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Multi-valent mRNA vaccines against monkeypox enveloped or mature viron surface antigens demonstrate robust immune response and neutralizing activity

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Monkeypox was declared a global health emergency by the World Health Organization, and as of March 2023, 86,000 confirmed cases and 111 deaths across 110 countries have been reported. Its causal agent, monkeypox virus (MPV) belongs to a large family of double-stranded DNA viruses, *Orthopoxviridae*, that also includes vaccinia virus (VACV) and others. MPV produces two distinct forms of viral particles during its replication cycles: the enveloped viron (EV) that is released via exocytosis, and the mature viron (MV) that is discharged through lysis of host cells. This study was designed to develop multi-valent mRNA vaccines against monkeypox EV and MV surface proteins, and examine their efficacy and mechanism of action. Four mRNA vaccines were produced with different combinations of surface proteins from EV (A35R and B6R), MV (A29L, E8L, H3L and M1R), or EV and MV, and were administered in Balb/c mice to assess their immunogenicity potentials. A dynamic immune response was observed as soon as seven days after initial immunization, while a strong IgG response to all immunogens was detected with ELISA after two vaccinations. The higher number of immunogens contributed to a more robust total IgG response and correlating neutralizing activity against VACV, indicating the additive potential of each immunogen in generating immune response and nullifying VACV infection. Further, the mRNA vaccines elicited an antigen-specific CD4$^+$ T cell response that is biased towards Th1. The mRNA vaccines with different combinations of EV and MV surface antigens protected a mouse model from a lethal dose VACV challenge, with the EV and MV antigens-combined vaccine offering the strongest protection. These findings provide insight into the protective mechanism of multi-valent mRNA vaccines against MPV, and also the foundation for further development of effective and safe mRNA vaccines for enhanced protection against monkeypox virus outbreak.

monkeypox virus, enveloped and mature viron, multi-valent mRNA vaccines, immune response, neutralizing antibody

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INTRODUCTION

Monkeypox virus (MPV) which was first identified in non-human primates in Africa in the 1950s, can cause smallpox-like disease in humans (Bunge et al., 2022; Magnus et al., 1959). Since the first monkeypox infection in humans was diagnosed in the Republic of Congo in the 1970s, it has spread across the African continent, mainly Central and Western Africa (Lum et al., 2022). Occasional breakouts of MPV infection were reported outside the African continent but were quickly contained due to inefficient transmission between humans (Fine et al., 1988; Huhn et al., 2005; Nolen et al., 2016). However, recent years saw an increased human-to-human transmission that lead to an escalation of monkeypox cases and spread to many countries worldwide. Monkeypox was declared a global health emergency by the World Health Organization in July 2022 (Kimball, 2022). And as of March 2023, more than 86,000 confirmed cases and 111 deaths in 110 countries have been reported (World Health Organization, 2023).

Monkeypox virus is a member of the *Orthopoxviridae*, a large family of double-stranded DNA viruses that also includes variola virus, vaccinia virus (VACV), cowpox virus, Akhmeta virus and Alaska pox virus (Rao et al., 2022). Phylogenetically MPV is separated into two clades: the Central African clade and the West African clade (Likos et al., 2005). Although they are highly homologous, the West African clade was found to cause the recent outbreak of MPV infection due to mutations in its immune evasion genes (Isidro et al., 2022; Weaver and Isaacs, 2008). Similar to the life cycles of many other members of the Orthopoxvirus family, MPV has two distinct forms of viral particles produced during its replication cycles: the enveloped viron (EV) and mature viron (MV) (McFadden, 2005). The former is released from infected host cells by exocytosis in the early stages of infection, while the latter is released by lysis of infected host in the late stages. Although both forms are capable of infecting new hosts, EV and MV particles have different compositions of surface proteins, thus conferring distinct immunogenicity and infectability that have not been fully characterized.

