Development of Interbilayer-Crosslinked Multilamellar Vesicles as a Potent Vaccine Platform

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Statement of Purpose: Currently licensed vaccine adjuvants promote immunity by primarily eliciting humoral immune responses, whereas the cellular arm of adaptive immunity is largely unaffected by these adjuvants. As strong cellular $CD8^+$ T cell responses may be required for vaccines against cancer or intracellular pathogens such as malaria, there is great interest in technologies to promote concerted humoral and cellular immune responses. In this work, we report development of novel interbilayer-crosslinked multilamellar vesicles (ICMVs) as antigen and adjuvant delivery vehicles that can promote exceptionally robust humoral and cellular immune responses *in vivo*.

Methods: In order to synthesize ICMVs, liposomes containing 50% maleimide-functionalized lipids were first formed by hydrating lipid films in the presence of cargo molecules. Liposomes were sonicated and induced to undergo fusion by addition of divalent cations such as Mg^{2+} and Ca^{2+}. The resulting multilamellar vesicles (MLVs) were incubated with dithiothreitol to conjugate apposing layers of maleimide-functionalized lipids and form crosslinked ICMVs. The particles were characterized with dynamic light scattering (DLS) and cryo-TEM. For encapsulation studies, ovalbumin (ova) modified with alexa-fluor 555 was used to quantify the amount encapsulated. Release of ova from these particles was assayed using dialysis membranes with MW cutoff of 100 kDa. For *in vitro* dendritic cell (DC) activation studies, splenic DCs were incubated with ICMVs encapsulating ova and a Toll-like receptor 4 agonist, monophorsphoryl lipid A (MPLA). CD8+ T cells from OT-1 transgenic mouse were co-cultured to assess cross-priming of antigen by DCs to T cells. Cells were analyzed by flow cytometry to examine the extent of DC activation and CD8+ T cell proliferation. Groups of C57Bl/6 mice were immunized s.c. in the tail base with ova and MPLA formulated as liposome, MLVs or ICMVs. Frequencies of ova-specific T-cells elicited by immunization were determined by flow cytometry analysis of peripheral blood mononuclear cells. Ova-specific antibody titers were measured with ELISA.

Results: ICMVs were synthesized by fusing unilamellar vesicles containing maleimide headgroup-functionalized lipids into MLVs by addition of divalent cations, followed by crosslinking of adjacent lipid layers with dithiols. The resulting particles had typical mean diameters of 250±40 nm with low polydispersity. CryoEM images taken of the particles revealed multilamellar structures comprised of electron dense bands thicker than single lipid bilayers, suggesting multilamellarity of the particles (Figure 1A). Using ova as a model protein antigen, we achieved superior encapsulation efficiency with ICMVs compared to traditional liposomes or PLGA particles (108- and 4-fold increases, respectively). ICMVs showed a significantly enhanced retention of protein, releasing only ~25% of their cargo by 7 d, and ~90% after d30, whereas liposomes and uncrosslinked MLVs quickly released their cargo within 4 d and 10 d respectively (Figure 1B).

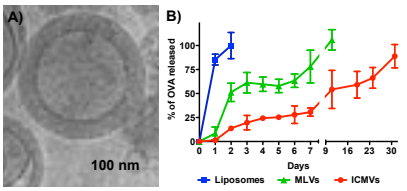

Figure 1. A) Cryo-EM image of ICMV particle. B) Release of ova in RPMI + 10% fetal bovine serum.

We next examined ICMVs as a platform for vaccine delivery. ICMVs loaded with ova and MPLA activated DCs *in vitro*, upregulating their expression levels of costimulatory molecules (e.g. CD40, CD80, CD86, and MHC II). Cross-priming of ova-specific CD8+ T cells was enhanced by DCs pulsed with ova-loaded ICMVs, compared to soluble formulations. To determine efficacy of ICMVs as vaccines *in vivo*, we vaccinated C57Bl/6 mice with equivalent doses of OVA, MPLA, and lipids (10 µg, 0.1 µg, and 142 µg, respectively) in the form of PEGylated unilamellar liposomes, MLVs, or ICMVs. On d7, there was a trend toward increasing frequency of antigen-specific CD8+ T-cells in blood in the order soluble ova< liposomes < MLVs < ICMVs (Figure 2A). At 3 weeks post-immunization, ICMV immunization generated a substantially stronger humoral response, ~1000-fold and ~10-fold greater than the soluble ova and non-crosslinked MLV immunizations, respectively (Fig. 2B). Furthermore, two additional boost immunizations with ICMVs elicited dramatically stronger $CD8^+$ T-cell responses, achieving a 14-fold greater frequency of $tetramer^+$ T-cells in the $CD8^+$ T-cell population compared to soluble OVA+MPLA by d41 (Figure 2C).

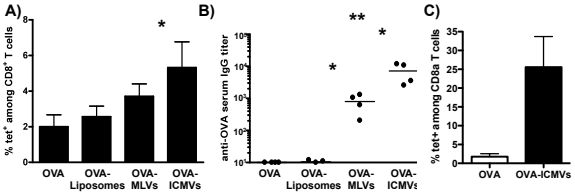

Figure 2. A) Frequency of ova-specific CD8+ T cells on d7 from a single immunization. B) Anti-ova serum IgG measured on d21 sera. C) Immunization regimen spanning 35 days (d0 prime, d21 boost, and d35 boost) resulted in dramatically enhanced T-cell responses in ICMV-immunized mice on d41.

Conclusions: In this study, we developed novel multilamellar lipid particles and demonstrated their versatility as a platform for vaccine delivery. These results suggest ICMVs as an effective vehicle for delivering antigens and adjuvants for immunizations.

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