Modulation of Dendritic Cells by Lipid Grafted Polyelectrolyte Microcapsules

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Polyelectrolyte microcapsules are fabricated by layer-by-layer deposition of dextran sulfate and poly-L-arginine layers at the surface of calcium carbonate template microparticles followed by core removal to produce hollow microcapsules. In the context of vaccination, these biodegradable LbL capsules emerge as promising antigen carriers and are believed to have potential for the co-delivery of antigens and immunomodulators associated within the same particle to enhance and steer the type of immune response. To this end, it is shown that LbL microcapsules can be functionalized at their surface with lipid layers containing immunopotentiators of lipid nature. The potency of the different lipid modified microcapsules to activate dendritic cells (DCs) is demonstrated by increased expression levels of the migration marker CCR7 and the maturation markers CD40 and CD86. Additionally, the DCs cytokine secretion profile is evaluated. The findings reveal that the lipid grafted microcapsules are superior to non-modified microcapsules in DC activation and suggest their potential as immune modulating antigen delivery systems.

1. Introduction

Dendritic cells (DCs) are widely regarded as the most potent antigen presenting cells (APC) for the initiation of an adaptive immune response. Immature DCs present in the peripheral tissues are specialized in screening the environment for foreign material. They do so by continuous sampling and are therefore characterized by high phagocytic capacity. Upon the encounter, uptake and processing of antigen or particle associated antigen, the DCs become activated. They differentiate into a so-called "mature state" and migrate to lymphoid nodes to encounter T cells. This maturation process is characterized by a loss of phagocytic activity and an up-regulation of co-stimulatory molecules (e.g., CD40, CD80, CD86), MHC molecules and chemokine receptors involved in DC migration (e.g., CCR7).[1–4]

In the field of vaccination, earlier use of the traditional killed or attenuated whole-cell vaccines gradually evolved to the use of the safer subunit vaccines. The improved safety profile, however, is accompanied by a decrease in immunogenicity since these formulations lack the immunostimulatory components present in whole-cell vaccines such as pathogen-associated molecular patterns (PAMPs). Since maturation of DCs is crucially important for efficient antigen presentation and subsequent priming of T cells, different particulate antigen delivery systems have been exploited for subunit vaccine delivery.[5–9] Association of antigens with particles has shown to enhance their uptake by antigen presenting cells and was reported to be more efficient in eliciting immune responses than soluble proteins. As an additional advantage, particulate vaccines may allow the simultaneous delivery of antigen and immunostimulatory molecules to the same APC. Immunopotentiators such as toll-like receptor (TLR) ligands stimulate immune cells through specific receptors or pathways.[10–12] Therefore, simultaneous delivery of an antigen and immunopotentiators accommodated in one particle represents an attractive strategy in vaccine development. The feasibility and benefits of co-delivery has been demonstrated for a variety of particulate delivery systems.[13–19]

In recent years, LbL-assembled microcapsules emerged as a novel type of particle for antigen delivery.[20–23] In the present study, we investigated the possibility to tailor the surface of LbL microcapsules with lipids possessing immunostimulatory properties as illustrated schematically (**Scheme 1**). Furthermore, we examined the immunostimulatory potential of lipids grafted to the surface of biodegradable, polyelectrolyte microcapsules for antigen delivery to DCs by examining the capacity of these capsules to stimulate DC maturation and the production of cytokines in vitro.

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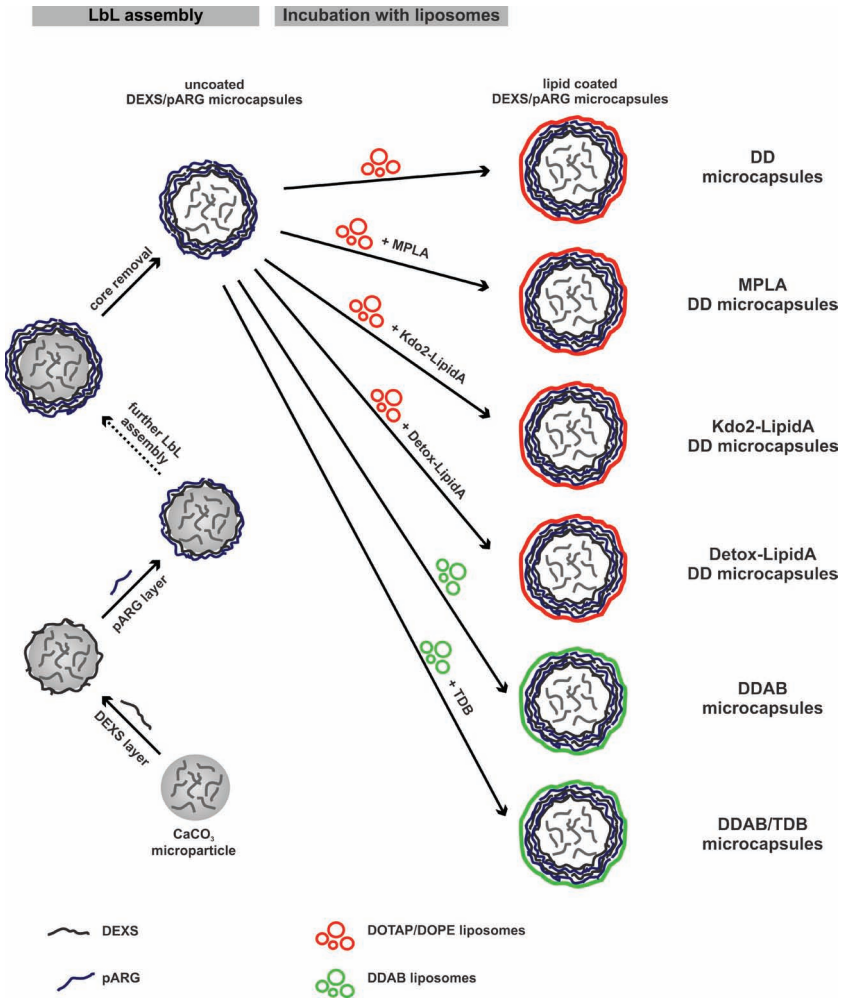