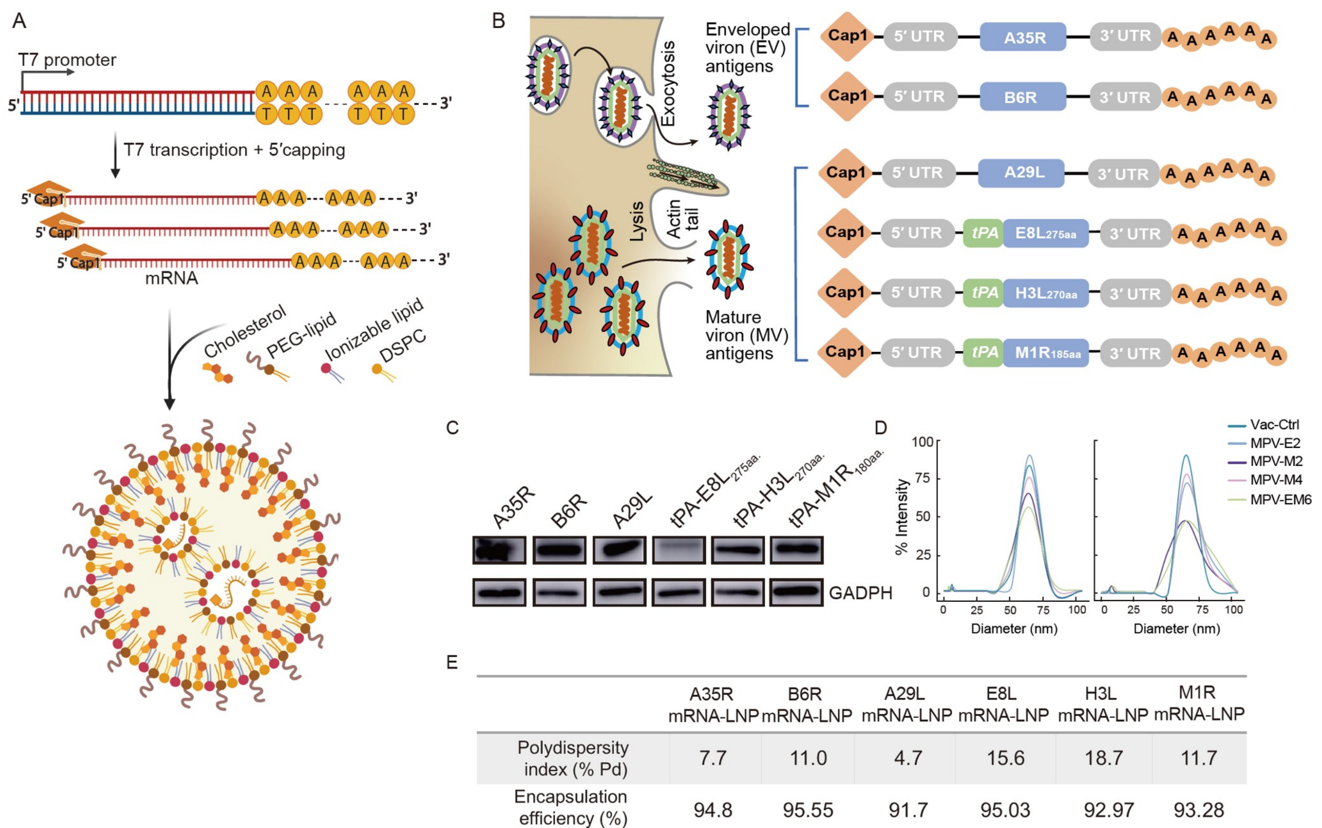
It has been reported that infection of Orthopoxvirus or immunization with its vaccines provided cross-protection against other members of the genus (Petersen et al., 2016), due to the conserved nature of protein-coding genes among the members of *Orthopoxviridae*, especially for the viral surface proteins (Fogg et al., 2004; Hooper et al., 2004). For example, the smallpox vaccine was reported to protect animals from infection by other orthopoxviruses, including MPV and VACV (Hatch et al., 2013; Hooper et al., 2004; Wyatt et al., 2004). Facing the global health emergency, the US Food and Drug Administration (FDA) approved two smallpox vaccines, the JYNNEOS vaccine (Frey et al., 2014) and ACAM2000 (Nalca and Zumbrun, 2010), for conditional use in order to prevent the spread of MPV. They are either modified live or live attenuated vaccines, which may offer only partial protection against MPV, in addition to other safety or effectiveness concerns (Thornhill et al., 2022; Zaeck et al., 2023). Breakthrough MPV infection has been observed in the population who received the first or second dose of the JYNNEOS vaccine, indicating the limited cross-protection provided by it (Hazra et al., 2022). Thus, facing the threat of MPV epidemic it is imperative and urgent to develop MPV-specific vaccines to understand their efficacy and mechanism, and alleviate associated safety concerns.

