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Encapsulation of two different TLR ligands into liposomes confer protective immunity and prevent tumor development

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ABSTRACT

Nucleic acid-based Toll-like receptor (TLR) ligands are promising adjuvants and immunotherapeutic agents. Combination of TLR ligands potentiates immune response by providing synergistic immune activity via triggering different signaling pathways and may impact antigen dependent T-cell immune memory. However, their short circulation time due to nuclease attack hampers their clinical performance. Liposomes offer inclusion of protein and nucleic acid-based drugs with high encapsulation efficiency and drug loading. Furthermore, they protect cargo from enzymatic cleavage while providing stability, and enhancing biological activity. Herein, we aimed to develop a liposomal carrier system co-encapsulating TLR3 (polyinosinic-polycytidylic acid; poly(I:C)) and TLR9 (oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs; CpG ODN) ligands as immunoadjuvants together with protein antigen. To demonstrate that this depot system not only induce synergistic innate immune activation but also boost antigen-dependent immune response, we analyzed the potency of dual ligand encapsulated liposomes in long-term cancer protection assay. Data revealed that CpG ODN and poly(I:C) co-encapsulation significantly enhanced cytokine production from spleen cells. Activation and maturation of dendritic cells as well as bactericidal potency of macrophages along with internalization capacity of ligands were elevated upon incubation with liposomes co-encapsulating CpG ODN and poly(I:C). Immunization with co-encapsulated liposomes induced OVA-specific Th1-biased immunity which persisted for eight months post-booster injection. Subsequent challenge with OVA-expressing tumor cell line, E.G7, demonstrated that mice immunized with liposomes co-encapsulating dual ligands had significantly slower tumor progression. Tumor clearance was dependent on OVA-specific cytotoxic memory T-cells. These results suggest that liposomes co-encapsulating TLR3 and TLR9 ligands and a specific cancer antigen could be developed as a preventive cancer vaccine.

1. Introduction

Innate immune cells recognize microbial (bacterial and viral) components via pattern recognition receptors (PRRs) through pathogen-associated molecular patterns (PAMPs) and differentiate self from non-self [1,2]. Toll-like receptors (TLRs) are the most extensively studied PRRs. TLR family members are sub-divided to cell membrane-associated and endosome-associated receptors. Endosomal TLRs are specialized to sense nucleic acids. While TLR3 and TLR7/8 recognizes double and single-stranded RNA, TLR9 recognizes bacterial DNA or single-stranded synthetic oligodeoxynucleotides (ODN) expressing unmethylated CpG motifs (CpG ODNs hereafter) [3–6]. Multiple TLR triggering could synergistically activate immune response upon multiple agonists engagement [7].

CpG ODNs, which are found frequently in bacterial and viral genome but rare in mammalian DNA, enhances innate immune response. Among different ODN classes, D-ODNs (also known as CpG-A type) synthesized as a mixed backbone ODN contain purine/pyrimidine/CpG/purine/pyrimidine motif and poly G-tail at both ends. This ODN type induces secretion of type I interferons (IFNs) from plasmacytoid dendritic cells (pDCs), and IFN-γ from natural killer (NK) cells [8,9]. It activates nuclear factor kappa B (NF-κB) and interferon regulatory factor 7 (IRF7) via myeloid differentiation primary response gene 88 (MyD88) dependent pathway. Polyinosinic-polycytidylic acid (poly(I:C) hereafter), a synthetic analog of double-stranded RNA initiates signaling cascade through TLR3 and induces type I IFNs along with proinflammatory cytokines mediated by NF-κB and IRF3 via TRIF-dependent (MyD88-independent) pathway [3,6,10]. Both TLR ligands trigger *anti*-viral and anti-bacterial immune responses and mimic viral and bacterial infections. The simultaneous sensing of these two endosomal TLR ligands by PRRs boosts innate immune response in a synergistic manner and leads to more pronounced type I IFNs [11], proinflammatory cytokines, and nitric oxide production [12] with elevated cytotoxic T

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lymphocyte (CTL) activity [13,14]. When delivered together these ligands may help to improve better antigen dependent immunity and antitumor activity [15]. Nucleic acid-based TLR ligands are promising candidates as type 1T helper cell (Th1) specific vaccine adjuvants [8,16], *anti*-cancer [17] or anti-allergic therapeutic agents [18], however, when given in non-encapsulated forms, their clinical performance is hampered mainly due to in vivo degradation by nucleases or rapid clearance by serum protein adsorption [19]. To overcome this problem, several strategies were proposed such as polymer-based nanoparticles [20,21], polysaccharide complexes [22], liposomes [23–25] aiming to protect these labile molecules until they reach their target cells upon administration. In our previous studies, we have demonstrated that encapsulation of TLR ligands within liposomes not only increases their stability and protects them from digestion but also enhances their immunostimulatory and immunotherapeutic breadth [24,26]. The present work, aims to extend our previous experience and planned to co-encapsulate dual TLR ligands (CpG ODN and poly(I:C)) in liposomes and test their vaccine potency leading to long-term prevention against cancer. We provide evidence that dual ligand encapsulating liposomal vaccine improved ligand internalization, enhanced APC function, promoted a strong Th1-biased antigen-specific immune response and subsequently prevented animals to develop tumors eight months after immunization. We also demonstrated that the dual ligand co-encapsulating liposomal vaccine induce a long-lasting antigen specific $CD8^+$ memory T-cell immunity, critically contributing tumor clearance of immunized mice.

2. Materials and methods

2.1. Reagents

Cholesterol (Chol) and L-α-phosphatidylcholine (PC), lipids that were used in liposomes preparation was purchased from Sigma Aldrich (St. Louis, MO, USA) and Avanti Polar Lipids (Alabaster, AL, USA), respectively. TLR9 ligand, CpG ODN D35 5′-GGtgcatcgatgcaggggGG-3′ was kindly provided by Dr. Ken Ishii (IFReC, Japan) [27]. Bases shown in capital letter have phosphorothioate linkage and those in lower case have phosphodiester linkages. CpG motif is underlined. Cy5-labeled CpG ODN was synthesized in CBER/FDA core Facility and provided by Dr. Dennis M. Klinman TLR3 ligand poly(I:C) was from Amersham (Piscataway, NJ, USA) and fluorescein labeled poly(I:C) was from Invivogen (Toulouse, France).

All cell culture media components were from Gibco (NY, USA) and Lonza (Lonza, Walkersville, MD, USA). Cytokine ELISA reagents: recombinant cytokines, monoclonal unlabeled and biotinylated antibodies against mIL-6, mIL-12 and mTNF-α were purchased from Biolegend (San Diego, CA, USA), while streptavidin-alkaline phosphatase, hIFN-α and mIFN-γ were purchased from Mabtech (Cincinnati, OH, USA) and hIP10 was from BD (San Jose, CA, USA). *P*-nitrophenyl phosphate disodium salt substrate (PNPP) was purchased from Thermo Scientific (San Jose, CA, USA). Immunoglobulin ELISA reagents; goat *anti*-mouse total IgG, IgG1, IgG2c, and monoclonal antibodies conjugated with alkaline phosphatase (AP) were from Southern Biotech (Birmingham, AL, USA). FACS antibodies conjugated to fluorescent chromophores were obtained from Biolegend. B16-Blue IFN-α/β cells that allow the detection of bioactive murine type I IFNs were obtained from Invivogen and used according to the manufacturer's protocol.

2.2. Methods

2.2.1. Preparation of liposomes

Phospholipid stocks were prepared in chloroform and stored at −20 °C until use. Liposomes were prepared as described earlier [24,26]. Briefly, lipids (PC:Chol, 1:1 molar ratio, 20 μmol total lipid in 2 ml chloroform) were mixed and film was formed using rotary evaporator (ILMVAC, Ilmenau, Germany). Vesicles were generated by hydrating film with 1× PBS. These were sonicated five times for 30 s on and 30 s off at 4 °C using a Vibra Cell Sonicator (Sonics and Materials, Danbury, CT, USA) to generate smaller unilammellar vesicles (SUV).