Scheme 1. Schematic illustration showing preparation of lipid-coated LbL capsules. First, capsules are prepared by coating $CaCO_3$ microparticles alternately with dextran sulfate (DEXS) and poly-L-arginine (pARG), followed by core decomposition resulting in hollow LbL capsules. In the next step, DEXS/pARG capsules are incubated with various liposomal formulations based on DOTAP/DOPE (DD) or DDAB yielding lipid-coated capsules.

2. Results

2.1. Characterization of Lipid-Coated Microcapsules

To modify the LbL capsules' surface with lipidic immunostimulators, we selected several lipid combinations. We chose DOTAP/DOPE (DD) in combination with different lipid A derivatives, specifically MPLA, Kdo2-Lipid A, or detox-Lipid A, and DDAB in combination with TDB. The zeta-potential values presented in **Figure 1**A demonstrate that all liposomal formulations, used for microcapsules coating, were cationic. The plain DD liposomes exhibited a zeta-potential of 58.8 ± 1.56 mV. Inclusion of negatively charged MPLA, Kdo2-Lipid A or detox-Lipid A resulted in a slight reduction of the zeta-potential. The surface charges of DDAB and DDAB/TDB liposomes were 61.0 ± 1.82 mV and 63.0 ± 1.55 mV, respectively.

The cationic liposomes were used to coat DEXS/pARG microcapsules. Uncoated microcapsules served as a reference. Their zeta-potential was –31.4 ± 1.56 mV (Figure 1B). After incubation with the different cationic liposomes, all coated microcapsules showed a significant increase in zeta-potential values indicating the presence of lipids on the surface. These results were confirmed by confocal microscopy. As shown in Figure 1C, fluorescently labeled lipids clearly mark the surface of capsules indicating the successful binding of the lipids.

2.2. Uptake of Lipid-Coated Microcapsules by DCs

The uptake of the different capsule formulations by DCs was first evaluated by confocal microscopy. As shown in **Figure 2** all the formulations were efficiently internalized by DCs. Flow cytometry was further used to quantify the uptake of the FITC-dextran labeled capsules by DCs. As shown in **Figure 3**, the non-modified DEXS/pARG capsules were internalized to a lower extent than any of the different lipid-coated capsule formulations. The DD coated microcapsules were internalized by 75% of DCs, which was not significantly changed by addition of any immunostimulatory lipid. The DDAB and DDAB/TDB coated microcapsules were internalized by 60% of DCs.

2.3. Effect of Internalized Microcapsules on the DC Phenotype and Maturation Status

Upon activation, DCs mature and become efficient antigen presenting cells and T-cell stimulators. Maturation of DCs is accompanied by up-regulated expression of several cell surface molecules such as CD40, CD80, CD86 and MHC class II molecules.[1]

To determine the effect of internalized microcapsules on the DC phenotype and maturation, we evaluated the expression levels of the cell surface markers CD40, CD86 and CCR7. To that end, DCs were incubated with lipid-coated microcapsules. 24 h after the incubation surface molecule expression was evaluated by flow cytometry and compared to appropriate controls. Untreated immature DCs and DCs incubated with non-lipid-coated microcapsules served as negative controls. DCs incubated with LPS served as a positive control.

As shown in **Figure 4**A, addition of LPS to immature DCs clearly induced their maturation as evidenced by the significant up-regulation in the expression of CD40, CD86 and CCR7 compared to the untreated cells. Levels of the maturation markers in the cells incubated with the non-coated microcapsules showed only a limited increase as compared to the untreated DCs. This indicates that non-modified DEXs/pARG microcapsules barely affect the DC maturation status.

