LETTER TO THE EDITOR

Polyvalent mRNA vaccination elicited potent immune response to monkeypox virus surface antigens

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Dear Editor,

The global monkeypox (Mpox) outbreak leads to a surge in demand for vaccine. The Mpox vaccine in short supply is JYNNEOS (modified vaccinia ankara, MVA) due to less side effects, while ACAM2000 supply is abundant because of the strategic stockpile in U.S.[1] The ACAM2000 replaced the first-generation smallpox vaccine Dryvax that has a questionable safety profile. However, the ACAM2000 is associated with high rate of myocarditis and pericarditis,[1] and can cause severe illness in immunocompromised patients, foreshadowing the need to update the strategic stockpile.

The mRNA vaccine technology has catalyzed the tremendous success of COVID mRNA vaccine and can be adopted to develop mRNA vaccines against SARS-CoV-2-evolving variants[2–4] and other infectious diseases, including Mpox. The need for strategic stockpiling to prevent future outbreak, and adaptability of mRNA vaccine prompt the idea of developing Mpox-targeting mRNA vaccine. A number of important questions await to answer in the development of a monkeypox virus (MPXV)-based mRNA vaccine. For example, how different are the MPXV and MVA antigens? Could an mRNA vaccine against MPXV elicit potent antibody response? Would an mRNA vaccine encoding these antigens be generally safe?

To answer these questions, we designed a polyvalent mRNA vaccine candidate, MPXVac-097, against 2022 Mpox in USA and characterized its immunogenicity in mice. Five MPXV antigens, A29L, E8L, M1R, A35R and B6R, were linked in tandem by 2A peptides and codon optimized. Their vaccinia homologs A27L,[5] D8L,[5,6] L1R,[7] A33R[8] and B5R[9] have been identified as targets of neutralizing antibodies. Their alignment with MVA homologs showed over 90% sequence identity (Supplementary information, Tables S1, S2), and revealed dozens of viral species-specific residues (Fig. 1a; Supplementary information, Figs. S1, S2), some of which are located on neutralizing antibody interface[6,8] and are speculated to decrease antibody affinity. The MPXV mRNA vaccine adopts the circulating MPXV antigens and avoids residue mismatch of MVA with MPXV.

To test whether MPXVac-097 could express proteins, a GFP reporter was appended to the end of MPXVac-097 (Supplementary information, Fig. S3). The GFP expression in 293T cells suggests successful translation of all residues in MPXVac-097. Confocal imaging was performed to visualize localization of MPXV antigens with C-term mScarlet, of which expression was confirmed by flow cytometry (Fig. 1b; Supplementary information, Figs. S4, S5). The SARS-CoV-2 BA.5 spike as a control was primarily localized on plasma membrane. A35R, B6R and E8L were localized to plasma membrane, perinuclear structures and endomembrane. M1R was enriched in plasma membrane or inclusion body-like structures. A29L that lacks transmembrane domain, was predominantly in the cytosol. To enable quantification of surface antigen using the same antibody with high specificity, a Flag tag was added to the extracellular terminus of each MPXV antigen that was either expressed alone or co-expressed in MPXVac-097 (Fig. 1c; Supplementary information, Figs. S6–S8). For example, the Flag tag is added to the C-terminus of A35R and denoted as A35R Flag, as the C-term Flag is at extracellular side (Fig. 1a). Cells expressing MPXV antigens were either surface stained or fixed/permeabilized then stained with PE anti-Flag antibody. A35R and E8L showed a surface positive rate closest to BA.5 spike, while A29L's surface positive rate is minimal. Staining after fixation/permeabilization revealed significant expression of all MPXV antigens including A29L. When compared with single antigen expression, tandem co-expression in MPXVac-097 significantly increased surface display of four MPXV antigens except for A29L (Supplementary information, Fig. S7). The effect of tandem expression and surface display pattern of MPXV antigens were recapitulated by dual staining of surface and total Flag antigens expressed alone or in MPXVac-097 (Supplementary information, Fig. S8). The confocal and flow cytometry data showed cellular expression and localization of five MPXV antigens.