Ligand loading within lipid vesicles were performed as described earlier [24,26]. Briefly, ligands (1 mg of each ligand) and preformed SUVs (20 μmol) were mixed and snap frozen in liquid nitrogen, and freeze-dried overnight (VirTis benchtop K, Bieleveld, Germany). Dried lipid/ODN mixture was rehydrated (1/10 initial volume) using nuclease-free ddH_2O and vortexed for 15 s every 5 min for 30 min at room temperature. Equal volume of PBS was added to the mixture to adjust the tonicity of the resulting liposomes. Liposomes were washed twice with 1× PBS (pH: 7.4) to remove the unloaded molecules and centrifuged at 16000 *g* for 1 h. Final concentration of liposomes is 20 μM lipid/mg ligand. Liposome formulations were stored at 4 °C until use.

2.2.2. In vitro stimulation

Splenocytes isolated from wild type C57BL/6 mice by mashing spleen with syringe plunger were seeded at 2×10^6 cell/ml (250 μl) on 96-well cell culture plates and stimulated with non- or liposome-encapsulated CpG ODN or poly(I:C) or their combinations (2 μg/ml per ligand; 20 μmol lipid/1 mg ligand) for 36 h at 37 °C in a 5% CO_2 incubator. After stimulation, supernatants were collected. mIL-6 and mIFN-γ secretion was analyzed by ELISA. Additionally, IFN-α/β was assessed using B16-Blue IFN-α/β reporter cells as described previously [28]. For uptake and binding experiments, splenocytes were incubated at 37 °C with labeled non-encapsulated ligands or liposomes encapsulating fluorescein-labeled poly(I:C) and/or Cy5-labeled CpG ODN for 2 h. CpG ODN and/or poly(I:C) positive cells were recorded using BD Accuri C6 flow cytometer. Cell viability was assessed using cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

All human peripheral blood mononuclear cell (hPBMC) experiments were conducted following approval from the Bilkent University Human Studies Ethical Committee (Approval #: BILHSEC NO:2014-09-02-02) and with the informed consent of all participants. hPBMCs collected from healthy donors and purified using Ficoll density centrifugation were seeded at 1×10^6 cell/ml (200–250 μl) on 96-well cell culture plates. Cells were stimulated for 24 h. After incubation, supernatants were collected and used to detect hIP-10 and hIFN-α via ELISA and cells were stained with fluorescent-labeled *anti*-CD83 and anti-HLA-DR, and analyzed by flow cytometry.

RAW 264.7 macrophage cell line (0.5×10^6 cell/ml) was stimulated with non-encapsulated or liposome-loaded CpG ODN or poly(I:C) or their combinations (2 μg/ml per ligand; 20 μmol lipid/1 mg ligand) and TNF-α level was analyzed via ELISA.

Antigen processing was analyzed addition of non-encapsulated or co-encapsulating ligands (2 μg/ml per ligand; 20 μmol lipid/1 mg ligand) together with DQ™-Ovalbumin (2 μg/ml, DQ™-OVA, Molecular Probes) into APCs (0.5×10^6; BM-DCs, BM-DMs and RAW cells). After 6 h incubation, fluorochrome released upon proteolytic processing were analyzed by flow cytometry

2.2.3. Generation and stimulation of bone marrow-derived dendritic cells (BM-DCs) and macrophages (BM-DM)

BM-DCs were generated from bone marrow isolated from the femur and tibia of C57BL/6 mice incubating with GM-CSF and IL-4 as described previously [29]. The 6–8-day old immature BM-DCs (1×10^6 cell/ml) were stimulated with non-encapsulated or liposomes (20 μM lipid/mg ligand) encapsulating CpG ODN (2 μg/ml) or poly(I:C) (2 μg/ml) or their 1:1 combination at 37 °C in a 5% CO_2 incubator. Following 24 h of incubation, the supernatants were collected and used for measurement of cytokines by ELISA and by B16-Blue IFN-α/β reporter cells. The expression levels of CD11b, CD11c, MHC-II, CD80 and CD86 in BM-DCs was analyzed by flow cytometry. Generated BM-DCs were approximately 80% $CD11b^+/CD11c^+$ double positive (Fig. S1A). In addition, propidium iodide (PI) staining was performed to analyze cell viability of stimulated BM-DCs.

BM-DMs were generated from bone marrow isolated from the femur and tibia of C57BL/6 mice incubated with 30% L929-conditioned medium (L929-CM) for 6 days. BM-DMs (0.5×10^6 cell/ml) were stimulated with non-encapsulated or liposome-encapsulated CpG ODN or poly(I:C) or their combinations (0–18 μg/ml from each ligand, 20 μM lipid/mg ligand) and supernatants were used for nitric oxide (NO) analysis via Griess reagent (Sigma, St. Louis, MO, USA) and cytokine ELISA.

2.2.4. Immunization study

Animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled ambient conditions (22 ± 2 °C) regulated with 12 h light and 12 h dark cycles. They were provided with unlimited access of food and water. All animal studies were conducted with prior approval of the animal ethics committee of Bilkent University (Approval #: BILAEC NO:2014/32).

Female C57BL/6 mice (6–8 week old) were injected twice intraperitoneally (i.p.) two weeks apart with non-encapsulated or encapsulated CpG ODN or poly(I:C) (10 μg each ligand 0.20 μmol liposomes/injection) or their 1:1 combination plus ovalbumin (OVA, 5 μg/injection). Blood was collected from tail vein of mice at 1st and 8th month after booster injection. IgG subtypes were analyzed by end-point ELISA from collected sera with proper titrations of each samples.

At the end of eight month post-booster injection, animals were subcutaneously inoculated with 4×10^6 E.G7-OVA thymoma cells (EL4 cells stably expressing OVA, ATCC) which were cultured in complete medium containing G418 (1 μg/ml) before injection. Tumor size was measured daily by digital caliper and recorded for 14 days. Then, animals were euthanized and spleens were recovered to isolate splenocytes. IFN-γ-producing $CD8^+$ T-cells against SIINFEKL peptide were determined by ELISPOT assay.

2.2.5. ELISA and ELISPOT assays

Immulon 2 HB or 1B microtiter plates (Thermo Scientific, San Jose, CA, USA) were coated with *anti*-cytokine or anti-IgG antibodies (Ab), respectively, and then blocked with PBS-BSA. Serially diluted standards and culture supernatants or serially diluted mouse sera were added to plates for overnight at 4 °C. Cytokine was detected using biotinylated *anti*-cytokine Ab followed by phosphatase-streptavidin whereas bound IgG subclasses were detected using alkaline phosphatase streptavidin-conjugated anti-IgG antibodies and developed by the addition of PNPP substrate.

Anti-IFN-γ Ab coated Immulon 2 HB plates were blocked with PBS-BSA in ELISPOT assay. Splenocytes were serially diluted and seeded to pre-coated plates either with SIINFEKL supplemented (SIINFEKL:OVA 257-264 peptide, Anaspec Inc., San Jose, CA, USA) or just media containing wells. Cells were incubated for further 18 h at 37 °C in a 5% CO_2 incubator. IFN-γ^+ $CD8^+$ T cell spots were detected using biotinylated *anti*-IFN-γ Ab followed by alkaline phosphatase streptavidin and BCIP dissolved in low melting agarose solutions (Prona, Reducta, Poland; 1/4, *v*/v). Spots were counted under binocular stereo microscope.

2.2.6. Statistical analysis

Statistical significant differences between groups were determined using one-way or two-way ANOVA following Tukey's or Bonferroni post-hoc comparison and Student's *t*-test analysis using GraphPad Prism software (version 5, San Diego, CA, USA). *P*-values <0.05 were considered as significant.

3. Results

3.1. IL-6 and interferon type I/II productions were synergistically enhanced by co-encapsulating poly(I:C) and CpG ODN in neutral liposomes

Our main aim was to combine and enhance the synergistic immunostimulatory activity of the two nucleic acid-based TLR ligands known to trigger MyD88 and TRIF dependent signaling cascades by co-encapsulating them in liposomes thereby achieving simultaneous ligand delivery to innate immune cells. The prospect of this approach was assessed by incubating splenocytes either with non-encapsulated or encapsulated poly(I:C) and CpG ODN at 2 μg/ml dose of each ligand. Data implied that, co-encapsulating poly(I:C) and CpG ODN significantly increased both IL-6 and IFN-γ and IFN-α/β levels over either non-encapsulated alone, non-encapsulated dual combined or separately encapsulated ligands ($p < 0.05$, one-way ANOVA, Tukey's HSD post hoc test, Fig. 1A–C). To rule out the toxicity of ligand stimulation on spleen cells, proliferation assay was conducted using Cell Counting Kit-8. Results revealed that there was no significant difference between groups (one-way ANOVA, Tukey's HSD Fig. 1D).

