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Co-delivery of a CD4 T cell helper epitope via covalent liposome attachment with a surface-arrayed B cell target antigen fosters higher affinity antibody responses

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ABSTRACT

Liposomal vaccines incorporating adjuvant and CD4 T cell helper peptides enhance antibody responses against weakly immunogenic B cell epitopes such as found in the membrane proximal external region (MPER) of the HIV-1 gp41 subunit. While the inclusion of exogenous helper peptides in vaccine formulations facilitates stronger and more durable antibody responses, the helper peptide incorporation strategy per se may influence the overall magnitude and quality of B cell target antigen immunogenicity. Both variability in individual peptide encapsulation as well as the potential for liposome surface-associated helper peptides to misdirect the humoral response are potential parameters impacting outcome. In this study, we used MPER/liposome vaccines as a model system to examine how the mode of the potent LACK T helper peptide formulation modulates antibody responses against the MPER antigen. We directly compared liposome surface-arrayed palmitoyl LACK (pLACK) versus soluble LACK (sLACK) encapsulated in the liposomes and free in solution. Independent of LACK formulation methods, dendritic cell activation and LACK presentation were equivalent *in vivo*. The frequency of MPER-specific GC B cells promoted by sLACK was higher than that stimulated by pLACK formulation, a finding associated with a significantly greater frequency of LACK-specific GC B cells induced by pLACK. While there were no significant differences in the quantity of MPER-specific serological responses, the MPER-specific antibody titer trended higher with sLACK formulated vaccines at the lower dose of LACK. However, pLACK generated relatively greater MPER-specific antibody affinities than those induced by sLACK-formulated vaccines. Overall, the results suggest that liposomal surface-associated LACK enhances immunogenicity of LACK through better engagement of LACK-specific B cells. Of note, this is not detrimental to the induction of MPER-specific immune responses; rather, the elicitation of higher affinity anti-MPER antibodies benefits from augmented help delivered via covalent linkage of the pLACK CD4 T cell epitope in conjunction with MPER/liposome presentation.

1. Introduction

Modern vaccine strategy is moving away from conventional approaches based on live-attenuated or inactivated forms of microbial pathogens in favor of more safe subunit antigens. This shift focuses the immune response on protective or highly conserved antigenic determinants, that may not elicit a potent response during natural infection or by vaccination with inactivated pathogens. Currently marketed hepatitis B and human papillomavirus vaccines are two successful examples of protein subunit vaccines [1–3]. Today, effective glycoconjugate vaccines are available for *Haemophilus influenzae* [4], pneumococcus [5,6], and the meningococcus types A, C, W, and Y [7–10]. While vaccines have been effective in protecting against pathogens with a low degree of antigen variability, challenges still remain with many of the important pathogens for which no effective vaccine exists including malaria, HIV-1, tuberculosis, and various bacteria [11].

Abbreviations: LLPC, Long-lived plasma cell; sLACK, liposomes packaged with free LACK peptide; pLACK, liposomes packaged with palmitoylated LACK peptide; iLN, inguinal lymph node; BM, bone marrow; DC, dendritic cell; APC, Antigen-presenting cell; ASC, Antibody-secreting cell; GC, germinal center; Tfh, follicular T helper cell; Tfr, follicular T regulatory cell.

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Unlike live-attenuated or inactivated vaccines, which are direct mimics of the natural immunity induced by the pathogens, subunit antigens alone are poorly immunogenic, requiring immunostimulatory molecules to elicit robust humoral and cell-mediated immunity. To elicit long-term humoral immunity, antigenic polysaccharide and peptide vaccines require formulations with MHCII-presented epitopes to engage $CD4^+$ helper T cells for induction of robust, high affinity antibody responses [12–14]. Therefore, protein conjugation or particulate systems along with new technology have been pursued as a means to mediate efficient delivery and activation of innate and adaptive immune cells, shaping the magnitude and quality of the humoral immune response [15,16]. In that regard, the biophysical properties of vaccine formulation are important determinants of antigen-specific antibody responses affecting the quantity and the quality of immune responses. Immunogenicity of polysaccharide antigens was suppressed by vaccination with multiple glycoconjugate antigens, or by pre-existing immunity against the same carrier due to immunodominance directed to carrier protein [17,18]. Further, antigen conjugation strategies or encapsulation methods in particulate vaccine formulations have been reported to influence the outcome of immunogenicity of target antigen [19–21], suggesting the importance of understanding immunological responses influenced by vaccine formulation.