After confirming antigen expression, we advance the MPXVac-097 to lipid nanoparticle (LNP) formulation and characterize immunogenicity of MPXVac-097 LNP mRNA in mice. The size distribution of MPXVac-097 LNP mRNA was determined by dynamic light scattering, which showed a monodispersed distribution and an average radius of 49 nm (Supplementary information, Fig. S9). Mice were immunized with four doses of 8 μg MPXVac-097 LNP mRNA on days 0, 14, 28, 126. The retro-orbital blood was collected on days 0, 14, 20, 28, 42, 126, 140 (Fig. 1d; Supplementary information, Fig. S10). After the second dose, antibody titers of A35R and E8L significantly increased in all mice. Interestingly, the antibody titer against M1R, that had no change post prime, started to increase moderately post boost in one mouse. Minimal antibody response was detected for either A29L or B6R on day 42. Next, we sought to ask whether plasma antibodies elicited by MPXVac-097 can recognize the antigens expressed on cell surface. The five MPXV antigens were separately expressed in 293T cells and surface antigens were detected using plasma antibody from day 20 mice by flow cytometry (Supplementary information, Fig. S11). Expression of A35R and E8L led to an increase of 293T cells that were bound to the mouse plasma antibodies, consistent with findings in ELISA.

To characterize MPXV antibody titer change over time, we collected mouse plasma on day 126. After ~3 months, anti-E8L and anti-A35R antibody titers decreased by 40% compared to titers on day 42 (Fig. 1d). To evaluate response to re-immunization after longer periods of time, a fourth dose was given to the mice on day 126 and plasma samples were collected after two weeks. A significant titer increase (~9 fold) was found in antibodies against A35R, while the increase in E8L, A29L and M1R antibodies was

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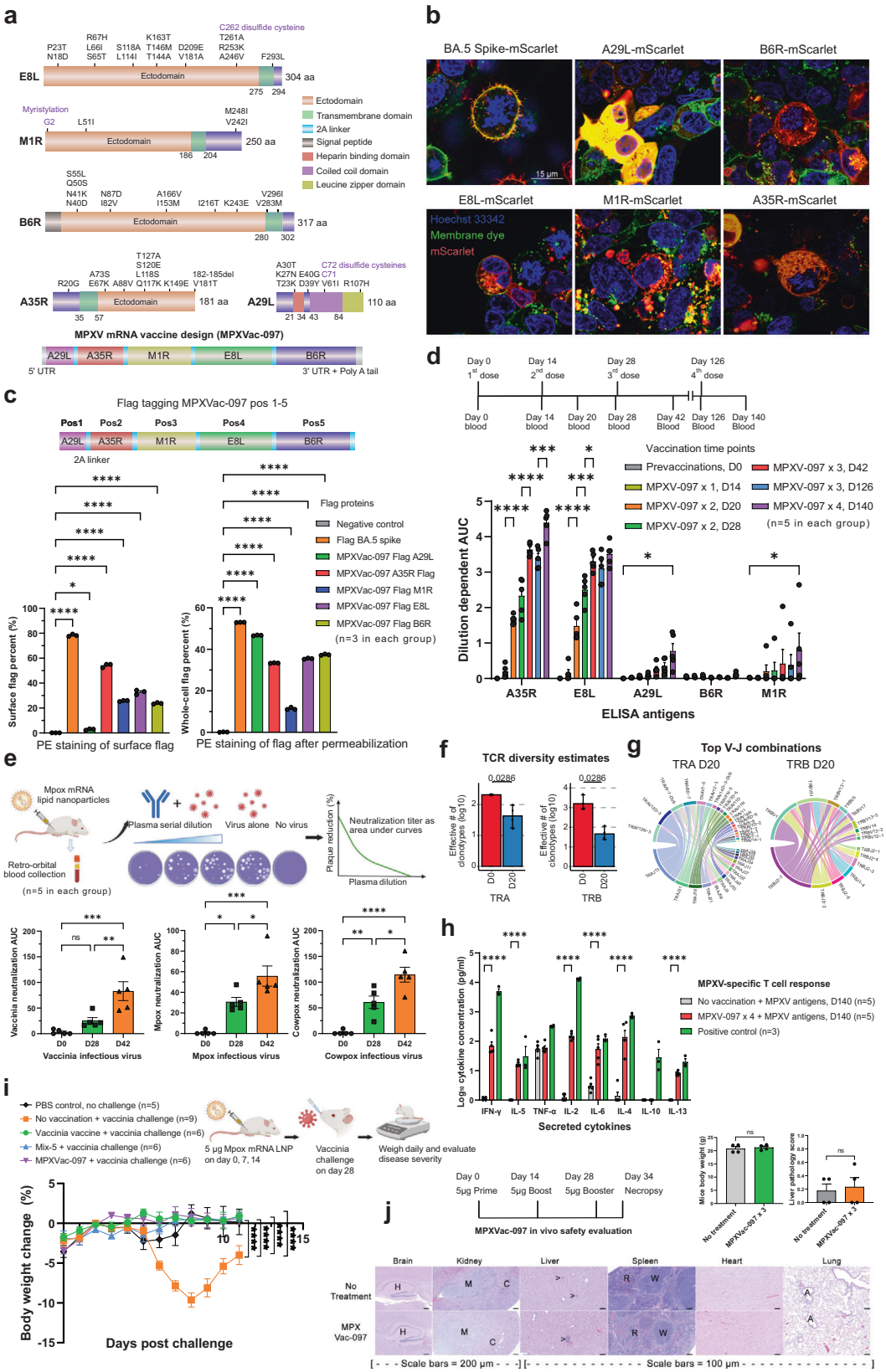

modest (Fig. 1d). This re-immunization likely reveals MPXVac-097-achievable plateaus of antibody titers against A35R and E8L.