Next, ex vivo immunostimulatory potential of dual TLR ligand co-encapsulated liposomes were checked following i.p. injection to C57BL/6 mice (Fig. 1E). Four hours after i.p. treatment, spleen cells were incubated for further 24 h in cell culture with no additional stimulation and co-stimulatory (CD86) molecule expressions were analyzed by flow cytometry. Even though the injected initial amounts of non-encapsulated ligands were 2.5 fold more (25 μg each ligand) than the dual ligand-loaded liposomal group (10 μg each ligand), cells that received liposomal poly(I:C) plus CpG ODN surpassed non-encapsulated combination response in terms of CD86 upregulation (Fig. 1E). Of note, 10 μg injection of each non-encapsulated ligands did not initiate any detectable CD86 upregulation. These findings revealed that only liposomes co-encapsulating both TLR ligands are potent immune inducers than their i) single or ii) dual non-encapsulated ligand counterparts both in culture and upon in vivo administration.

3.2. Liposomes co-encapsulating TLR3 and TLR9 ligands enhanced APC function

Upregulation of MHC class II as well as co-stimulatory molecules along with cytokine production is a direct evidence of improved APC activity/function. We next assessed the potential of liposomes co-encapsulating poly(I:C) and CpG ODN for cellular activation and maturation of antigen presenting cells (APCs). We found that indeed liposome formulations mediate superior effect than the non-encapsulated ligand treatments. BM-DCs were generated in the presence of GM-CSF and IL-4 for six days in culture. Following harvest they were incubated with non-encapsulated or liposome-loaded TLR ligands. After 24 h cells were fixed and the number of $CD11c^+$ BM-DCs positive for MHC-II, CD86 or CD80 was determined by flow cytometry. Our cell gating strategy throughout these investigations is presented in Fig. S1B. When the surface marker upregulation levels of non-encapsulated and liposome-loaded single CpG ODN or poly(I:C) groups were analyzed there were no significant difference (Fig. S1C). Strikingly, when TLR3 and TLR9 ligands were co-encapsulated within liposomes, it led to significantly higher levels of co-stimulatory molecule (CD80 and CD86) upregulation (Fig. 2A–B and Fig. S1D–E). According to three independent experiments, MHC-II^+/$CD86^+$ double positive $CD11c^+$ BM-DCs was higher in co-encapsulated group (74.0 ± 2.0%) than non-encapsulated or liposome-loaded poly(I:C) (57.5 ± 2.6% and 55.0 ± 1.5%, respectively) or non-encapsulated or liposome-encapsulated CpG ODN (30.5 ± 2.0% and 35.0 ± 2.0%, respectively) or their separately encapsulated combination (62.5 ± 3.5%) (Fig. 2A). This data suggested that when dual ligands were encapsulated within liposomes higher MHC-II^+/$CD86^+$ double positive BM-DCs population were obtained compared to non-encapsulated ligand combination treated BM-DCs (Fig. 2B). Of note, in order to rule out that ligand treatments induce non-specific cell death, at the end of stimulation, cell viability was analyzed using PI staining and no significant difference between groups were observed (one-way ANOVA, Tukey's HSD, $p < 0.05$; Fig. S1F).

When, IL-12 production, a cytokine known to skew immune response towards Th1 from BM-DCs in response to ligand treatments was analyzed from cell supernatants after treatment, we detected that dual ligand co-encapsulated group generated six fold more IL-12 from

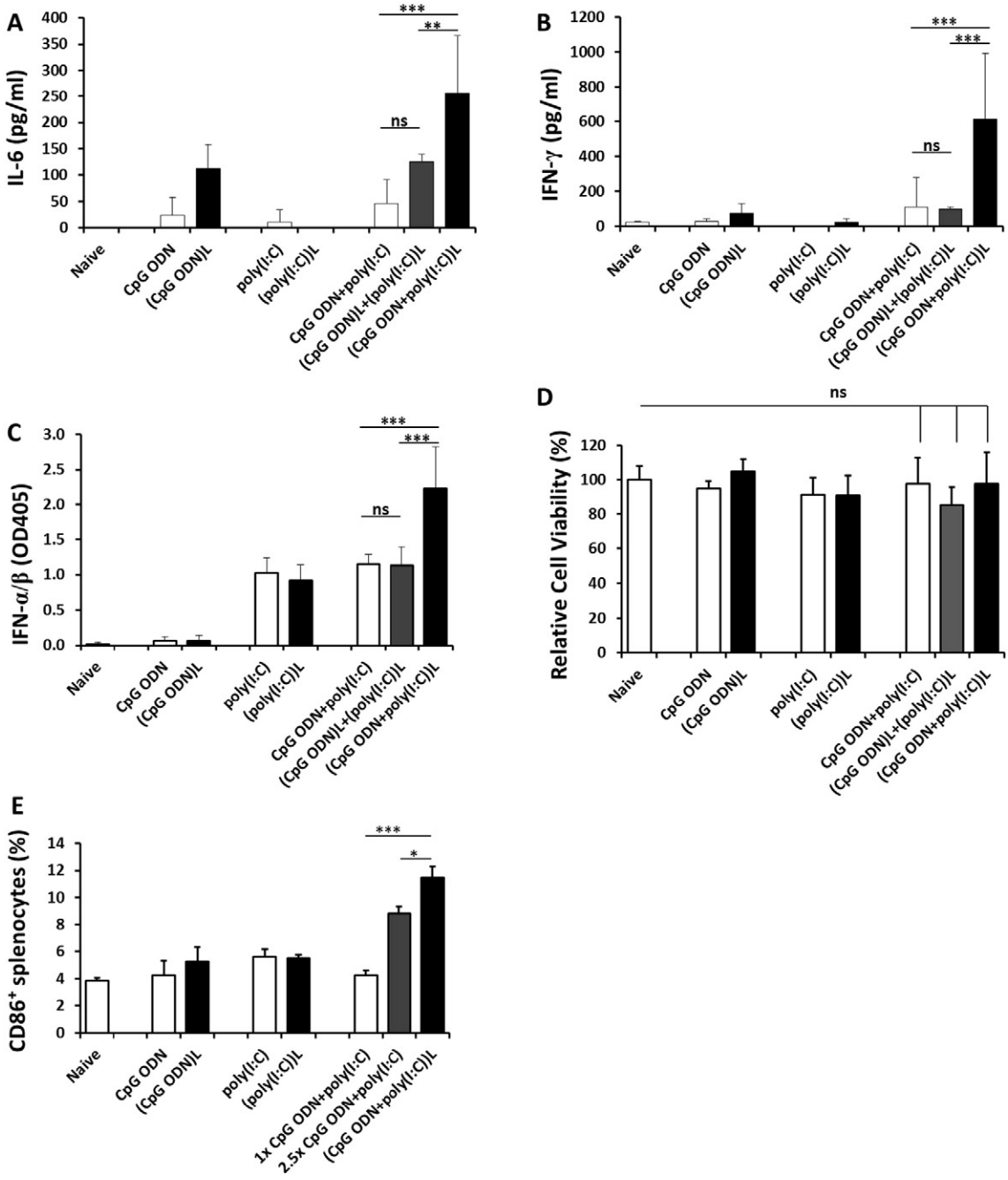


Fig. 1. Liposomes co-encapsulating CpG ODN and poly(I:C) improves in vitro *and* ex-vivo immunostimulatory effect. Splenocytes were stimulated with non-encapsulated or liposome-encapsulated (20 μM lipid/mg ligand) CpG ODN and/or poly(I:C) (2 μg/ml from each ligand) for 36 h. Supernatants were collected to detect (A) IL-6 and (B) IFN-γ cytokine levels by ELISA and (C) IFN-α/β level from reporter cell line (B16-Blue™ IFN-α/β Cells). (D) Cell viability was detected using Cell Counting Kit-8 and absorbance was measured at 450 nm. Relative cell viability was determined according to naive group. (E) Cells from mice following i.p. injection were harvested and incubated for further 24 h in cell culture. CD86 positivity was assessed by flow cytometry. Results are the average of two independent experiments done in triplicate (mean ± SD). Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

the supernatants, whereas separately encapsulated ligands yielded only ~4 fold more IL-12 over non-encapsulated ligand combination treated groups (Fig. 2C). Furthermore, type I IFN secretion from BM-DCs treated with co-encapsulated CpG ODN plus poly(I:C) was found to be ~2.0 fold and ~1.7 fold more compared to non-encapsulated combination treated group and separately encapsulated CpG ODN and poly(I:C) group, respectively (Fig. 2D).