Vaccines are important public health tools offering protection against a variety of infectious pathogens (Li et al., 2023). During the COVID-19 pandemic, the mRNA vaccine technology was widely applied, demonstrating its effectiveness and reliability with a swift response, short production cycle, and excellent safety profile (Fang et al., 2022; Pardi et al., 2018). Development of mRNA vaccines targeting MPV is the primary choice here, but also faces a number of challenges. Unlike SARS-CoV-2 with a membrane-bound spike protein as a main target for neutralizing antibodies due to its unique function in receptor recognition and cell membrane fusion (Xu et al., 2020), MPV has more than 20 membrane-bound proteins, and two distinct forms of viron particles, both capable of infecting host cells (McFadden, 2005). A great deal of knowledge about their immunogenicity was obtained based on studies of other orthopoxvirus members (Golden et al., 2008; Hooper et al., 2003; Sakhatskyy et al., 2006). In the current study, we first selected six MPV surface proteins, two (A35R and B6R) from EV and four (A29L, E8L, H3L, and M1R) from MV, based on the data of previous orthopoxvirus studies, and assessment of the immunogenicity potential of these surface proteins using *in silico* computation approach. We then designed four different combinations of mRNA vaccines with EV-specific, MV-specific, or EV and MV surface antigens, to investigate the efficacy and safety of multi-valent mRNA vaccines. Our study showed the robust immune response to the multi-valent mRNA vaccines by activating antigen-specific T cells in vaccinated mice. Further, the multi-valent mRNA vaccines with different combinations of surface antigens can effectively protect a mouse model from a lethal dose of VACV challenge. While this manuscript was under review, other studies reported that mRNA vaccines using four or five MPV surface proteins induced immune response and neutralizing antibodies in mice (Fang et al., 2023; Zhang et al., 2023). Our current study provided detailed analysis of a wider range of MPV surface antigens and their immune response dynamics *in vivo*, and dissection of the protective effects with EV-only, MV-only, or EV and MV combined immunogens in a mouse model challenged with a lethal dose of VACV.

RESULTS

Design and production of mRNA vaccines against monkeypox EV- or MV-surface antigens