Next, we determined plasma neutralization activity using half-maximal plaque reduction neutralization assays (PRNT50) (Fig. 1e). Plasma from mice vaccinated with MPXVac-097 exhibited neutralizing activity against cowpox, vaccinia virus and MPXV 2022, in a dose number-dependent fashion. These data provide evidence that MPXVac-097 immunization induces a neutralizing antibody response in mice.

Fig. 1 MPXVac-097 elicits potent antibody response, T cell response and protection against vaccinia virus challenge in mice. a Five neutralizing targets of monkeypox mRNA vaccine candidate are connected by 2A linkers and translated from the same mRNA transcript, MPXVac-097. Antigen sequence difference between MPXV and MVA is shown. **b** 293T live cells expressing mScarlet (red)-tagged MPXV antigens were co-stained with CellBrite-Green membrane dye (green) and Hoechst 33342 (blue) under confocal microscope. **c** Flow cytometry of surface-stained 293T cells or 293T cells stained after fixation/permeabilization ($n = 3$). These cells co-expressed MPXV antigens in tandem tagged by Flag at extracellular end of each gene position in MPXVac-097 (one position only). Flag was added after the signal peptide if any, and its N-term or C-term position was indicated by its appearance before or after the antigen, respectively. The Flag was added to N-terminus of BA.5 spike after its signal peptide. **d** After being immunized with MPXVac-097 LNP-mRNA, mice showed significantly increased antibody titers against A35R and E8L ($n = 5$). Comparisons were made between adjacent time points or day 0 vs day 140. The dose of mRNA is 8 μg. **e** MPXVac-097 LNP-mRNA elevated MPXV, vaccinia virus and cowpox neutralizing antibody titers ($n = 5$). **f** Bar plots of the estimated TCR diversity in TRA and TRB repertoires between pre-vaccination (D0) and MPXV post-boost (D20) mice. **g** Replicate-pooled circos plots of the top 20 TRA and TRB V–J gene combinations for MPXVac-097-vaccinated mice on day 20. **h** Secreted cytokine quantification to assess functional T cell response to MPXV antigen stimulation in mice with or without MPXVac-097 vaccination on day 140. PBMCs stimulated by eBioscience™ stimulation cocktail as positive controls were not included in comparison. **i** MPXVac-097 and Mix-5 vaccination offered protection against vaccinia virus challenge. **j** Pathological analysis of mice with and without vaccination of 5 μg MPXVac-097 ($n = 4$). H, hippocampus; >, foci of hepatic EMH; C, cortex; M, medulla; R, red pulp; W, white pulp; A, airway. Data on bar plots are shown as means ± SEM with individual data points in plots. All micrographs are at the same magnification. Statistics information is described in Supplementary methods. Illustrations in Fig. 1e and i are created with Biorender.

A natural question that follows is whether T cell response is induced in these animals. To address this question, we performed bulk TCR sequencing of peripheral blood mononuclear cells (PBMCs), which revealed V–J combination and clonality maps of TCRs from mice at baseline and 20 days post MPXVac-097 vaccinations (Fig. 1f, g; Supplementary information, Figs. S12–S14). The clonal diversity of samples post boost was significantly reduced as compared to vaccine-naive animals (Fig. 1f). MPXVac-097 vaccination resulted in increased use of *TRAJ13, TRAV12N-3, TRAV12D-3* in α chain and *TRBJ1-4, TRBJ2-2, TRBV1* in β chain (Fig. 1g). There was significant reduction of low-frequency clones and increase in hyperexpanded clones (Supplementary information, Figs. S13c, S14d), indicating clonal expansion after vaccination.

To validate whether induced T cell response is MPXV-specific, PBMCs were collected on day 140 and stimulated with five MPXV antigens. After 16 h of stimulation, culture media were collected for quantification of Th1 and Th2 type cytokines. The MPXVac-097 vaccination prompts the secretion of Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5 and IL-13) cytokines that are 10–100-fold higher than those of vaccination-naive mice (Fig. 1h; Supplementary information, Fig. S15).

