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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.