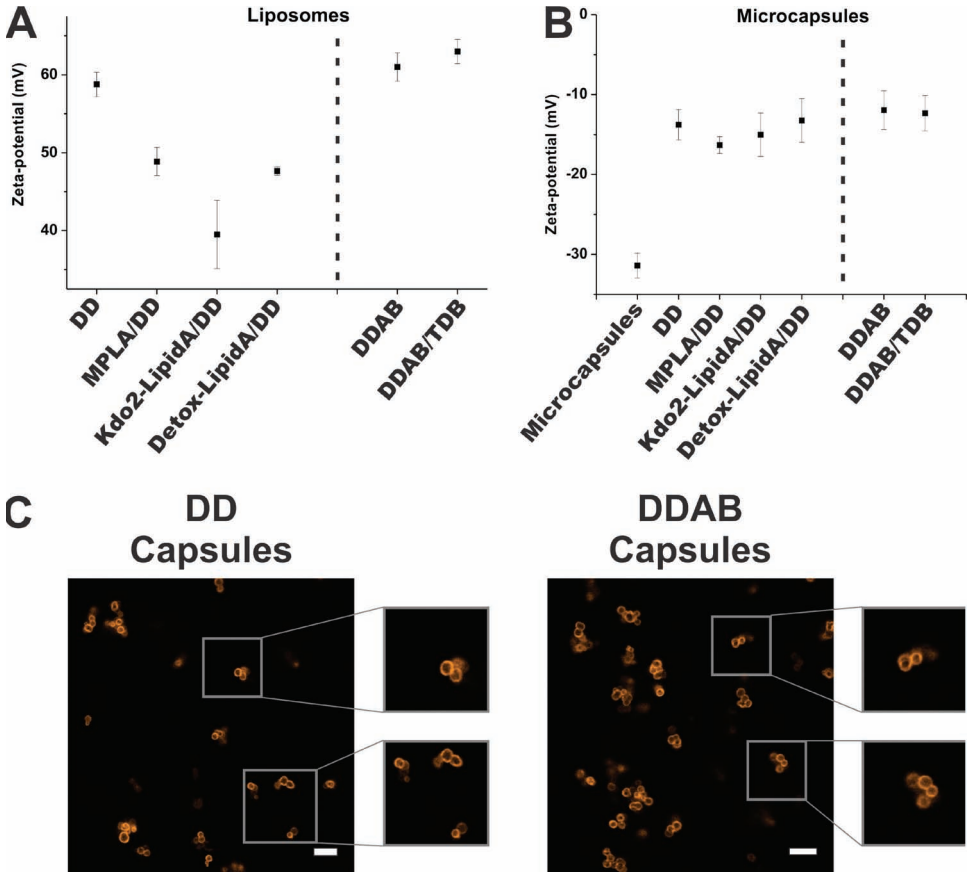

Figure 1. Preparation of lipid-coated microcapsules. Zeta-potential values of different liposomes (A) and LbL capsules (B). Data are expressed as mean of $n = 3$ preparations per formulation. C) Confocal images of lipid-enveloped LbL capsules. The microcapsules' shell was labeled fluorescently red, which originates from TRITC-PE present in the lipid coating. The concentration of capsules used to acquire confocal pictures was relatively high. Therefore, we could see some aggregation. The concentrations employed for in vitro experiments were lower, resulting in a lower level of aggregation as demonstrated by very efficient capsule uptake by dendritic cells presented in Figures 2,3. The scale bars represent 10 μm.

All DEXS/pARG microcapsules modified with a MPLA/DD, Kdo2-Lipid A/DD or detox-Lipid A/DD layer induced up-regulation of CD40 and CD86 molecules on the DC surface (Figure 4B). However, it should be noted that the microcapsules coated with DD induced maturation of the DCs, indicating that the DD coating itself already triggers DC maturation.

DEXS/pARG microcapsules functionalized with DDAB, with or without TDB, showed a clear increase in expression levels of CD40 and CD86.

The results in Figure 4A show that incubation of DCs with non-modified LbL capsules did not change the CCR7 expression level. By contrast, all lipid-grafted LbL microcapsules resulted in increased CCR7 levels. This up-regulation is necessary for the migration of the DCs to the lymph nodes where they can prime T cells.

2.4. Effect of Internalized Microcapsules on the Cytokine Secretion Profile

To gain more insight in the polarization of the immune response upon uptake of the lipid-functionalized microcapsules, we analyzed the expression levels of different inflammatory mediators released in the culture medium. Untreated cells and cells incubated with LPS served as negative and positive controls, respectively. As shown in **Figure 5**, 24 h after incubation of DCs with non-modified LbL microcapsules, the IL-6 and TNF-α levels were not increased compared to the untreated cells. In case of the microcapsules coated with DD (Figure 5A), DDAB (Figure 5B) or DDAB/TDB (Figure 5B), we observed a slight but significant increase in levels of both cytokines compared to those observed for the non-coated microcapsules ($p < 0.05$). The microcapsules coated with MPLA/DD, detox-LipidA/DD or Kdo2-LipidA/DD all strongly enhanced expression levels of both IL-6 and TNF-α ($p < 0.01$) (Figure 5A).

Furthermore, we observed that non-modified microcapsules did not lead to significant up-regulation of IFN-γ expression. Incubation of the cells with DD, DDAB or DDAB/TDB modified microcapsules enhanced levels of IFN-γ production by DCs. MPLA/DD, detox-LipidA/DD and Kdo2-LipidA/DD coated microcapsules induced the highest level of IFN-γ production. We also assessed the MCP-1 release upon incubation with the particles. The non-modified LbL microcapsules did not cause a change in the MCP-1 expression. However, all lipid-modified