Next, we sought to compare immunogenicity of MPXVac-097 with simple mix of five individual LNP mRNAs each encoding an MPXV antigen (Mix-5). We are also interested in testing whether a shortened vaccination schedule induces potent binding and neutralizing antibodies. Mice were immunized with 5 μg MPXVac-097, 5 μg Mix-5 (1 μg single antigen LNP mRNA x 5 antigens) or 1 μg single antigen LNP mRNA on days 0, 7 and 14. We observed that all three types of LNP-mRNAs elicited potent antibody response to A35R and E8L (Supplementary information, Figs. S16, S17), of which final titers were comparable between vaccination groups and to those in 42-day vaccination (Fig. 1d). These data suggest that MPXVac-097 and Mix-5 led to similar binding antibody responses. For A29L and B6R antigen ELISA panels, only single antigen LNP-mRNAs, but not MPXVac-097 or Mix-5, induced modest antibody titers on day 28 (Supplementary information, Fig. S17a). Of note, three doses of MPXVac-097, but not Mix-5 or single antigen LNP mRNA, led to moderate antibody response to M1R. Compared to non-vaccinated control, increased neutralizing antibody titers against vaccinia virus were observed in mice 28 days post vaccination of Mix-5 or MPXVac-097 (Supplementary information, Fig. S17b).

Next, we evaluated whether polyvalent Mpox LNP mRNAs could provide protection against vaccinia virus challenge. Mice were intramuscularly immunized with 5 μg MPXVac-097 or Mix-5 LNP mRNA on days 0, 7 and 14 followed by intranasal vaccinia virus challenge on day 28 (day 0 post challenge). Mice subcutaneously immunized with a low dose of vaccinia virus were used as vaccination positive controls. After viral challenge, unvaccinated mice had significant body weight loss (Fig. 1i), which started 3 days post challenge and reached maximum loss on day 8 before gradual recovery. Importantly, following challenge, all vaccinated groups had no significant change in body weight as compared to no-challenge control. Animals were scored according to criterion of disease severity (details in Supplementary methods). Unvaccinated, virally challenged mice all showed higher clinical scores by day 6 (Supplementary information, Fig. S17c). Mild symptoms were observed for MPXVac-097-, Mix-5- or vaccinia virus-vaccinated mice. In conclusion, both MPXVac-097 and Mix-5 mRNA vaccines provided protection against vaccinia virus challenge.

In addition to vaccine efficacy, we investigated MPXVac-097 safety. A new batch of mice vaccinated with MPXVac-097 were necropsied for a safety evaluation. All vaccinated mice show no gross toxicity-related appearance with 3× weekly monitoring. Mice were weighed prior to euthanasia and there were no significant differences in body weight by treatment (Fig. 1j). These mice were submitted blind to treatment for comprehensive histology and pathology review. There were no significant gross or microscopic changes in any organs between MPXVac-097 and control groups. As extramedullary hematopoiesis (EMH) was found in liver, the liver microscopic findings were translated to a pathology score, which was determined to be insignificant after the treatment groups were decoded. The analysis of complete blood count and plasma liver enzyme reveal no difference between treatment groups (Supplementary information, Fig. S18), and these data are within normal limits.[10] Pathologist review of all data suggested that, MPXVac-097 vaccination in mice is well-tolerated and does not result in any significant pathologic changes.

In summary, our study compared immunogenicity of monovalent and multivalent Mpox mRNA vaccine candidates, including MPXVac-097, Mix-5 and single antigen LNP mRNAs. MPXVac-097 elicits broad neutralizing antibodies, MPXV-specific T cell response and protection against vaccinia virus challenge. The immunogenicity and efficacy of MPXVac-097 are comparable to Mix-5. Given its simpler production procedure, antigen tandem co-expression remains an attractive direction. MPXVac-097 vaccination did not cause significant pathologic changes in mice. Together, these data provided evidence for developability of a multivalent MPXV mRNA vaccine.

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DATA AVAILABILITY

All data including source data and statistics in this study are included in main text or Supplementary information, Data S1. NGS data has been deposited to GEO (GSE225183). Other materials and custom codes are available via reasonable requests from the corresponding authors.

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AUTHOR CONTRIBUTIONS

S.C. and Z.F. conceptualized and designed the study. Z.F., V.S.M. and C.L. performed most of experiments and analyzed the data. P.A.R., X.S., and K.S. assisted data analysis. X.L. and M.B. assisted experiments. Y.X. and A.L. provided scientific support. C.J.B. performed mouse necropsy and histopathologic analysis. S.C. and C.L. supervised the study. Z.F., P.A.R., C.J.B., C.L. and S.C. wrote the manuscript with feedback from all authors.

COMPETING INTERESTS

A patent has been filed related to this study by Yale University. S.C. is a founder of EvolveImmune, Cellinfinity, Chen Consulting, and Chen Tech, all unrelated to this study.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41422-023-00792-5.

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