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Dual TLR agonist nanodiscs as a strong adjuvant system for vaccines and immunotherapy

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Recent studies have shown that certain combinations of Toll-like receptor (TLR) agonists can induce synergistic immune activation. However, it remains challenging to achieve such robust responses in vivo in a manner that is effective, facile, and amenable for clinical translation. Here, we show that MPLA, a TLR4 agonist, and CpG, a TLR9 agonist, can be efficiently co-loaded into synthetic high-density lipoprotein nanodiscs, forming a potent adjuvant system (ND-MPLA/CpG) that can be readily combined with a variety of subunit antigens, including proteins and peptides. ND-MPLA/CpG significantly enhanced activation of dendritic cells, compared with free dual adjuvants or nanodiscs delivering a single TLR agonist. Importantly, mice immunized with physical mixtures of protein antigens ND-MPLA/CpG generated strong humoral responses, including induction of IgG responses against protein convertase subtilisin/kexin 9 (PCSK9), leading to 17–30% reduction of the total plasma cholesterol levels. Moreover, ND-MPLA/CpG exerted strong anti-tumor efficacy in multiple murine tumor models. Compared with free adjuvants, ND-MPLA/CpG admixed with ovalbumin markedly improved antigen-specific CD8+ T cell responses by 8-fold and promoted regression of B16F10-OVA melanoma ($P < 0.0001$). Furthermore, ND-MPLA/CpG admixed with E7 peptide antigen elicited ~20% E7-specific CD8+ T cell responses and achieved complete regression of established TC-1 tumors in all treated animals. Taken together, our work highlights the simplicity, versatility, and potency of dual TLR agonist nanodiscs for applications in vaccines and cancer immunotherapy.

1. Introduction

Vaccination is a powerful medical intervention that is proven to be effective in the settings of infectious diseases, cancer, and many pathologies [1–3]. Compared with live attenuated or killed vaccines, vaccines based on subunit antigens, such as recombinant proteins or peptide antigens, are more attractive due to their safety and ease of manufacturing and quality control [4–7]. However, because of their low immunogenicity, subunit antigens need to be administered with immune-stimulating adjuvants to promote immune responses [8]. Among various adjuvant molecules, Toll-like receptor (TLR) agonists have been studied extensively [9,10]. TLR agonists can activate dendritic cells to upregulate costimulatory ligands and secrete pro-inflammatory cytokines, thereby providing critical signals to the adaptive immune system for induction of cellular and humoral immune responses [11].

Notably, recent studies have indicated synergy between various combinations of TLR agonists [12,13]. Specifically, monophosphoryl lipid A (MPLA, a TLR4 agonist) and CpG-rich oligonucleotide (CpG, a TLR9 agonist) activate dendritic cells (DCs) via two distinct pathways. MPLA triggers the interferon regulatory factor 3 (IRF3) pathway via Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) and is currently used in the clinic [10,14,15]. CpG activates nuclear factor kappa B (NF-KB) via myeloid differentiation primary response gene 88 (MyD88) [16–18], and a hepatitis B vaccine (HEPLISAV-B™, Dynavax Technologies Corporation, Berkeley, CA) containing CpG oligonucleotide-1018 as an adjuvant was approved by the US Food and Drug Administration (FDA) for clinical use on November 9, 2017 [19]. Importantly, potent synergy between TLR4 and TLR9 agonists in activation of antigen-presenting cells has been documented in vitro

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[12,13], and their in vivo efficacy to generate adaptive immune responses have been recently reported using adjuvant delivery systems [20–23]. Despite these advances, there is still a need for a general methodology that can achieve potent immune activation with combinations of TLR agonists, especially in a manner that is effective, facile, and amenable for clinical translation.

Here, we propose a simple strategy for co-delivering multiple TLR agonists in vivo that can be readily formulated with a variety of subunit antigens. Previously, synthetic high-density lipoprotein (sHDL), either made from recombinant apolipoproteins or obtained from endogenous plasma has been examined as delivery vehicles for various cargo molecules [24–27]. In our own prior work, we have reported the development of sHDL nanodiscs (ND), composed of biocompatible phospholipids and apolipoprotein A1 (ApoA1)-mimetic peptide [28]. We have demonstrated their versatility to deliver a wide range of therapeutics, including chemotherapeutics, imaging agents, and nucleic acids [28–32]. Additionally, we have recently shown that ND loaded with peptide antigens and a TLR9 agonist efficiently drained to local draining lymph nodes and induced strong antigen-specific T cell responses against cancer cells [33]. Here, we show that ND serves as an effective delivery platform for dual TLR agonists and demonstrate their adaptability and potency with a range of subunit antigens, including protein and peptide antigens.

In this current study, we report that sHDL ND allowed for efficient incorporation ($>$ 80% efficiency) of MPLA. Additionally, as sHDL ND is a good acceptor for cholesterol [28], we have employed cholesterol-modified CpG for loading into sHDL ND and achieved $>$ 95% incorporation efficiency for CpG. The resulting ND co-loaded with the dual adjuvants (ND-MPLA/CpG) was more effective at activation and maturation of DCs, compared with free dual adjuvants or even ND containing either MPLA or CpG. Immunizations with ND-MPLA/CpG physically mixed with protein antigens generated strong humoral immune responses in vivo, including induction of antibody responses against protein convertase subtilisin/kexin 9 (PCSK9), leading to 17–30% reduction of the total plasma cholesterol levels in mice. Importantly, ND-MPLA/CpG also served as a potent adjuvant system for elicitation of cellular immune responses in vivo. Compared with free adjuvants, ND-MPLA/CpG admixed with a model antigen protein, ovalbumin (OVA), significantly improved antigen-specific CD8+ T cell responses in B16F10-OVA tumor-bearing mice, inducing regression of established melanoma tumors. Finally, we have also confirmed these results using the TC-1 tumor cell line expressing the E7 oncogene from human papillomavirus (HPV) type 16. Immunizations with ND-MPLA/CpG admixed with E7 antigen peptide elicited ~20% E7-specific antigen-specific CD8+ T cells and exerted potent anti-tumor efficacy against established TC-1 tumors. Overall, our results demonstrate that ND-MPLA/CpG is a promising adjuvant system for vaccination and immunotherapy.