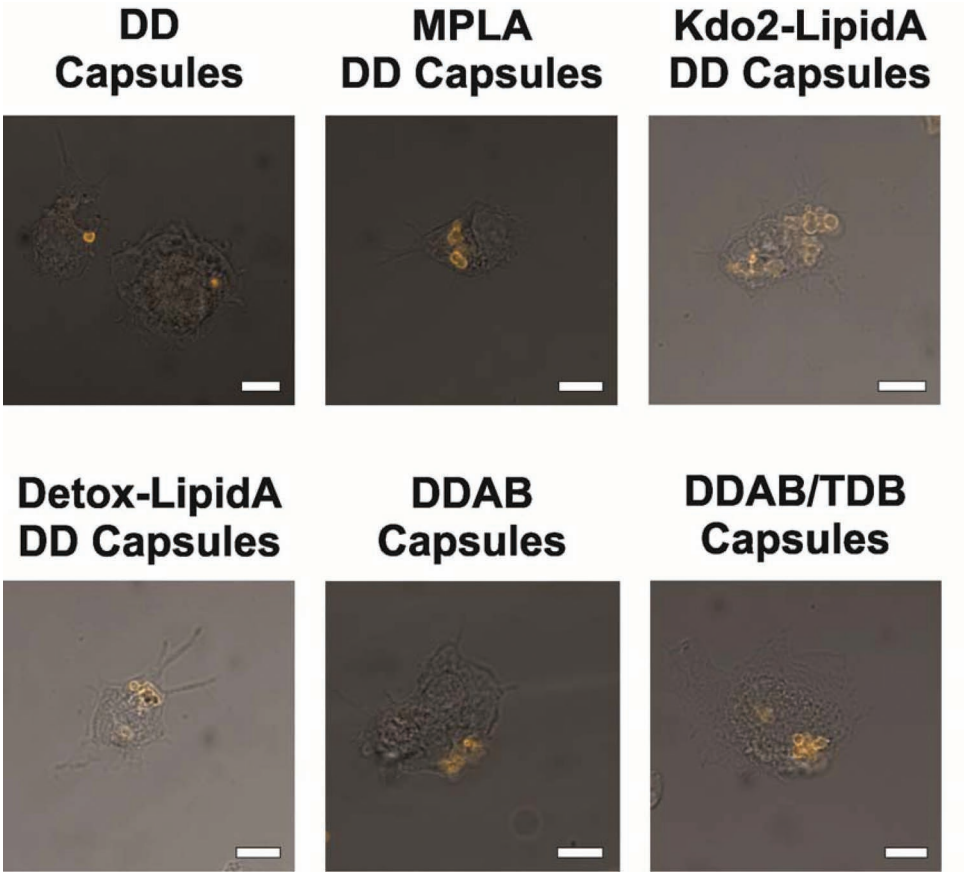

Figure 2. Confocal images of the cellular uptake of different lipid-grafted microcapsules by BM-DCs in vitro (overlay of transmission, red fluorescence). In the CLSM images, the lipid-coated capsules appear in red (red fluorescence due to TRITC-PE in the lipid coat), the scale bar equals 10 µm. Pictures represent a cross section through the cell to distinguish between the internalized capsules and those attached to the cell surface.

microcapsules induced MCP-1 secretion to the same levels as LPS.

3. Discussion

In recent years, LbL microcapsules have emerged as interesting candidates for antigen delivery.[20–23] As the trend in vaccine development shifted towards the use of subunit vaccines, the need for immunopotentiators rose. In this study, we investigated whether the LbL microcapsules could be employed to co-deliver lipid-based immunopotentiators. Up to now, only charged and water soluble immunopotentiators have been included in LbL capsule formulations for antigen delivery. Inclusion of the TLR3 ligand poly(I:C) in LbL capsules has been reported. Co-delivery of HIV-1 p24 antigen and poly(I:C) has induced enhanced immune responses both in vitro and in vivo.[18]

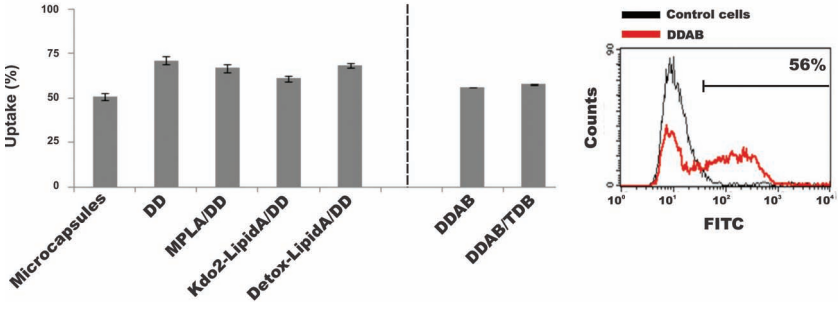

Figure 3. DCs were incubated with different fluorescently labeled microcapsule formulations for 3 h and uptake by DCs (CD11c gating) was measured by flow cytometry. Data represent means ± standard deviation ($n = 3$). A representative histogram of the uptake of FITC-labeled DDAB-coated microcapsules by DCs is shown.

Our aim was to coat polyelectrolyte microcapsules with lipidic immunopotentiators. This strategy was applied to ensure co-delivery of both entities to the same cell. So far, lipid layers were deposited on LbL capsules for various purposes, for example to modulate the LbL shell permeability for slow release of encapsulated substances,[25] to coat them with viral lipids for bead-based multiplex assays.[26,27] The potential of lipid-coated microcapsules for antigen delivery has not been investigated yet. We selected several lipids with known adjuvant activity. Even though lipopolysaccharides are known