In a separate experiment, bone marrow derived macrophages (BM-DM) were generated and incubated with non-encapsulated or liposomes co-encapsulating ligands for 24 h. Nitric oxide (NO), and IL-12 levels were determined by Griess reagent, ELISA, respectively. While IL-12 production in co-encapsulating group, (CpG ODN + poly(I:C))L, was consistently higher than non-encapsulated combination at all doses except at 18 μg/ml dose, NO production was elevated only in co-encapsulated group at 18 and 6 μg/ml doses (Two-way ANOVA, Bonferroni; Fig. 3A and B). Moreover, 2 fold more TNF-α production was detected from (CpG ODN + poly(I:C))L treated cells (Fig. 3C).

Effective antigen presentation could only be achieved when both antigen and adjuvant simultaneously internalized by APCs. Liposomes are excellent candidates for simultaneous delivery of their cargo. To address this, DQ™-OVA, was used either with non-encapsulated or with dual ligand co-encapsulated liposomes. DQ™-OVA is an ovalbumin protein

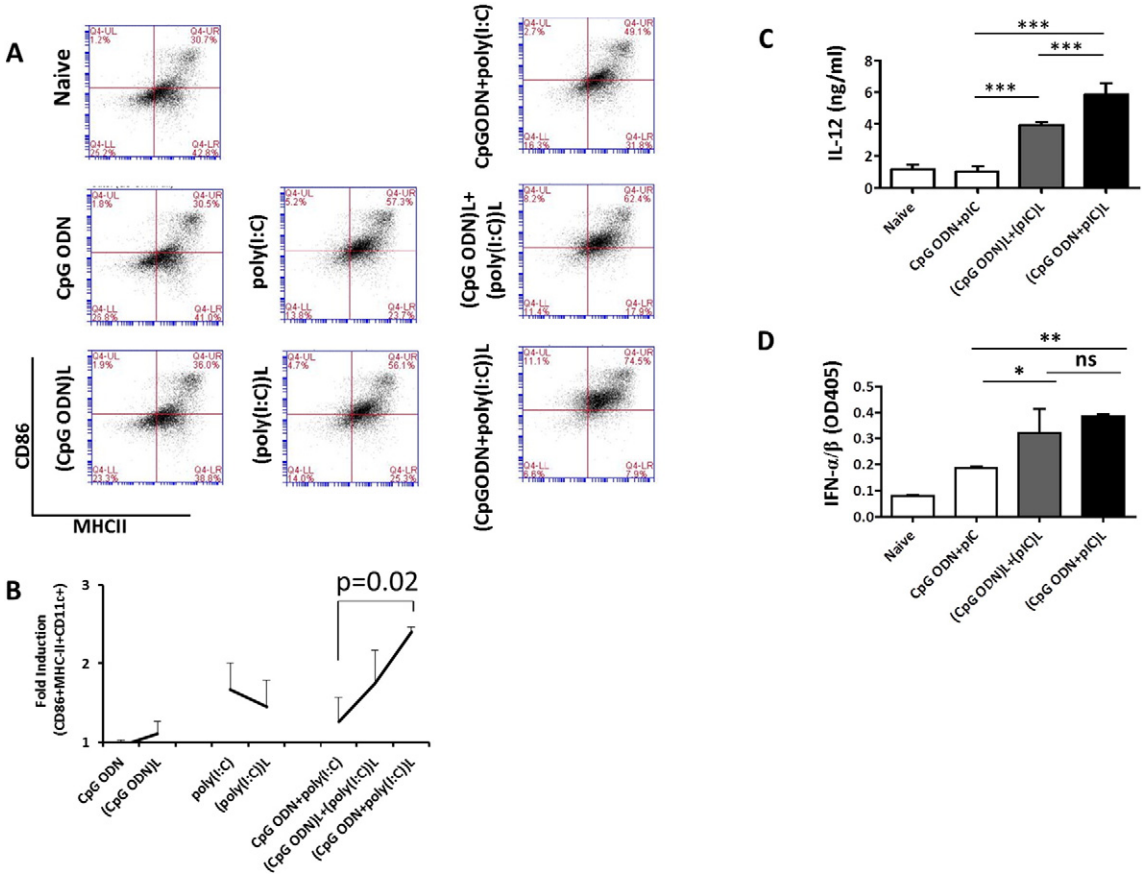


Fig. 2. Liposomes co-encapsulating CpG ODN and poly(I:C) promoted BM-DC maturation. Immature BM-DCs were incubated at 37 °C with non-encapsulated or liposomal CpG ODN and/or poly(I:C) (2 μg/ml from each ligand) for 24 h. Expression levels of CD86 and MHC-II were analyzed by flow cytometry: (A) representative dot plots. (B) Fold induction profiles of double positive cells. Average of three independent experiment are presented (Student's *t*-test, $p = 0.02$). Following stimulation, supernatants were collected to determine (C) IL-12 levels by ELISA and (D) IFN-α/β levels by reporter cell line. Results are average of two independent experiments done in triplicate (mean ± SD). Statistical significance was determined by ANOVA followed by Tukey's (HSD) test. ns: non-significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

conjugated to a pH insensitive self-quenching dye. Upon internalization, fluorescence could be detected when ovalbumin is hydrolyzed. We assessed antigen delivery and processing ability of liposomal constructs following incubation with BM-DC, BM-DM and RAW 264.7 cells (Fig. 3D). All data was normalized to degradation level of each treatment group incubated at 4 °C. We found that 6 h after incubation, fluorescence signal was significantly high for liposome treated cells compared to non-encapsulated ligand treated cells (*t*-test, $p < 0.001$).

When the findings from Figs. 2 and 3 were taken together, liposomes co-encapsulating CpG ODN and poly(I:C) i) induced robust cytokine production, ii) magnified bactericidal activity, iii) upregulated costimulatory molecules, and iv) improved APC function.

3.3. Co-encapsulation of poly(I:C) and CpG ODN within liposomes augmented ligand binding by immune cells

The mechanism of the enhanced synergistic innate immune activation could be due to effective internalization of liposomal ligands by the target cells.

To verify whether augmented immune activity correlated with the improved cellular uptake, non-encapsulated and liposomal forms of Cy5-labeled CpG ODN and fluorescein-labeled poly(I:C) ligands were used to stimulate mouse immune cells and ligand specific flurochrome positivity was analyzed by flow cytometry (Fig. 4). After 2 h incubation, non-encapsulated dual ligand treated cells had 5.2% and 1.3% (for CpG ODN and poly(I:C) signal, respectively) positivity, whereas dual ligand co-encapsulated treatment gave 12.5% and 6.5% CpG ODN and poly(I:C) positivity, respectively (Fig. 4A). Consequently, co-encapsulation by liposomes provided ~2.5 fold more CpG ODN and ~5 fold more poly(I:C) binding. When relative MFI values of the treatments were analyzed, this improvement in ligand binding was more pronounced (Fig. 4B). Thus, the improved uptake ability of both ligands via liposome encapsulation led to a robust synergistic immune activity. Furthermore, this finding revealed that rather than encapsulating TLR3 and TLR9 ligands in different liposomes, co-encapsulating them within a single carrier is more preferable (Fig. 4C).

