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Caveolin-mediated endocytosis of the *Chlamydia* M278 outer membrane peptide encapsulated in poly(lactic acid)-Poly(ethylene glycol) nanoparticles by mouse primary dendritic cells enhances specific immune effectors mediated by MHC class II and $CD4^+$ T cells

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Responses to Reviewers' Comments

Reviewers' comments:

Reviewer #1: In the article "Caveolin-mediated endocytosis of the Chlamydia M278 Outer Membrane Peptide encapsulated in poly(lactic acid)-poly(ethylene glycol) nanoparticles by mouse primary dendritic cells enhances specific immune effectors mediated by MHC Class II and CD4+ T cells," the authors examined the possible mechanisms of a previously developed nanoparticle formulation in mediating anti-Chlamydial responses. The efforts and a wide range of techniques, including flow cytometry, quantitative PCR and immunofluorescence microscopy employed in this study are deeply appreciated. While the formulation, which consists only of peptide-loaded PLGA nanoparticles, offers little novelty, the extensiveness of the work could be of value of to the field of biomaterials development. However, there are several issues in this manuscript that require further clarifications.

We appreciate the reviewer's comment in helping to make our manuscript stronger and relevant to the field. Our laboratory currently employs both PLGA and PEGylated-PLA as delivery systems for development of chlamydial nanovaccines. However, in this manuscript, we are using PEGylated-PLA instead of PLGA to encapsulate the M278 peptide because it has a slower degradation rate than PLGA. This feature is very attractive for prolonged antigen presentation and delivery of nanovaccine candidates.

1. The authors observed correlations between PPM stimulation and upregulation of markers, such as Cd1d2, Fcgr1, Tlr2, Nod, and Cav1, and suggested that these molecules play a part in the capturing PLA vaccine. However, correlation does not necessarily mean causation, so that increased expressions of certain markers cannot be used as evidence for their interaction with nanoparticles. It is of particular interest to further dissect the role of Cav1 using Cav1 inhibitor, as this would be a more definite proof showing that the nanoparticle uptake is mediated by the Cav1-dependent mechanism. Instead of directly showing filipin III-inhibited nanoparticle capture using quantitative assays or immunofluorescence imaging, the data of marginally downregulated cytokines and costimulatory molecules are rather indirect and unconvincing. Moreover, the inhibition of Cav1 by filipin III primarily relies on its effect on caveolae disruption, therefore it should have nothing to do with the Cav1 expression level shown in Fig. 8C.

Response: There are not many inhibitors for Cav1 that act directly on it. Mechanistically, "filipin-III binds specifically to cholesterol in the caveolae structures and in so doing disrupts the integrity of caveolae and uptake through caveolae" (Voigt et al, 2014). Thus, we focused on filipin III that is widely used and the methods from the recent PNAS paper" *Voigt J, Christensen J, Shastri VP. Differential uptake of nanoparticles by endothelial cells through polyelectrolytes with affinity for caveolae. Proc Natl Acad Sci U S A. 111 (2014) 2942-2947*. Of importance to mention is that it is impossible to eliminate basal levels of Cav1 since it is expressed in DCs and high dose of filipin III is toxic to cells. Thus, to observe drastic suppression in the expression of cytokines and receptors will be suicidal for cells. We tried different scenarios to avoid toxicity and the best one, was when DCs were only pretreated for 30 min with filipin III and removed by washing prior to stimulation for 24 with PPM, M278 and PPP to avoid toxicity.