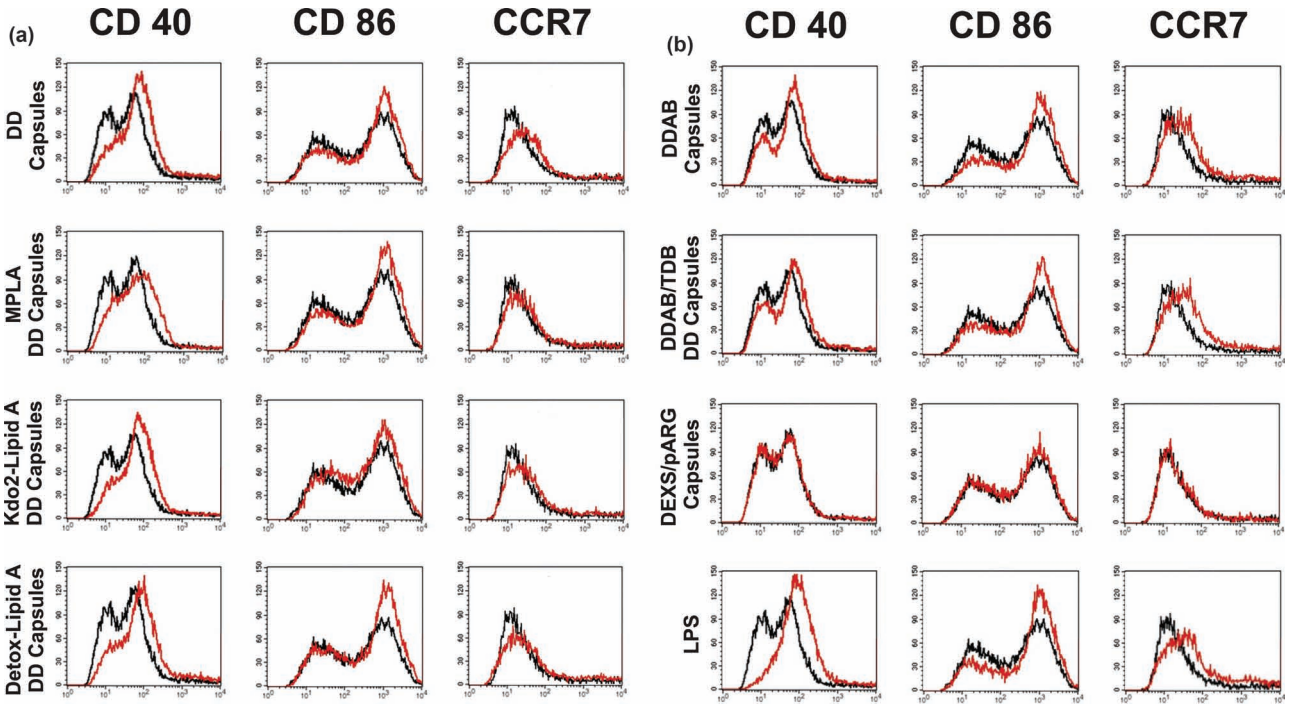

Figure 4. A) Representative histograms of the phenotypical maturation of DCs. BM-DCs were incubated with different DDAB-based microcapsule formulations (red curves). 24 h post incubation, expression of maturation markers CD40, CD86 and migration marker CCR7 was measured by flow cytometry. DCs were gated on CD11c staining. Untreated DCs (black curve) and LPS stimulated DCs served as negative and positive controls, respectively. B). Representative histograms of the phenotypical maturation of DCs. BM-DCs were incubated with different DD-based microcapsule formulations (red curves). Untreated DCs served as negative control (black curve). 24 h post incubation, expression of maturation markers CD40, CD86 and migration marker CCR7 was measured by flow cytometry. DCs were gated on CD11c staining.

as potent immune stimulators via TLR4 activation, their use has been limited due to toxicity. Moreover, due to heterogeneity of LPS derived from native Gram-negative bacteria, its chances of clinical application are remote.[28] Therefore, lipid A derivatives with reduced toxicity and well defined structure have been developed. For our study we selected MPLA, detox-Lipid A and Kdo2-Lipid A. MPLA is a constituent of AS01 and AS02 adjuvants (GSK) which are currently tested in clinical trials.[29] We have chosen DDAB with or without TDB because these lipids have known adjuvant activity.[13] Before coating MPLA, detox-Lipid A and Kdo2-Lipid A were incorporated in DD liposomes. The reduced zeta-potential of these liposomes compared to those made from DD was expected since the lipid A derivatives have a negatively charged phosphate group. Although the zeta-potential was reduced, the liposomes were still cationic. This was important since LbL capsules used in this study were negatively charged (–31.4 ± 1.56 mV). Lipid-coated microcapsules exhibited a zeta-potential between –16.3 and –12.0 mV. This shift is compatible with the presence of a lipid layer on the microcapsule surface. LbL capsules will likely determine the amount and distribution of lipids to be incorporated. It is conceivable, therefore, that irrespective of the number of lipid molecules present in the liposomes only a limited amount of charged molecules can be incorporated into the final microcapsule structure. That is likely why the zeta potential values of different formulations are almost the same. Successful coating was confirmed by confocal microscopy: fluorescently labeled lipids clearly mark the capsule surface.