3.4. Antigen loading together with dual TLR ligands within liposomes provides magnified and sustained antigen specific IgG response

After observing that poly(I:C) and CpG ODN co-encapsulated within liposomes boosted Th1-biased immune activation and improved APC function, we next investigated the potential of this system as a preventive vaccine (Fig. 5A). After injecting C57BL/6 mice twice (primary @ d:0 and booster @ d:14) with indicated formulations (Fig. 5B), sera were collected from animals following tail bleedings and *anti*-OVA IgG levels were detected by end-point ELISA. Serum titrations for total IgG, IgG1 and IgG2c subtypes were analyzed following 1st and the 8th month post-booster injections (Fig. 5B–D). The rationale of the immunization study was to identify *anti*-OVA antibody persistence among groups. As seen in Fig. 5B, (CpG ODN + OVA)L+(poly(I:C) + OVA)L and (CpG ODN + poly(I:C) + OVA)L groups gave the highest total IgG titers at the end of eight months post-booster injection. If one expects to raise a Th-1 biased anti-OVA immunity upon immunization, the vaccine candidate should reduce IgG1 and promote IgG2c (or IgG2a in Balb/c mice). When our results by the end of eight months of antibody titer is

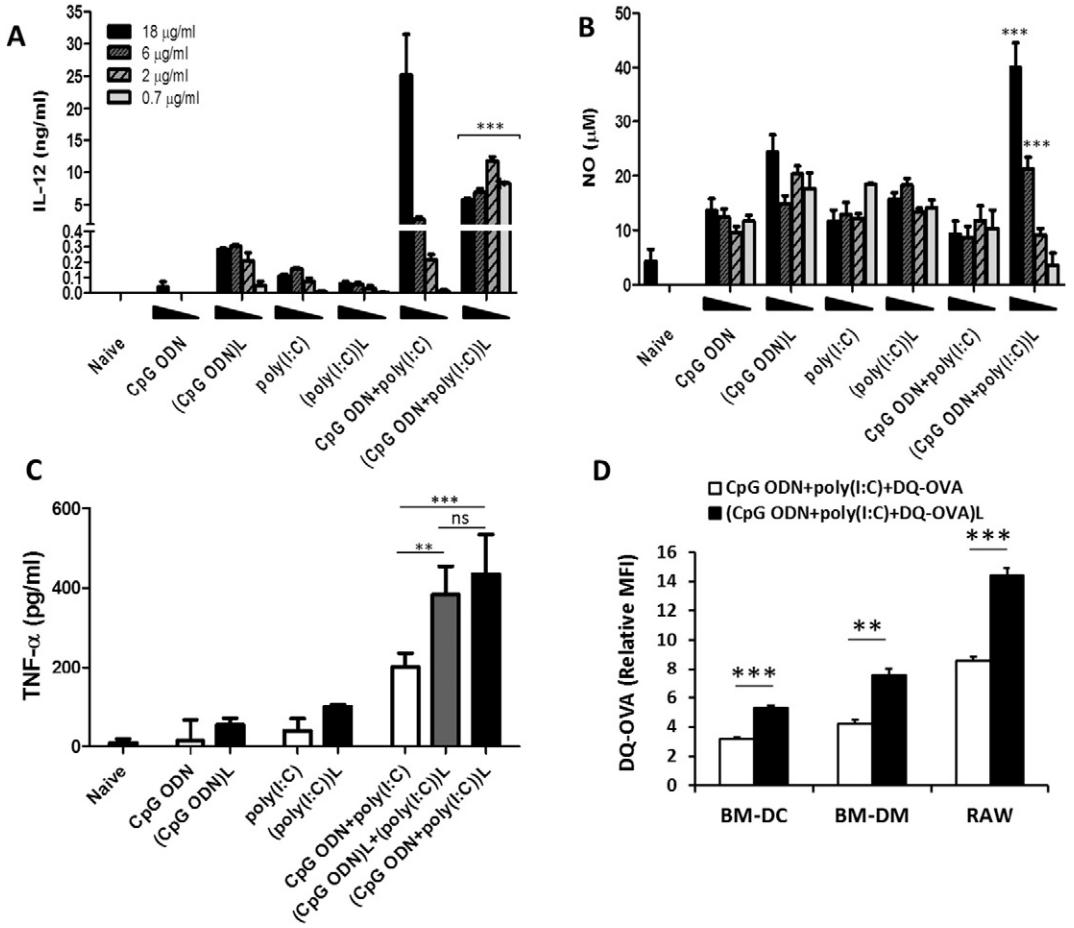


Fig. 3. Liposomes co-encapsulating CpG ODN and poly(I:C) induce pronounced macrophage activation. BM-DM were generated with 30% L929 supplemented medium and stimulated with non-encapsulated or liposome co-encapsulating CpG ODN and poly(I:C) (0–18 μg/ml per each ligand). (A) IL-12 and (B) NO levels were determined by ELISA and Griess reagent, respectively. Statistical significance was determined by two-way ANOVA followed by Bonferroni test (non-encapsulated vs. co-encapsulated liposome). ns: non-significant; $^{**}p < 0.01$; $^{***}p < 0.001$. (C) RAW 264.7 cells were stimulated with non-encapsulated ligands or co-encapsulated CpG ODN and poly(I:C) liposomes for 24 h and TNF-α secretion was determined by ELISA (one-way ANOVA, Tukey's HSD test). (D) Antigen processing was analyzed after the addition of non-encapsulated ligands or co-encapsulated ligands (2 μg/ml from each ligand) together with DQ™-OVA (2 μg/ml) onto BM-DCs, BM-DMs or RAW 264.7 cells by flow cytometry (non-encapsulated ligands vs co-encapsulated ligands; Students *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Results are represented as average of two independent experiments done in triplicate (mean ± SD).

considered, Only (CpG ODN + poly(I:C) + OVA)L group gave the highest IgG2c titers and lower IgG1 titer (compare Fig. 5C and D last three bars).

Collectively this data pointed out that antigen and dual adjuvants co-encapsulated within liposomes achieved simultaneous antigen + adjuvant delivery to APC and triggered long-lasting, sustained anti-OVA specific Th1-biased immunity.

3.5. Vaccine formulations containing both CpG ODN and poly(I:C) generated persisting memory CD8$^+$ T cells and prevented tumor formation

We then sought whether OVA immune animals could effectively control or eradicate tumor inoculation. At the end of eight month post-booster injection, mice were subcutaneously (s.c.) inoculated with 4×10^6 E.G7 cells (EL4 cells transduced to express OVA) and were followed for 14 days. The individual tumor development was followed daily with electronic caliper. In all animals that were immunized with saline, tumor formation was observed 4 days post-E.G7 inoculation. The median of tumors in naive group reached 402.5 mm^3. The tumor sizes at day 14 post-inoculation are presented in Fig. 6A. Animals immunized with liposomal formulations showed reduced tumor sizes. Mice that received (CpG ODN + poly(I:C) + OVA)L vaccine and challenged 8 months later gave 75% ($n = 6/8$) tumor free animals at the end of d = 14. All groups were significantly different than naive group (one-way ANOVA, $p < 0.01$) and the variance between co-encapsulated group and independent ligands encapsulated liposome group were significantly different (F test, $p = 0.0019$).

OVA specific memory T-cells is one of the major contributing cells to eliminate OVA-expressing tumor cells. The tumor specific CD8$^+$ T-cell numbers were analyzed by IFN-γ ELISPOT assay. The splenocytes from immunized animals were treated with CD8$^+$ T cell specific OVA peptide (SIINFEKL) for 18 h. The data revealed that co-administration of ligands + OVA to mice either with separately encapsulated or co-encapsulated liposomes significantly increased IFN-γ$^+$ CD8$^+$ T cell numbers (Fig. 6B). Surprisingly, (CpG ODN + poly(I:C) + OVA)L vaccine treated animals did not generate significantly higher CC8+ memory T-cell number compared to (CpG ODN + OVA)L+(poly(I:C) + OVA)L vaccine treated group.

Collectively, our findings implied that a combination of poly(I:C) and CpG ODN adjuvants in liposomal formulations induced stronger and persistent humoral and cellular *anti*-OVA specific immune response sufficient to eradicate tumors.