2. Materials & methods

2.1. Reagents

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Nippon Oils and Fats (Osaka, Japan). ApoA1 mimetic peptide 22A (PVLDLFRELLNELLEALKQKLK) was synthesized by GenScript Corp (Piscataway, NJ). MPLA was purchased from Avanti Polar Lipids (Alabaster, AL). 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) was purchased from Invitrogen. CpG1826 modified with cholesterol at the 3′ end (labeled as CpG throughout this manuscript) was synthesized by Integrated DNA Technologies (Coralville, IA). Ovalbumin (OVA) was purchased from Worthington (Lakewood, NJ). Recombinant human protein convertase subtilisin/kexin 9 (hPCSK9) was purchased from BioLegend (San Diego, CA). Recombinant mouse PCSK9 (mPCSK9) was purchased from Abcam (Cambridge, MA). E7 (GQAEPDRAHYNIVTFCCKCD) peptide was synthesized by Anaspec (Fremont, CA). Fetal bovine serum (FBS), penicillin-streptomycin, β-mercaptoethanol and ACK lysis buffer were purchased from Life Technologies (Grand Island, NY). Granulocyte-macrophage colony stimulating factor (GM-CSF) was from GenScript Corp. (Piscataway, NJ). Anti-mouse CD16/32, CD80, CD86 were from eBioscience (San Diego, CA). Anti-mouse CD8α-APC was from BD Bioscience (San Jose, CA). Tetramer H-2Kb-SIINFEKL-PE was purchased from Beckman Coulter (Brea, CA). Tetramer H-2Db-RAHYNIVTF-BV421 was kindly provided by the NIH Tetramer Core Facility (Atlanta, GA).

2.2. Preparation of ND-MPLA/CpG

sHDL nanodiscs (ND) were prepared by using the lyophilization method, as we reported previously [29,31,32]. To load MPLA into ND, MPLA was co-dissolved with DMPC and 22A at the weight ratio of 0.01:2:1 in acetic acid, followed by lyophilization and hydration with PBS to form ND-MPLA. To load CpG in ND-MPLA, CpG1826 modified with cholesterol at the 3′ end (i.e. CpG) was incubated with pre-formed ND-MPLA for 30 min at room temperature. In some experiments, 0.3% mol of DiD was mixed with lipids to prepare DiD-labeled nanodiscs.

2.3. Characterization of ND-MPLA/CpG

The loading efficiency of MPLA in ND-MPLA was measured by HPLC equipped with an evaporative light scattering detector (ELSD) as described before [34]. The loading efficiency of CpG was measured by gel permeation chromatography (GPC), as we reported previously [33]. Briefly, ND samples were injected in a Shimadzu HPLC system equipped with a TSKgel G2000SWxl column (7.8 mm ID × 30 cm, Tosoh Bioscience LLC), and the amount of CpG was quantified with the detection wavelength set at 280 nm. The particle size of ND-MPLA/CpG was measured by dynamic light scattering (DLS) on a Malvern Zetasizer (Westborough, MA). The ND morphology was assessed by transmission electron microscopy (TEM). Properly diluted ND sample solution was deposited on a carbon film-coated 400 mesh copper grid (Electron Microscopy Sciences) and dried for 1 min. The ND samples were then negatively stained with 1% (w/v) uranyl formate, and the grid was dried before TEM observation. All specimens were imaged on a 100 kV Morgagni TEM equipped with a Gatan Orius CCD.

2.4. Cell culture

Bone marrow-derived dendritic cells (BMDCs) were prepared as described previously [35]. Briefly, femur and tibia were harvested aseptically from C57BL/6 mice, and the bone marrow was flushed into a petri dish using a 5 mL syringe (26 G needle) loaded with BMDC culture media (RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol, and 20 ng/mL GM-CSF). Cells were collected by passing the cell suspension through a cell strainer (mesh size = 40 μm), followed by centrifugation. Cells were seeded into non-tissue culture treated petri-dish at a density of 2×10^5 cells/mL, cultured at 37 °C with 5% CO_2. Culture media were refreshed on days 3, 6, 8, and 10, and BMDCs were used for the following assays on days 8–12. B16F10-OVA cells were kindly provided by Dr. Darrell Irvine at Massachusetts Institute of Technology MIT (Cambridge, MA). TC-1 cells were kindly provided by Dr. T. C. Wu at Johns Hopkins University (Baltimore, MD). The TC-1 tumor model was generated by transformation of primary lung epithelial cells from C57BL/6 mice with active Ras together with HPV-16 E6 and E7 oncogenes [36]. Both cell lines were maintained in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. HEK cells transfected with TLR2, TLR4, or TLR9 (InvivoGen, San Diego, CA) were incubated with nanodiscs containing 0.5 μg/mL CpG and/or 0.05 μg/mL MPLA for 24 h, and activation of TLR signaling pathways was detected by following the manufacturer's instructions.

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