Since the surface characteristics of particles are likely to determine their interaction with the cells, we next evaluated the cellular uptake of the different microcapsule formulations. Interestingly, diversely composed microcapsules were taken up by DCs with distinct efficiencies. Of the various lipid-modified microcapsules, the DD containing microcapsules were more readily internalized than non-modified and the DDAB coated capsules. This observation indicates that application of a lipid outer layer may not only enable tailoring of microcapsules for co-delivery of lipid adjuvants, but it can also improve their uptake and in consequence result in a better activation of the DCs. Our results are in agreement with earlier studies demonstrating that LbL capsules coated by a lipid outer layer are more efficiently internalized by MCF-7 breast cancer cells than non-modified polyelectrolyte capsules.[30]

Upon maturation, the DCs' phenotype changes and results in an up-regulation of the expression of some surface molecules involved in T cell priming. These molecules interact with receptors on T cells to enhance adhesion and signaling (co-stimulation) thereby promoting T cell activation. Therefore in this study, we evaluated whether and to what extent the different types of lipid-modified LbL capsules were capable to induce higher expression levels of CD40 and CD86 in DCs. Furthermore, we evaluated the expression of CCR7 (CD197) on the DC surface. This is important since, upon antigen uptake and activation, the DCs have to reach the lymph nodes, where they will encounter naïve T cells and initiate a specific immune response. It has been shown in vivo in CCR7 deficient mice

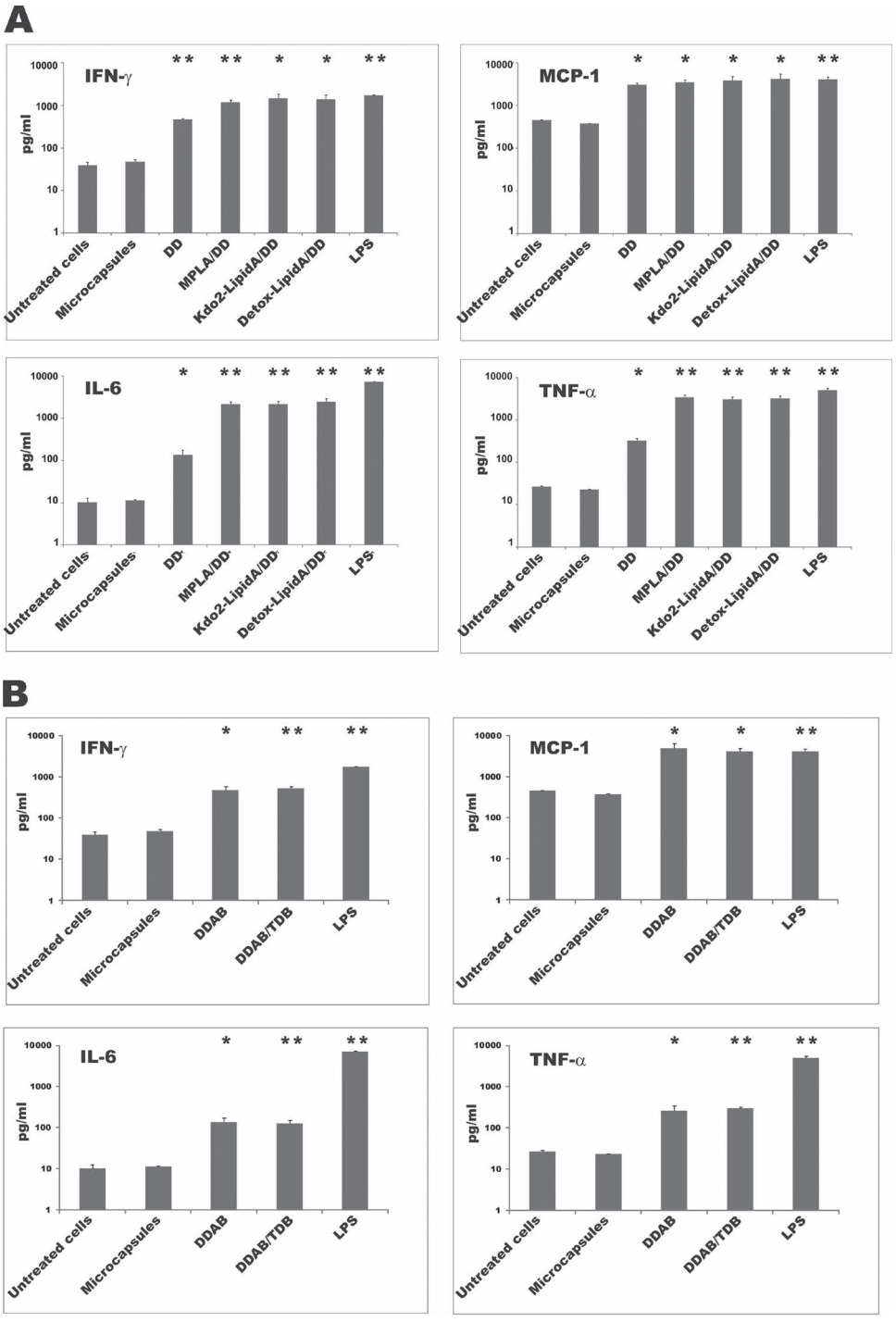

Figure 5. In vitro IFN-γ, MCP-1, IL-6 and TNF-α cytokine production from DCs 24 h post incubation with DOTAP/DOPE- (DD) (A) or DDAB (B)-based microcapsule formulations. Untreated cells and LPS stimulated cells served as negative and positive controls, respectively. Data reported are the mean ± standard deviation ($n = 3$), ($*p < 0.05$, $**p < 0.01$ as compared to non-modified microcapsules).

that CCR7 expression on DCs is crucial for this migration process.[31,32]

Our results presented in Figure 3 show that incubation of DCs with non-modified LbL capsules barely changed the expression levels of CD40, CD86 and CCR7. By contrast, incubation of DCs with all lipid-grafted LbL microcapsules resulted in increased levels of CD40, CD86 and CCR7. It should be noted, however, that capsules coated with DD only already induced DC maturation.