In the initial design, we sought to construct mRNA vaccines with EV-surface antigens, MV-surface antigens, or a combination of both EV- and MV-surface antigens. Based on the following analysis, we selected and synthesized mRNAs for two EV-antigens: A35R and B6R, and four MV-antigens: A29L, E8L, H3L and M1R (Figure 1A and B). Due to a lack of information for MPV surface proteins regarding their immunogenicity, we performed analyses based on previous studies on orthopoxvirus surface immunogens that are homologous to the MPV-surface proteins. Proteome investigations of serological response in human recipients of vaccinia or smallpox showed that the homologs to the six MPV-surface proteins induced predominate IgG response (Davies et al., 2007). A recent survey of humoral immune responses in 1,037 smallpox vaccine recipients indicated positive responses to the homologs of the six MPV-surface proteins in more than 50% of human subjects (Kennedy et al., 2022). With the help of *in silico* computational tools, we further evaluated the binding affinity of potential immune epitopes from the six selected MPV-surface proteins: A35R, B6R, A29L, E8L, H3L and M1R, to human high frequent MHC I and the MHC II molecules in the Allele Frequency Net Database (Gonzalez-Galarza et al., 2020) using NetMHCpan and NetMHCIIpan (Reynisson et al., 2020). The six MPV-surface antigens were also evaluated for B-cell receptor binding. The number of B-cell receptor binding epitopes was predicted using ABCPred (Saha and Raghava, 2006), for which the counts of the predicted high-affinity epitope in the six MPV-surface proteins were summarized (Table S1 in Supporting Information). Taking into account the data from both literature and computational analyses, the six MPV-surface proteins were chosen with high confidence



	A35R mRNA-LNP	B6R mRNA-LNP	A29L mRNA-LNP	E8L mRNA-LNP	H3L mRNA-LNP	M1R mRNA-LNP
Polydispersity index (% Pd)	7.7	11.0	4.7	15.6	18.7	11.7
Encapsulation efficiency (%)	94.8	95.55	91.7	95.03	92.97	93.28

Figure 1 Design of mRNA vaccine and characterization of the monkeypox virus surface antigens. A, Schematic representation for preparation of mRNA vaccines by LNP encapsulation. B, Design of mRNAs encoding the multiple surface antigens from MPV. The full-length sequences of A35R, B6R and A29L were used with addition of 5′ and 3′ UTR sequences (Materials and Methods; Figure S1 in Supporting Information). For E8L, H3L and M1R, regions encoding their extracellular domains were fused to the N-terminal signal peptide tPA. tPA, human tissue plasminogen activator signal peptide. C, Expression of MPV antigens by transfecting mRNA into HEK 293T cells. At 20 h post transfection, cell lysates were analyzed by SDS-PAGE and Western blot. GADPH was used as the internal protein loading control. D and E, Characterization of mRNA vaccines encapsulated in LNP. The mRNA was analyzed by agarose gel electrophoresis (Figure S2 in Supporting Information). The size distribution of mRNA-LNP vaccines in two preparations (D), including Vac-Ctrl (non-coding mRNA), MPV-E2 (A35R and B6R mRNA), MPV-M2 (H3L and M1R mRNA), MPV-M4 (A29L, E8L, H3L and M1R mRNA), MPV-EM6 (A35R, B6R, A29L, E8L, H3L and M1R mRNA). The polydispersity index and encapsulation efficiency of LNP (E) were determined as described (Materials and methods).

for further evaluation in our multi-valent mRNA vaccine study.

The correct expression of each antigen protein using synthesized mRNA was validated by transfection of their mRNA into HEK 293T cells and detection with Western blot analysis (Figure 1C). We encapsulated the immunogen mRNAs in lipid nanoparticles (LNP) using formulations previously developed (Ramachandran et al., 2022). The LNP particles were found to have encapsulation rates between 91.7% and 95.6%. They had a similar average size and polydispersity indexes that are less than or equal to 18.7%, determined by dynamic light scattering (Figure 1D and E).

Multi-valent mRNA vaccines with different combinations of EV- and MV-surface antigens inducing robust IgG antibody response in mice