3.6. Synergistic immune activation elucidated in hPBMCs with liposomes co-encapsulating poly(I:C) and CpG ODN

In mice, due to strong in vivo anti-tumoral immune response generated upon co-encapsulated (CpG ODN + poly(I:C) + OVA)

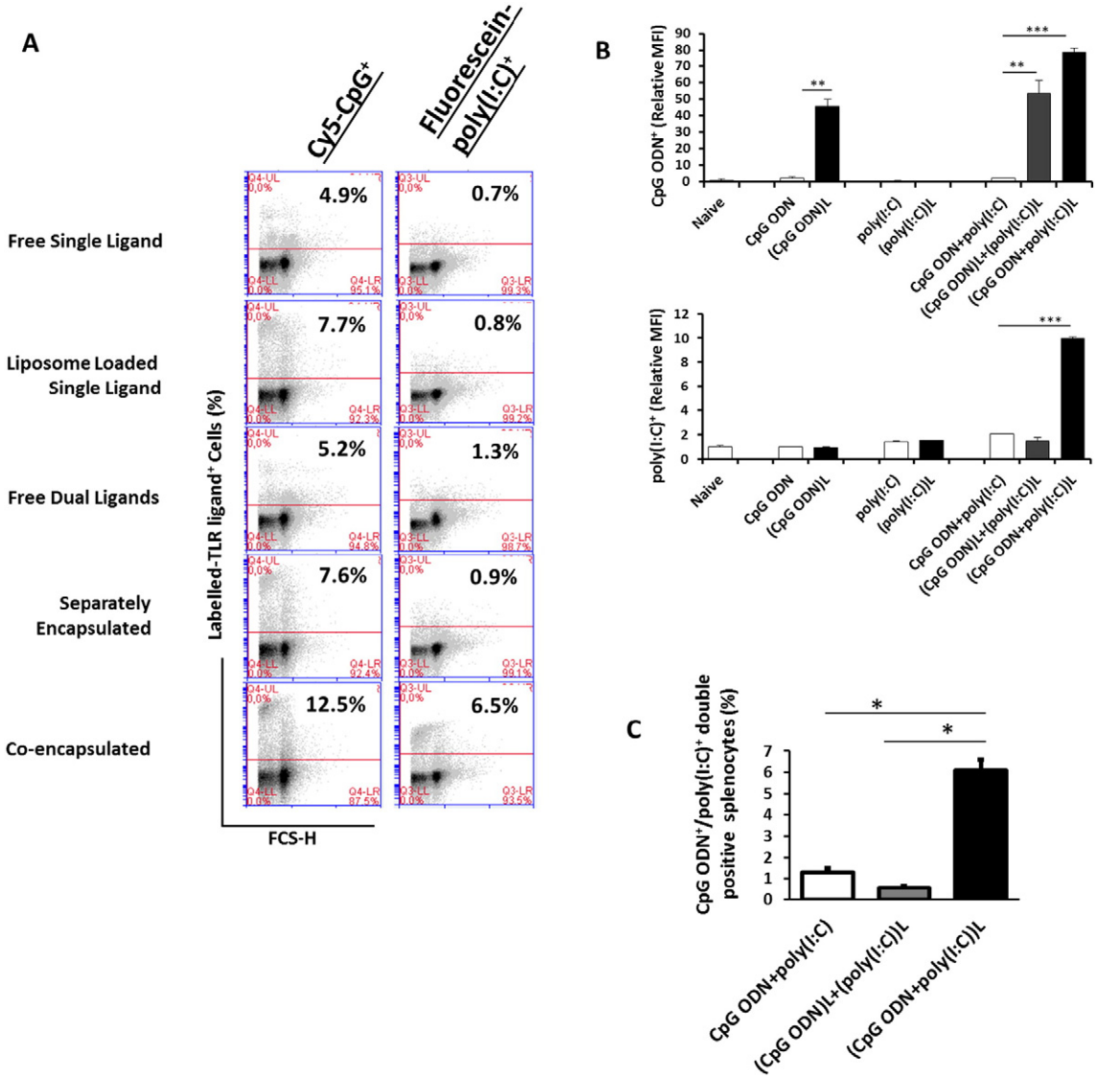


Fig. 4. Liposomes co-encapsulating CpG ODN plus poly(I:C) improves cellular uptake. Splenocytes were incubated at 37 °C with non-encapsulated ligands or liposomes encapsulating fluorescein-labeled poly(I:C) and/or Cy5-labeled CpG ODN (2 μg/ml each) for 2 h. (A) Representative dot-plots and (B) relative MFI values of uptake & binding of CpG ODN or poly(I:C). (C) Cy5-CpG ODN and fluorescein-poly(I:C) double positive cell percent after non-encapsulated ligand or liposomal ligand treated cells for 2 h (Student's *t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ between groups). Data represents average of two independent experiments done in triplicate (mean ± SD).

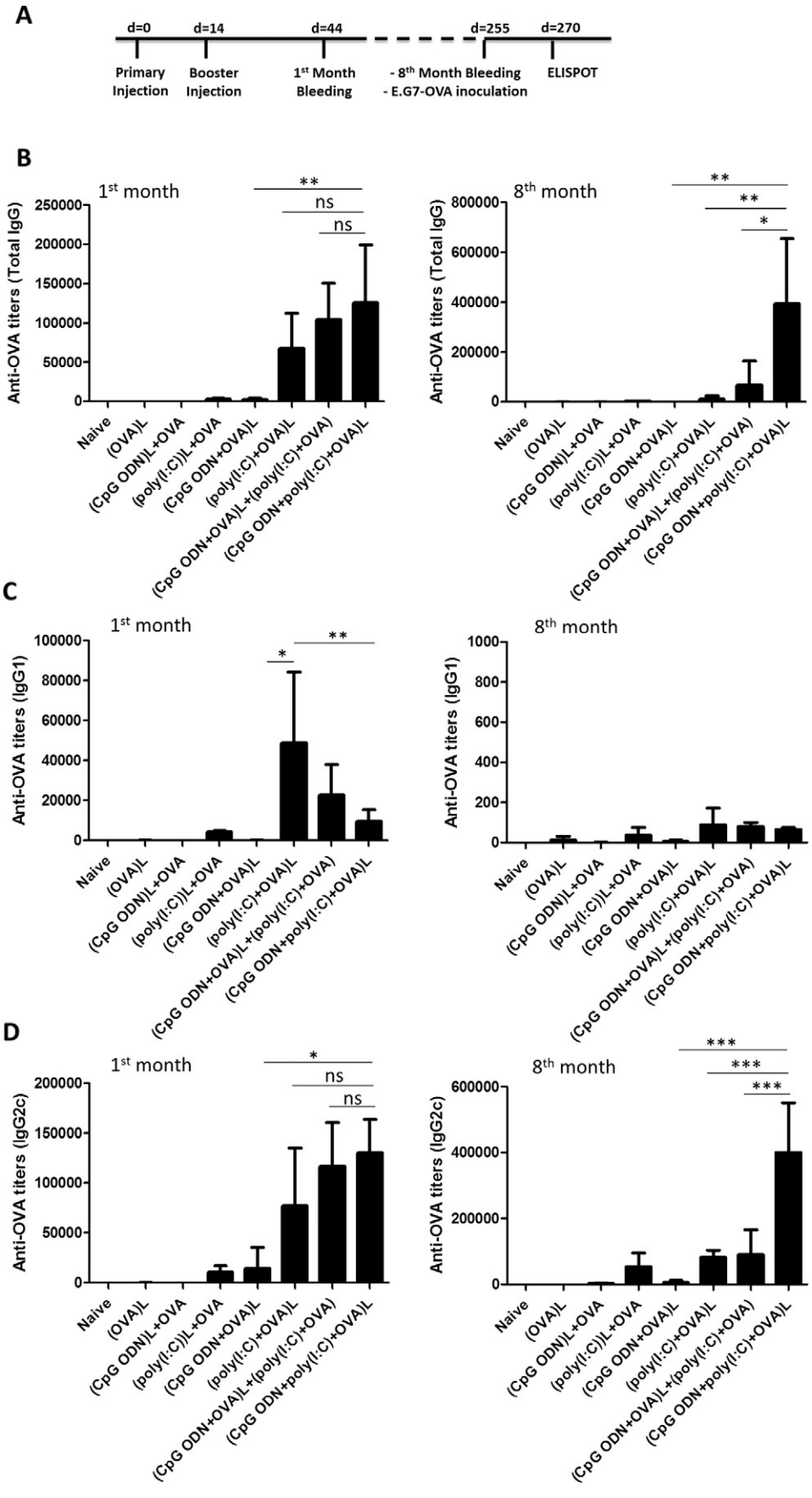
immunization, 75% of mice showed complete protection from tumor challenge. We next sought to establish whether this formulation triggers hPBMCs to the same extent that we observed in mice. Therefore, hPBMCs were collected from healthy donors and stimulated with i) non-encapsulated, ii) encapsulated and iii) co-encapsulated CpG ODN and/or poly(I:C) for 24 h. All hPBMCs produced significantly higher levels of IFN-α (A, left panels) when treated with (CpG ODN + poly(I:C))L group. Similarly, the IP10 production was more with co-encapsulated liposomal group compared to non-encapsulated or separately encapsulated ligand combinations (Fig. 7A, right panels). In addition, percentages of HLA-DR^{+}/CD83^{+} hPBMCs were significantly higher in cells treated with dual ligands co-encapsulated into liposomes (one-way ANOVA, Tukey's HSD, $p < 0.05$ between non-encapsulated vs co-encapsulation, and between separately vs co-encapsulation groups; Fig. 7B).