A clear increase in CD40 and CD86 levels in DCs incubated with microcapsules grafted with DDAB and TDB is not

surprising since both lipids are known for their immonostimulating potential.[13] In conclusion, the observed up-regulation of CD40, CD86 and CCR7 induced by differently composed microcapsules is desirable in the light of DCs' functions as outlined above.

Depending on the DC maturation status, different cytokine expression profiles are observed. The cytokines expressed by mature DCs will ultimately influence the Th1/Th2 polarization of the immune response. Increase of IL-6 and TNF-α is desirable for induction of inflammation, IFN-γ is relevant in steering the response toward the cellular arm of immunity while MCP-1 has chemotactic activity. Incubation with non-modified microcapsules did not alter the cytokine profile secreted by DCs. This outcome is in accordance with our previous observation that these microcapsules do not induce up-regulation of maturation markers. DDAB and DDAB/TDB modified microcapsules significantly augmented IFN-γ and MCP-1 levels and induced slightly though still significantly higher expression of the inflammatory cytokines IL-6 and TNF-α when compared to control microcapsules. DD-coated microcapsules induced a cytokine release pattern comparable to that caused by DDAB and DDAB/TDB modified microcapsules. However, when a lipid A derivative was included in the DD layers, the DCs were very strongly activated as evidenced by the significantly increased cytokine response, in case of IFN-γ and MCP-1 comparable with that achieved by LPS. In general, our results indicate that the application of a lipid A derivative/DD layer on the surface of microcapsules generated the strongest activation of the DCs. DDAB and DDAB/TDB functionalized microcapsules also resulted in enhanced DC activation as compared to plain polyelectrolyte microcapsules but still to a lower extent than those functionalized with DD. However, this phenomenon may be explained by the lower uptake of these capsules by DCs.

4. Conclusions

Concomitant delivery of antigen and immunopotentiator by means of particulate vaccine formulations represents an appealing strategy to enhance the immunogenicity of subunit vaccines and to steer the immune response in the desired direction. In this study, we have shown that the surface of LbL microcapsules can be furnished with a lipid coating exposing immunostimulating capacities. These findings offer perspectives toward modulation of DC maturation by depositing different lipids on the LbL microcapsule surface. This might be relevant with regard to the required type and strength of the immune response to be generated depending on the specific applications. To the best of our knowledge, this is the first report on functionalization of the LbL microcapsules surface with lipid-based immunopotentiators. Our findings may encourage further investigation of the immune modulating effects of lipid-modified microcapsules.

5. Experimental Section

Preparation of Polyelectrolyte Microcapsules: $CaCO_3$ microparticles were prepared by mixing equal volumes of Na_2CO_3 (1 M, Sigma Aldrich) and $CaCl_2$ (1 M, Sigma Aldrich) under stirring. The $CaCO_3$ particles were subsequently coated with the biodegradable polyelectrolytes: dextran sulfate (DEXS, Sigma Aldrich) and poly-L-arginine (pARG, Sigma Aldrich) by the LbL technique as described earlier.[24] Briefly, the $CaCO_3$ template microparticles were dispersed in a DEXS solution (1 mg/mL) containing NaCl (0.5 M, Sigma Aldrich). After adsorption of the first DEXS polyelectrolyte layer, the particles were centrifuged for 3 min at 300g and washed twice with water to remove non-adsorped polyelectrolytes. In the next step, the microparticles were incubated in a pARG solution (1 mg/mL) in NaCl (0.5 M) followed by centrifugation and washing. This LbL procedure was repeated until an additional bilayer was deposited. Hollow polyelectrolyte microcapsules, composed of 2 DEXS/pARG bilayers, were obtained by removing the $CaCO_3$ core with a EDTA (0.2 M, Merck) solution.

Preparation of Liposomes: 1,2-Dioleolyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), Dimethyldioctadecylammonium (Bromide Salt) (DDAB), D-(+)-trehalose 6,6'-dibehenate (TDB), Lipid A Detoxified (detox-Lipid A, *Salmonella Minnesota R595*), Monophosphoryl Lipid A (MPLA) and Di[3-deoxy-D-manno-octulosonyl]-lipid A (ammonium salt) (Kdo2-Lipid A) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (TRITC-PE) were purchased from Avanti Polar Lipids.

Cationic liposomes composed of DOTAP/DOPE (DD) (50:50) were prepared in the presence or absence of a lipid A derivative, specifically Lipid A Detoxified or MPLA or Kdo2-Lipid A. Appropriate amounts of lipids dissolved in chloroform were mixed in a round-bottom flask. The solvent was removed with a rotavapor under reduced pressure and the resulting lipid film was flushed with nitrogen for 10 min at room temperature. The dried lipid film was hydrated with 20 mM HEPES-buffer (pH 7.4, Sigma Aldrich) in the presence of glass beads. The resulting liposomal suspension was sonicated for 10 min with a Branson2510 sonicator.

DDAB liposomes were prepared with or without addition of TDB. Briefly, appropriate amounts of DDAB (1.25 mg/ml in chloroform) and TDB (0.25 mg/ml in chloroform:methanol 9:1) were pipetted in a round-bottom flask and organic solvents were removed by rotary evaporation, followed by flushing with nitrogen. The obtained lipid film was hydrated with a 10 mM Tris buffer (pH 7.4, Sigma Aldrich) and the suspension was kept at 60 °C for 30 min, which was followed by sonication for 10 min.

