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Enhancement of antigen-specific CD4$^+$ and CD8$^+$ T cell responses using a self-assembled biologic nanolipoprotein particle vaccine

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ABSTRACT

To address the need for vaccine platforms that induce robust cell-mediated immunity, we investigated the potential of utilizing self-assembling biologic nanolipoprotein particles (NLPs) as an antigen and adjuvant delivery system to induce antigen-specific murine T cell responses. We utilized OT-I and OT-II TCR-transgenic mice to investigate the effects of NLP-mediated delivery of the model antigen ovalbumin (OVA) on T cell activation. Delivery of OVA with the TLR4 agonist monophosphoryl lipid A (MPLA) in the context of NLPs significantly enhanced the activation of both CD4$^+$ and CD8$^+$ T cells *in vitro* compared to co-administration of free OVA and MPLA. Upon intranasal immunization of mice harboring TCR-transgenic cells, NLPs enhanced the adjuvant effects of MPLA and the *in vivo* delivery of OVA, leading to significantly increased expansion of CD4$^+$ and CD8$^+$ T cells in lung-draining lymph nodes. Therefore, NLPs are a promising vaccine platform for inducing T cell responses following intranasal administration.

1. Introduction

Current vaccine development efforts have shifted largely toward developing subunit vaccines capable of generating both cellular and humoral immunity [1]. While subunit vaccines benefit from improved safety profiles, they lack the immunogenicity of live attenuated or heat-killed vaccines and require the use of stimulatory adjuvants to elicit long-lasting immunity [2]. Furthermore, vaccines capable of inducing robust cell mediated immune responses are necessary to combat intracellular pathogens and tumors [1]. However, the use of alum as an adjuvant provokes a strong Th2 response resulting primarily in antibody generation, rather than a robust Th1 cytotoxic T cell response, to the antigen [3,4]. Therefore, the development of vaccine formulations that integrate newly identified adjuvants and novel delivery platforms that can generate strong Th1 immune responses is paramount.

Nanoparticles have been widely explored as vaccine delivery platforms and offer a number of benefits including improved immunogenicity, reduced toxicities associated with high doses of adjuvants, improved pharmacokinetic profiles, and enhanced stability [2,5,6]. As soluble proteins are poorly internalized and have limited access to the cross-presentation pathway [7], strategies aimed at enhancing antigen uptake and processing by dendritic cells are critical for the development of subunit vaccines against intracellular pathogens and tumors. Nanoparticle-based vaccines that contain co-localized antigen and adjuvant provide two-fold benefits in this context. First, they present antigens in particulate form, which are internalized by dendritic cells more readily than soluble antigens [7]. Second, co-delivery of adjuvants serves to markedly enhance antigen processing, thus resulting in more efficient cross-presentation [8].

Despite a significant expansion in our understanding of innate and adaptive immunity, the development of vaccines comprised of new adjuvants and formulations remains largely unachieved [3,4]. While Toll-like receptor (TLR) ligands, such as lipopolysaccharide (LPS), function as potent adjuvants and may induce more balanced T cell responses than alum, the non-specific toxicity and widespread inflammation associated with natural TLR ligands has limited their clinical translation to date. As such, approaches to mitigate these limitations are critical.

Nanolipoprotein particles (NLPs) are self-assembling, discoidal particles comprising a lipid bilayer stabilized by scaffold proteins, i.e. apolipoproteins. NLPs are well-tolerated both *in vitro* and

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in vivo and can be delivered by multiple routes with intranasal administration resulting in retention for at least 24 h in the lungs [9]. Importantly, NLPs can be functionalized to incorporate TLR agonists which greatly enhances the activity and delivery of these compounds *in vitro* and *in vivo* [10]. Further, incorporation of both antigen and adjuvant onto NLPs significantly enhances antibody responses in mice compared with non-NLP conjugated control formulations [11].

To investigate the effects of NLPs on T cell responses, we utilized OT-I and OT-II transgenic mice, which have T cell receptors (TCRs) that are engineered to recognize peptides derived from the model antigen ovalbumin (OVA) displayed in the context of class I (OT-I) or class II (OT-II) MHC molecules. Since the specific TCRs expressed on OT-I and OT-II T cells are known, these cells can be easily identified by antibody staining, thus allowing the evaluation of $CD8^+$ and $CD4^+$ (respectively) T cell responses to OVA formulated with NLPs. The OVA and OT-I/OT-II model systems are well-characterized, robust models to assess T cell responses to vaccine formulations, including particle-based vaccines [12–14]. We investigated the intranasal route of immunization to evaluate the potential for NLP-based vaccines against inhaled pathogens. Delivery of OVA on NLPs significantly enhanced the activation of T cells both *in vitro* and *in vivo* upon intranasal immunization. *In vitro*, this effect correlated with increased uptake of OVA by APCs, while *in vivo*, the data suggest that NLPs enhance both antigen delivery and the adjuvant effects of MPLA.