In conclusion, co-encapsulation of poly(I:C) and CpG ODN within liposomes enhanced secretion of interferon alpha and maturation of APCs in human immune cells. A promising finding implicating that these combinations could be translated to clinical applications.

4. Discussion

There is no doubt that TLR therapeutics soon will be in the clinics. The endosomal-associated TLR ligands senses nucleic acid ligands and are one of the most promising candidate bio-drugs as immunotherapeutic agents. Clinical trials harnessing various nucleic acid TLR ligands are underway aiming to cure diseases like cancer, allergy, autoimmune or autoinflammatory and infectious diseases. One of the promising area for TLR mediated therapeutics is to utilize them as vaccine adjuvants or immunoprotective agents where there is no available vaccines

Fig. 5. Immunization with liposomes co-encapsulating CpG ODN, poly(I:C), and/or ovalbumin promotes Th-1 biased immunity. (A) Immunization schedule. C57BL/6 female mice (8/group) were immunized twice with 14 day intervals with liposome encapsulated CpG ODN (10 μg/animal) and/or poly(I:C) (10 μg/animal) or their combinations with ovalbumin (OVA, 5 μg/animal) and sera was collected from tail vein 1st and 8th month post-booster injection. (B) Diluted sera were used for OVA specific (B) total IgG, (C) IgG1 and (D) IgG2c by end-point ELISA (mean ± SD). Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.


A
d=0
d=14
d=44
d=255
d=270
Primary Injection
Booster Injection
1st Month Bleeding
- 8th Month Bleeding
- E.G7-OVA inoculation
ELISPOT
B
1st month
8th month
Anti-OVA titers (Total IgG)
C
Anti-OVA titers (IgG1)
D
Anti-OVA titers (IgG2c)
Naive
(OVA)L
(CpG ODN)L+OVA
(poly(I:C))L+OVA
(CpG ODN+OVA)L
(poly(I:C)+OVA)L
(CpG ODN+OVA)L+(poly(I:C)+OVA)
(CpG ODN+poly(I:C)+OVA)L
ns
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(including viral, bacterial and parasitic infections) [8,16,18,19]. Formulating these labile nucleic acid-based agents (i.e. ds RNA or CpG DNA) in stable form within proper depot systems improve their bioavailability and bioactivity at the of the target cell sites. We previously demonstrated that liposomes enhance in vivo immunostimulatory activity of TLR ligands along with ligand sparing effect and improved shelf-life [17,24,26]. In this study, liposomes are used to co-encapsulate two important TLR ligands, expanding their breadth of synergistic immune activity, therefore, improving their immunoadjuvant effect upon co-encapsulating antigen of interest. Here, we provide evidence that these liposomes encapsulating dual TLR ligands i) upregulates costimulatory molecules, ii) magnifies proinflammatory and inflammatory cytokine secretion, iii) provides more efficient ligand internalization, iv) improves macrophage bactericidal activity v) boosts APC function of DCs and vi) promotes development of Th1 type immunity and vii) in mice provides long lasting antigen specific humoral and cell-mediated immunity capable of preventing E.G7 tumor development even after eight months post-booster injection.

Low doses of non-encapsulated single TLR ligand could be insufficient to mount an appreciable degree of immune activation however; combination of ligands even at low doses (here, 2 μg/ml of each ligand within liposomes) could trigger synergistic immune response when given within liposomes. Using MyD88 independent and dependent ligands trigger multiple signaling pathways improves the breadth of immune activation [30]. Krummen et al. demonstrated that the synergistic immunostimulatory action between TLR ligands was more pronounced than their standalone counterparts when MyD88 and TRIF-dependent signaling, like CpG ODN and poly(I:C), respectively, was combined [31]. In addition, studies showed the elevated immune response when CpG ODN is administered together with poly(I:C) [11–14,29,32]. We encapsulated these two ligands to enhance their synergistic activation and utilized them as novel form of long term effective *anti*-cancer vaccine. The demonstration of synergy between CpG ODN and poly(I:C) following co-encapsulation within liposomes were tested on murine splenocytes. Co-encapsulating CpG ODN plus poly(I:C) led to higher levels of secretion of type I and II IFNs, and IL-6 than non-encapsulated or loaded single or combinational ligands by splenocytes (Fig. 1). Co-encapsulation was stronger than separately encapsulated ligands. This improved activity suggested that co-encapsulating TLR3 and TLR9 ligands can activate immune response even better than their non-encapsulated combinations.

Additionally, type I IFN and IL-12 secretion was significantly elevated by co-encapsulation when compared with non-encapsulated or separately encapsulated dual ligands. Supporting our findings, it was shown that, IL-12 production from BM-DCs was synergistically increased with non-encapsulated combination [31,32]. However, in those studies, non-encapsulated poly(I:C) plus CpG ODN could not achieve to synergistically increase neither CD86 surface marker nor type I IFN secretion.

Liposomes act as a depot delivery system and improve cellular uptake of the ligands. Previously, we reported that uptake of CpG ODN is higher by immune cells when it is encapsulated within sterically stabilized cationic liposomes (SSCL) [26]. In this study, we showed that CpG ODN uptake was even higher when administered together with poly(I:C) into liposomes. More importantly, we provide evidence that dual uptake of ligands amplified sixfold more than non-encapsulated or separately encapsulated dual ligands (Fig. 4). In a study, lipofectamine mixed phosphorothioate-modified CpG ODN plus poly(I:C) was demonstrated to block uptake of poly(I:C) by tumor cells [34]. Here, since our ligands are encapsulated rather than complexed with the nanocarrier, we did not observe any blockage of wither of the ligand uptake. Besides, the uptake increase would be explained by the decreased size of liposomes co-encapsulating dual ligands compared to unloaded liposomes (Fig. S2).

Simultaneous presentation of adjuvants and antigen plays an important role in the quality and quantity of immune response against protein antigen [35,36]. Encapsulation of antigen with adjuvant within delivery vehicle overcomes random antigen and adjuvant uptake by APCs, furthermore it increases the internalization of exogenous antigen [20,26,35–37]. We show that, higher IgG2c production was maintained over the course of 8 months only when mice were immunized with liposomes co-encapsulating antigen and adjuvants rather than standalone counterparts (Fig. 5).

In the present work, both co- and separately encapsulated groups protected immunized mice against tumor formation. However, when the content of the separately encapsulated liposome formulation, it contained two fold more antigen than co-encapsulated formulation (i.e. (CpG ODN + OVA)L+(poly(I:C) + OVA)L vs CpG ODN + poly(I:C) + OVA)L). Therefore, it is important to note that co-encapsulated group yielded more pronounced Th1-biased *anti*-OVA immunity with 2-fold less antigen.

In a study, effect of lipid structure and size of DDA:TDB liposomes were investigated on cellular and humoral responses and CpG ODN and/or poly(I:C) were co-encapsulated or complexed to enhance immunogenicity of liposomes. Results revealed that co-encapsulating dual ligands into DDA:TDB liposomes boosted total IgG and induced Ag-specific T cell activation in multilamellar vesicles (MLV) not in SUV [37]. In that study, three immunizations with 250 μg DDA:DSPC, 50 μg TDB, 20 μg CpG, 50 μg poly(I:C) and 20 μg OVA were employed for every injection. As DDA:TDB liposomes have immunogenic effect by its own [38], Our vaccine candidate composed of 10 μg CpG, 10 μg poly(I:C), and 5 μg OVA, in neutral liposomes is the first study demonstrating enhanced synergy of CpG ODN and poly(I:C) when co-encapsulated into non-immunogenic liposomes (Fig. S3) together with antigen that with immunization, both Th1 and CTL responses were sustained over the course of 8 months, and successfully protects immunized mice to develop tumors. The aim of the present study is to develop a cancer vaccine that can protect individuals long after they are vaccinated against a specific cancer antigen. It is true that much shorter tumor challenge intervals are used for therapeutic vaccinations. However, CpG ODN and poly(I:C) is known to mediate antigen independent clearance of tumor cells. In this study, since we aimed to address the contribution of memory CD8 + T-cells, we chose to challenge our animals eight month after booster injection, therefore, TLR ligands mediated non-specific innate immune activation could not be responsible from tumor elimination.