Preparation of Lipid-Coated Polyelectrolyte Microcapsules: DEXS/pARG microcapsules consisting of 2 bilayers were coated with lipids by incubation with the liposomes for 30 min at 37 °C (DD) or at 60 °C (DDAB), which was followed by three washings with HEPES-buffer or Tris buffer respectively. We have optimized the coating conditions for each formulation. The optimal liposome to capsule ratio was obtained experimentally. To that end, we made use of fluorescently labeled lipids. The presence of lipids inside the capsules was never observed.

Zeta-potential Measurement: The surface charge of the microparticles was determined by zeta-potential measurement using a Zetasizer Nano series (Malvern). To that end, the liposomes and lipid-coated microcapsules were dispersed in distilled water. The reported zeta-potential values are the average of three consecutive measurements ± SD.

Fluorescence Microscopy: Confocal microscopy images of lipid-coated DEXS/pARG microcapsules were acquired using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope. For that purpose a drop of capsule suspension was placed on a cover glass and analyzed with CLSM using a water immersion objective lens (Plan Apo 60×, NA 1.2, collar rim correction, Nikon). The fluorescence signal derived from the TRITC-PE (1 mol%, Avanti Polar Lipids) added during liposome preparation.

Mice: Female C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France) and housed in a specified pathogen-free facility. All the experiments were performed according to the regulations of the Belgian law and the local Ethical Committee.

Murine Bone Marrow-Derived DCs: DCs were isolated from bone marrow of C57Bl/6 mice. Mice were sacrificed and bone marrow was

flushed out of the femur and tibia. After red blood cell lysis (Pharm Lyse, BD Biosciences), cells were seeded at a density of 2×10^5 cells/ml and incubated at 37 °C in 5% CO_2. Cell culture medium was RPMI-1640 (Invitrogen) supplemented with 5% FCS (Hyclone), 1% penicillin/streptomycin (Invitrogen), 1% L-glutamine (Invitrogen) and 50 μM β-mercaptoethanol (Invitrogen), 10 ng/ml IL-4 (Peprotech) and 10 ng/ml GM-CSF (Peprotech). At days 2 and 6 of culture, the non-adherent cells were centrifuged, re-suspended in fresh medium and seeded in the same flask. On day 7 of culture, the non-adherent cells were harvested, counted and plated at a density of 1×10^5 cells/ml in 24-well plates.

Microcapsule Uptake by Dendritic Cells: Flow cytometry was used to assess the uptake of microcapsules by BM-DCs. At day 8 of the culture, the cells were incubated with various microcapsule formulations for 3h. The cells were vigorously washed with Stain buffer (BD Pharmingen) to remove non-internalized microcapsules and incubated with 5% goat serum (Invitrogen) in PBS on ice for 30 min. DCs were identified by CD11c-APC antibody staining (BD Pharmingen) for 1 h on ice, followed by washings. Finally, the cells were re-suspended in flow buffer and analyzed with a FACSCalibur flow cytometer (BD Pharmingen). Microcapsules were fluorescently labelled by incorporation of 200 kDa FITC dextran (Sigma Aldrich) in their lumen. FITC dextran was loaded in the capsules' lumen by so called "co-precipitation", i.e., FITC dextran present at the moment of the precipitation reaction of Na_2CO_3 and $CaCl_2$ is captured in the $CaCO_3$ microparticles. Data were analyzed using the CellQuest software.

Evaluation of Dendritic Cells Activation: At day 8 of culture, BM-DCs were incubated for 3 h with the lipid-coated microcapsules at a concentration equivalent to 0.1 μg/mL lipid A derivative or a 1:20 ratio for DDAB and DDAB/TDB-coated microcapsules. After 24 h, activation of the DCs was evaluated by means of flow cytometric analysis. Untreated cells and cells incubated with non-coated microcapsules served as negative controls. *E. Coli*-derived LPS (1 μg/mL; Sigma Aldrich) was included as a positive control.

Evaluation of Maturation and Migration Markers Expression: The effect of the internalised microcapsules on the DC maturation status was assessed by evaluating up-regulation of the maturation surface markers CD40 and CD86, and of the migration marker CCR7. 24 h after incubation of DCs with microcapsules, the cells were washed with Stain buffer (BD Pharmingen) and incubated with 5% goat serum (Invitrogen) in PBS on ice for 30 min. Then the cells were stained with a CD11c-FITC antibody in combination with staining with a CD40-PE antibody or a CD86-PE antibody or a CCR7-PE antibody (all BD Pharmingen) for 1 h on ice. This was followed by washings with Stain buffer. Finally, the cells were re-suspended in flow buffer and analyzed with a FACSCalibur flow cytometer (BD Pharmingen). Data were analyzed using the CellQuest software.

Analysis of Cytokine Production: Cell culture supernatants were analyzed for secreted cytokines 24 h after incubation of DCs with differently composed capsules or 1 μg/mL LPS (positive control). Cell culture medium of untreated DCs served as a negative control. Cytokine levels were measured using the Mouse Inflammation Kit (BD Pharmingen) according to the manufacturer's instructions. The samples were analyzed with a FACSCalibur flow cytometer (BD Pharmingen). Further data analysis was performed using the FCAP Array software.

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