RM mature DCs via TLR4 [10]. Surprisingly, OMPLL–OVA DNA and RMPLL–OVA DNA do not require TLR4 to mature DCs. However, maturation was determined to be MyD88 dependent, indicating that other TLRs might be involved in OMPLL–DNA and RMPLL–DNA induced maturation. Further investigations led to the finding that OMPLL and RMPLL matured DC via TLR2. TLR2 recognizes mannan containing bacterial/fungus cell wall components [40]. In addition, it is activated by peptidoglycan, which is a polymer consisting of sugars and amino acids that is found in abundance on the cell wall of Gram-positive bacteria [26]. Notably, ligands of TLR2 are the most diverse amongst all TLRs. This is due to TLR2 requiring hetero-dimerization with other TLRs, such as TLR1 and TLR6, to mediate a response, and different combinations bind to different ligands. Hence, TLR2 is a likely candidate for OMPLL and RMPLL to target and activate DCs. Besides DC maturation, TLR2 activation has been shown to induce production of pro-inflammatory cytokine and chemokines (such as TNF-α, IL-2, IL-6, IL-12 and MIP-2) that are essential in innate and subsequent adaptive responses [41,42]. The cytokine microenvironment at the site of APCs-T cell priming is a primary factor in dictating the downstream effector response generated against the antigen. This would include the presence or absence of various cytokines, the timing and the concentration of each cytokine secreted [43]. Here, it is shown that OMPLL–DNA and RMPLL–DNA are better stimulants in cytokine production from GM-CSF cultured DCs compared to DNA alone.

RMPLL–DNA induced higher IL-2 whilst OMPLL–DNA induced higher IL-2, IL-12, IFN-γ and TNF-α secretion compared to DNA alone stimulated DCs. TNF-α is an inflammatory cytokine. Its effect on DC maturation was shown in a study where fresh immature Langerhans cells from healthy human skin and exposed for two days to TNF-α under serum-free conditions, expressed up-regulated level of co-stimulatory molecules (CD40, CD54, CD86), maturation markers (CD83, DC-LAMP), CCR7 lymph node homing receptor in a dose-dependent manner [44]. This may explain why OMPLL induced stronger maturation than RMPLL as more TNF-α was produced in the former. The primary role of IL-2 is T-cell proliferation and differentiation. Its function in promoting cell-mediated immunity has been exploited to stimulate cell-mediated immunity and systemic change in T-cell responses to diseases such as lepromatous leprosy [45]. Furthermore, it has been shown to activate other immune cell types such as B cells [46], macrophages and natural killer cells [47]. For this reason, IL-2 has been included in DNA immunization regimes, to augment T-cell responses [48–51]. IL-12 and IFN-γ are cytokines that promote Th1 responses. The production of IL-12 and IFN-γ in OMPLL–DNA stimulated DCs are nearly twice the amount compared to that stimulated by RMPLL–DNA, which may account for the Th1 responses induced by OMPLL–DNA in vivo, noted in previous immunization studies [11,12].

5. Conclusion

In conclusion, the mechanism of processing leading up to antigen presentation between DNA alone and OM/RMPLL–DNA upon immunization is different on several levels. When viewed together, the data explain the increase in immunogenicity provided by the carriers and the skewing of Th1 and Th2 responses by OMPLL–DNA and RMPLL–DNA, respectively.

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