2. Materials and methods

2.1. Materials for NLP assembly

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and synthetic MPLA (PHAD™) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were ordered from Sigma-Aldrich (St. Louis, MO). N-Hydroxysuccinimide-polyethyleneglycol 4-dibenzylcyclooctyne (NHS-PEG_4-DBCO) was purchased from Click Chemistry Tools (Scottsdale, AZ). The C18-PEG_6-N_3 molecule was custom synthesized by Creative PEGworks (Winston Salem, NC).

2.2. Preparation and characterization of adjuvanted NLPs and OVA conjugation

The NLP scaffold protein apoE422k (N-terminal 22 kDa fragment of apolipoprotein E4 (apoE4)) was expressed and purified using a similar protocol as previously described [15,16]. MPLA was incorporated into the NLP using published procedures [11]. To covalently conjugate OVA to the MPLA-adjuvanted NLPs using click chemistry, an azide-bearing lipidic molecule (C18-PEG_6-N_3) was incorporated into the NLP bilayer during assembly to form MPLA:N_3NLPs, as previously described [9]. Incorporation of MPLA and the N_3-bearing molecule into MPLA:N_3NLPs was assessed by reverse phase HPLC as described previously [10]. For additional detailed information regarding MPLA:N_3NLP assembly and MPLA quantification, as well as OVA conjugation and subsequent fluorescent labeling, please see Supplementary Materials.

2.3. Mice

All animal work was conducted in accordance with protocols approved by the Lawrence Livermore National Laboratory Institutional Animal Care and Use Committee. OT-I and OT-II breeding pairs were obtained from The Jackson Laboratory (Sacramento, CA) and were maintained in PHS-assured facilities. OT-I transgenic animals were maintained as hemizygous by crossing to wild-type C57BL/6, while OT-II mice were homozygous. C57BL/6 mice were obtained from Harlan Laboratories (Livermore, CA).

2.4. Activation of OT-I and OT-II T cells and splenic APCs

Spleens from OT-I, OT-II or wild-type C57BL/6 mice were collected in cold PBS and made into a single-cell suspension by manual dissociation as described previously [10]. Cells were plated into 96-well plates at a density of 4×10^6 cells/ml in 200 μl per well in complete RPMI medium (10% FBS, 1× L-gluatamine, and 1× penicillin/streptomycin (all cell culture reagents from Gibco/Life Technologies, Grand Island, NY) containing 2 ng/ml IL-7 (PeproTech, Rocky Hill, NJ) to promote survival of all T cells. Final concentrations of OVA and MPLA were 3 μg/ml and 0.41 μg/ml, respectively. Cells were incubated at 37 °C with 5% CO_2 for 4 or 24 h, then stained for flow cytometric analysis.

2.5. OT-I/II cell transfer and intranasal immunizations

One day before immunization, spleens from OT-I or OT-II mice were collected in cold PBS and made into a single-cell suspension by manual dissociation, as described above. Cells were diluted to 10^7 cells/ml, and 100 μl (10^6 OT-I or OT-II splenocytes) were transferred to naïve C57BL/6 recipients via intraperitoneal injection. The next day, mice were lightly anesthetized using isoflurane, and OVA preparations (or PBS control) were administered intranasally in 2 volumes of 15 μl each (one drop/nare; 30 μl/mouse). The doses administered per mouse ranged between 3–10 μg OVA and 0.41–0.5 μg MPLA (depending on the batch of particles used) or the equivalent conjugated to NLPs. Lymph nodes (LNs, mediastinal and inguinal) were collected seven days later and processed for staining. LNs were gently crushed into a single cell suspension in 2% FBS in PBS using a tissue homogenizer tube and pestle then lysed of RBCs, rinsed, and resuspended for staining (typically $0.5–1 \times 10^6$ cells per stain).

2.6. Flow cytometry

Lymph node cells or cultured splenocytes were resuspended in FACS Buffer (2% FBS in PBS with 0.1% NaN_3) containing 0.25–0.5 μl Mouse BD Fc Block (Clone 2.4G2, BD Pharmingen, San Diego, CA) and were kept on ice throughout staining. Surface-staining antibodies were added at dilutions of 1/200–1/2000, and cells were incubated on ice for 20–30 min. Cells were washed and resuspended in 200 μl FACS Buffer for analysis on a FACSCaliber (BD Biosciences, San Jose, CA). Antibodies against cell-surface antigens were obtained from either BD Biosciences or Biolegend (San Diego, CA).

2.7. Analysis of OVA uptake

Splenocytes from wild-type C57BL/6 mice were processed and incubated with fluorescently-labeled OVA formulations as described for the activation of OT-I/II cells. After an incubation of 1 h, cells were collected and surface stained as described above. Following surface staining, cells were incubated with anti-AF488 antibody from Life Technologies (Carlsbad, CA) at 1/100 for 30 min prior to analysis by flow cytometry. For permeabilized cell experiments, cells were stained first for surface makers then fixed and permeabilized using the BD FACSPerm kit as indicated in the manufacturer's instructions. Cells were then incubated with anti-AF488 antibody in PermWash buffer for 30 min prior to analysis by flow cytometry. APCs were defined as B cells (CD19+), macrophages (CD11b+ CD11c−) or dendritic cells (CD11c+).

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