In HIV-1 envelope protein subunit vaccine, immunodominance directed to non-neutralizing epitopes shifts the immune response away from neutralizing antigenic determinants, resulting in suboptimal induction of desirable epitope-specific antibody responses [22]. The membrane proximal external region (MPER) of the HIV-1 gp41 subunit is an attractive vaccine target due to its highly conserved linear sequence targeted by broadly neutralizing antibodies (bNAbs) [23,24]. MPER is poorly immunogenic during natural infection or by vaccination in the context of envelope protein gp160. While administration of MPER peptides mixed with adjuvant and CD4 T cell helper peptides generated minimal antibody responses, the MPER peptides anchored to the surface of liposome vaccine augmented MPER-specific antibody titers [25,26]. Although incorporation of CD4 T cell helper peptides are required to induce antigen-specific long-lived plasma cells (LLPCs) and memory B cell responses to peptide-liposome vaccine, the encapsulation efficiency of helper peptides could be suboptimal depending on the physicochemical properties of the peptide sequence and lipid composition [27–29]. In addition, helper peptides often contain B cell antigen determinants that extraneously compete with target B cell epitopes for help provided by CD4 T follicular helper (Tfh) cells during germinal center (GC) reactions creating a potential epitopic hierarchy. Studies showed that the size and quality of the GC response are directed by Tfh cells which provide growth and differentiation signals to GC B cells and mediate positive selection of high-affinity B cell clones in the GC, thus playing a central role in the production of long-lasting humoral immunity [30–33].

Utilizing a linear neutralizing epitope targeted MPER peptide/liposome vaccine as a model system, we aimed to define the B cell response competition and interplay following immunization with MPER/liposomes packaged with sLACK for encapsulation and pLACK for covalent linkage of LACK to the liposome surface. The $LACK_{156\text{-}173}$ peptide, a well characterized immunodominant CD4 T cell epitope presented by the $I\text{-}A^d$ (MHC class II) molecule, was derived from the *Leishmania major* RACK-like homolog of the WD protein family [34]. While the magnitude of MPER-specific serological antibody responses is independent of LACK formulation per se, higher affinity antibody induction facilitated by pLACK compared to sLACK suggests that the elicitation of high affinity protective antibody may benefit from co-delivery of lipid-anchored helper peptides with B cell antigen derived from pathogens with a high mutation rate.

2. Materials and methods

2.1. Animal care and use

All animal procedures were performed according to protocols approved by the Dana-Farber Cancer Institute and Harvard Medical School Animal Care and Use Committee Institutional Review Board. 8–10 week old naïve, wild type, female BALB/c mice were purchased from Taconic Biosciences (Hudson, NY, BALB/cAnNTac) and maintained in a specific pathogen-free facility at Dana-Farber Cancer Institute.

The following primary mouse samples were obtained: blood via tail vein puncture, inguinal lymph nodes (iLNs), spleens, and bone marrow (BM). Single-cell suspensions of the combined iLNs were generated by mashing lymph nodes through a 70 μm strainer into FACS buffer (0.5% BSA 2 mM EDTA PBS). Splenocytes were similarly mashed through a strainer; however, followed by a red blood cell lysis step before being resuspended in FACS buffer. BM was collected from the combined femurs and tibias by removing the ends of the bones and flushing the cells out with PBS. BM red blood cells were further lysed and the cells were resuspended in FACS buffer. Sera was collected from tail vein by isolation of ~50 μl blood from gently-warmed (under a heat lamp) mice. Blood was maintained at room temperature and was allowed to coagulate. Serum was then isolated by centrifugation for 5 min in a microcentrifuge at high speed. Supernatant was collected and stored at −20 °C until assayed.

2.2. Liposomes and peptides

MPER/liposomes were prepared as described previously [35]. In brief, the following components were mixed: MPER peptide, monophosphoryl lipid A (MPLA), 1,2-dioleoyl-sn-glycero-3-phos phocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids, Alabaster, AL) with or without N-terminally palmitoylated-LACK (pLACK) for the pLACK formulated MPER/liposome preparation. For free LACK (sLACK) formulated MPER/liposomes, organic solvents were fully evaporated and the following day the liposomes were rehydrated in PBS with the addition of sLACK. In addition to the sLACK and pLACK formulations above some liposomes were formulated with sLACK added following extrusion (post-extrusion) to ensure no encapsulation. For ELISA and calcium flux assays, liposomes consisted of 1:50 or 1:1000 palmitoylated peptide in DOPC: DOPG (4:1) lipids with 0.2% biotinylated polyethylene glycol (PEG) 2000. ELISPOT liposomes were formulated identically with exclusion of the PEG biotin. For fluorescent liposomes a peptide:lipid ratio of 1:200 was used with 4:1 DOPC:DOPG and either 1% biotin-polyethylene glycol-DSPE or 1% carboxyfluorescein-DOPE (all lipid reagents from Avanti Polar Lipids; Alabaster, AL) along with 3% or 4% polyethylene glycol (2000)-DOPE, respectively.

As described by others the LACK ($LACK_{156\text{–}173}$) sequence was (ICFSPSLEHPIVVSGSWD) [36]. The MPER peptide was an N-terminally palmitoylated $MPER_{662\text{-}683}$ peptide (ELDK-WASLWNWFNITNWLWYIK) synthesized at the Massachusetts Institute of Technology Biopolymers and Proteomics Core Facility (Boston, MA). For immunization studies, mice (5 mice per group) were administered with pLACK or sLACK formulated MPER/liposome vaccine (50 μl/injection, 2.52 mg of total immunization liposomes per mouse) intradermally at day 0 and again at day 30. MPER/liposomes for immunization were formulated as above and injected into mice to deliver palm-MPER at 1:200 with lipid, 17.5 μg of MPLA, and 40 μg of LACK if not noted otherwise.

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