5. Conclusion

Liposomes co-encapsulating CpG ODN and poly(I:C) boosted cellular uptake of the cargo, improved proinflammatory cytokine production along with type I/II IFN secretion, as well as co-stimulatory molecule upregulation leading to a pronounced APC function and antigen processing. The co-encapsulation of OVA antigen into liposome vesicles created a long-lasting *anti*-cancer immune response. The heightened Th1 immunity in addition to enhanced OVA-specific memory $CD8^+$ T cell response helped to control tumor development. Similar immune stimulatory activity was reproduced with human peripheral mononuclear cells, supporting the applicability for human use. In conclusion, the present liposomal vaccine candidate offers an effective antigen and adjuvants co-encapsulation approach and could be developed as preventive vaccine for human use against cancer or viral diseases.

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

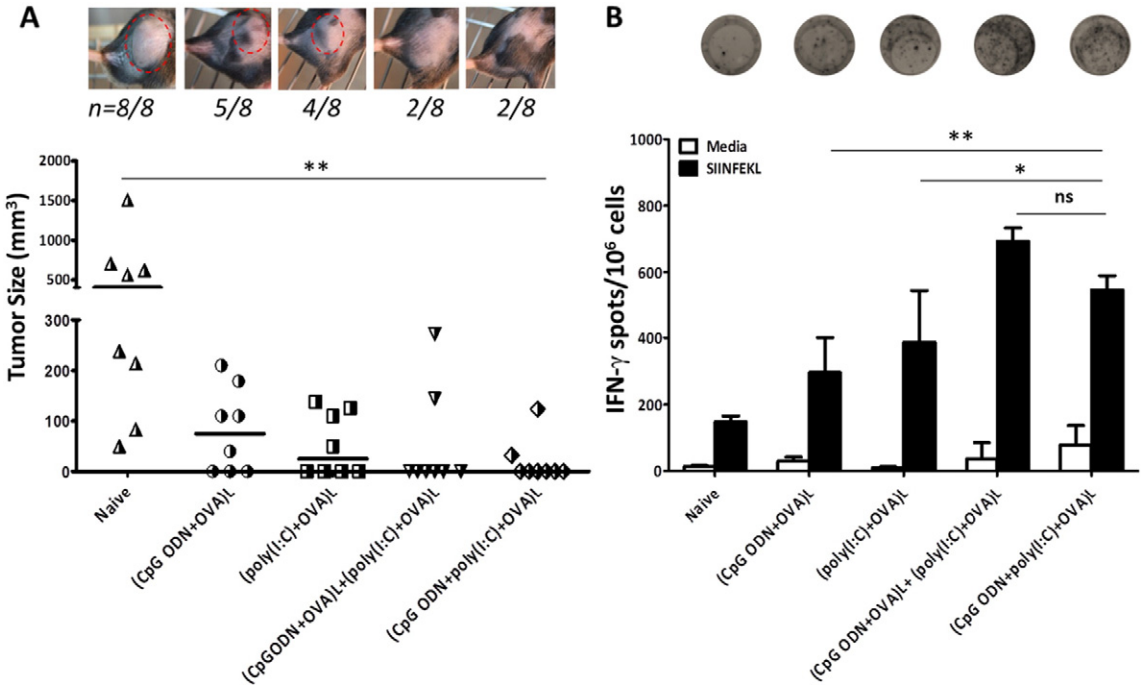

Fig. 6. Mice immunized with liposomes co-encapsulating CpG ODN + poly(I:C) + OVA resist tumor development long after booster injection. Following 8th month of booster injection, immunized animals were inoculated with OVA expressing E.G7 thymoma cells (4×10^6 cells/animal). (A) Tumor volume was measured daily for a duration of 14 days following the appearance of palpable tumor. (B) At the end of tumor follow-up mice were sacrificed and splenocytes were isolated and IFN-γ^+ $CD8^+$ T-cells were detected stimulating serially diluted splenocytes with OVA 257–264 peptide (SIINFEKL) for 18 h by ELISPOT method. Data were presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

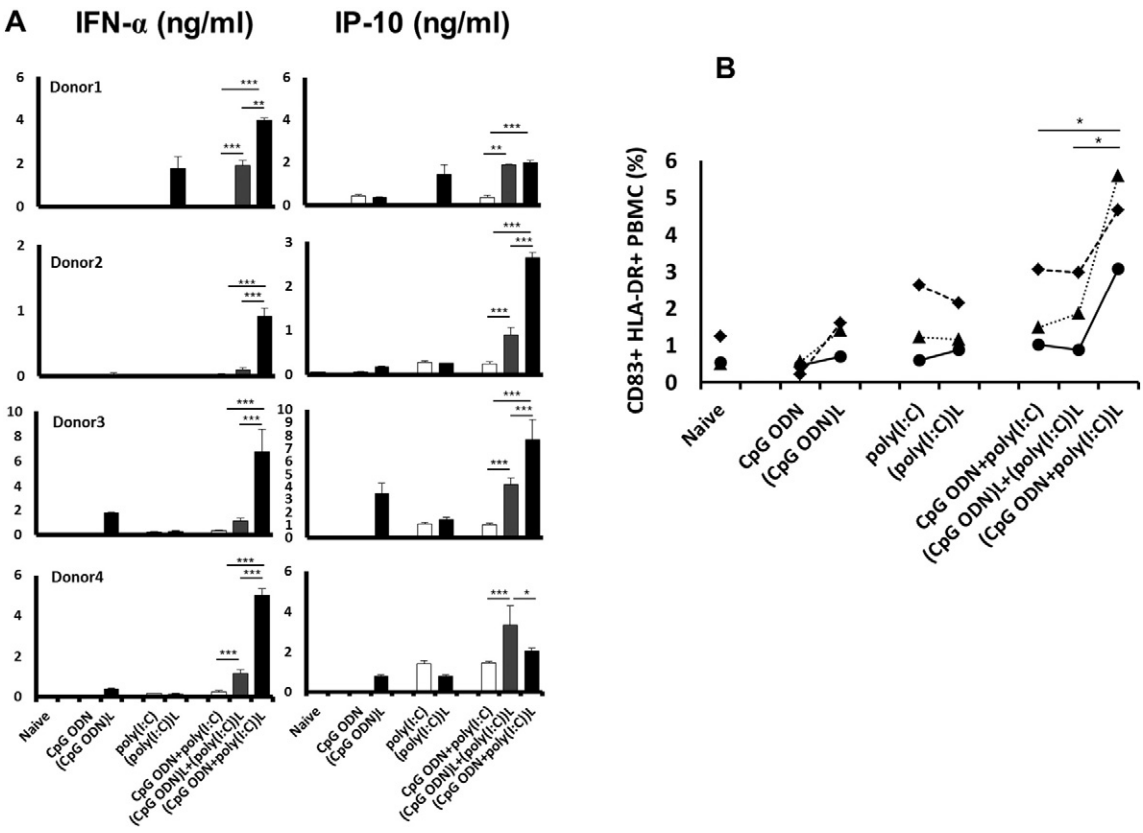

Fig. 7. Immune response of human PBMCs following treatment with liposome co-encapsulating CpG ODN plus poly(I:C). PBMCs were isolated from healthy donors' blood and stimulated with non-encapsulated or liposome encapsulating CpG ODN and/or poly(I:C) for 24 h. Supernatants were used for (A) IFN-α and IP-10 cytokine ELISA. Triplicate wells (mean ± SD) of four healthy donors are presented. (B) CD83 and HLA-DR positive PBMCs (three healthy donors) were analyzed via flow cytometry. Statistical significance was determined by one-way ANOVA followed by Tukey's (HSD) test. ns: non-significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.01.004.

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