To investigate the immunogenicity of different antigens and the effectiveness of multi-valent vaccines, we designed and tested four formulations with combinations of 2 EV-antigens (A35R and B6R), 2 MV-antigens (A29L and H3L), 4 MV-antigens (A29L, H3L, E8L, and M1R), and 6 EV+MV antigens (A35R, B6R, A29L, H3L, E8L, and M1R) (Figure 2A). Each formulation contained equal amounts of each antigen mRNA. The four combinational mRNA vaccines, namely MPV-E2, MPV-M2, MPV-M4, and MPV-EM6, and one control formulation, Vac-Ctrl, with noncoding RNA as placebo, were used to immunize BALB/c mice of 5-week old, with two intramuscular administrations on days 0 and 14 (Figure 2A). We then observed these immunized mice for possible side-effect. No local reaction at the administration sites, such as skin inflammation, was observed in all five groups. We also found no apparent difference in behavior between the immunized and the control animal groups—the different mouse groups had normal activities and feeding, and were consistent before and after inoculation. To evaluate the extent of the inflammatory response caused by the mRNA vaccines, we measured the number and proportion of neutrophils, monocytes, and other myeloid cells from blood samples of the mice vaccinated with MPV-EM6. The results showed that the proportion of monocytes significantly increased in the mouse blood samples after immunization (Figure S3 in Supporting Information), which indicated the extent of an inflammatory response in mice.

Blood samples were collected on days 0, 7, 14, 28, 43, and 57 from all groups, and antibody response against each immunogen was assessed by enzyme-linked immunosorbent assay (ELISA). ELISA was prepared with recombinant proteins as the capture antigen, i.e., A35R, B6R, A29L, E8L, H3L, and M1R that were produced in *E. coli* (Materials and methods). Compared with the sera from the control group (Vac-Ctrl) that generated only background level of A_{450} reading along the course of 57 days, antigen specific IgG response against A35R, M1R, E8L, and H3L was detected as soon as 7 days after initial immunization (Figure 2B). The antibody responses for all the EV- and MV-surface antigens were significantly boosted with the second immunization, which reached peak level 14 days after the second immunization and stayed at those levels. Interestingly, B6R mRNA induced a weak IgG response with two administrations of the MPV-E2 vaccine, but produced a much stronger response for MPV-EM6 (Figure 2B). Apart from B6R, the rest immunogens had consistent response dynamics between the different combinational mRNA vaccines.

The antisera collected on day 57 were evaluated for their ability to neutralize a VACV strain using plaque reduction neutralization test (PRNT) assay. Although the antisera from the control group (Vac-Ctrl) did not detect neutralization activity above the background level, significant neutralizing activities were observed for all the immunized groups (Figure 2C). The highest $PRNT_{50}$ titers were observed for MPV-EM6 which are between 215 and 664. Notably the neutralizing activity of each vaccine is in good correlation with its total IgG response (Figure 2B). The higher number of immunogens contributed to a more robust total IgG response and more potent neutralizing activity, indicating the additive effect of each immunogen in generating immune response and nullifying VACV infection.

mRNA vaccines against EV and MV-surface antigens eliciting antigen-specific T cell immune response in mice

To validate whether the different mRNA vaccines induced antigen-specific T-cell response in vaccinated mice, we performed T cell immunity assay on splenic lymphocytes collected from mice on day 7 after the second immunization with MPV-EM6 or from control mice administered with Vac-Ctrl (Figure 2A). Cultured splenic lymphocytes were stimulated for 10 h with each of the recombinant immunogens, A35R, B6R, A29L, E8L, H3L, and M1R, respectively. The cytokines, i.e., IFN-γ, IL-2, TNF-α, and IL-4 in T cells were detected with intracellular cytokine staining (ICS) assay and analyzed using flow cytometry (Materials and methods). When stimulated with each antigen separately, a significantly higher percentage of $CD4^+$ and $CD8^+$ T cells were found to be positive for IFN-γ, IL-2, TNF-α, but not IL-4 in mice vaccinated with MPV-EM6 than in control mice (Figure 3A and B). Note each antigen was able to induce IFN-γ, IL-2, and TNF-α production, but not IL-4 in $CD4^+$ T cells, indicating that the mRNA vaccine induced an antigen-specific $CD4^+$ T cell response biased towards Th1.

mRNA vaccines against EV and MV-surface antigens protecting mice from lethal VACV challenge

We used a mouse mode that can be infected with a vaccinia

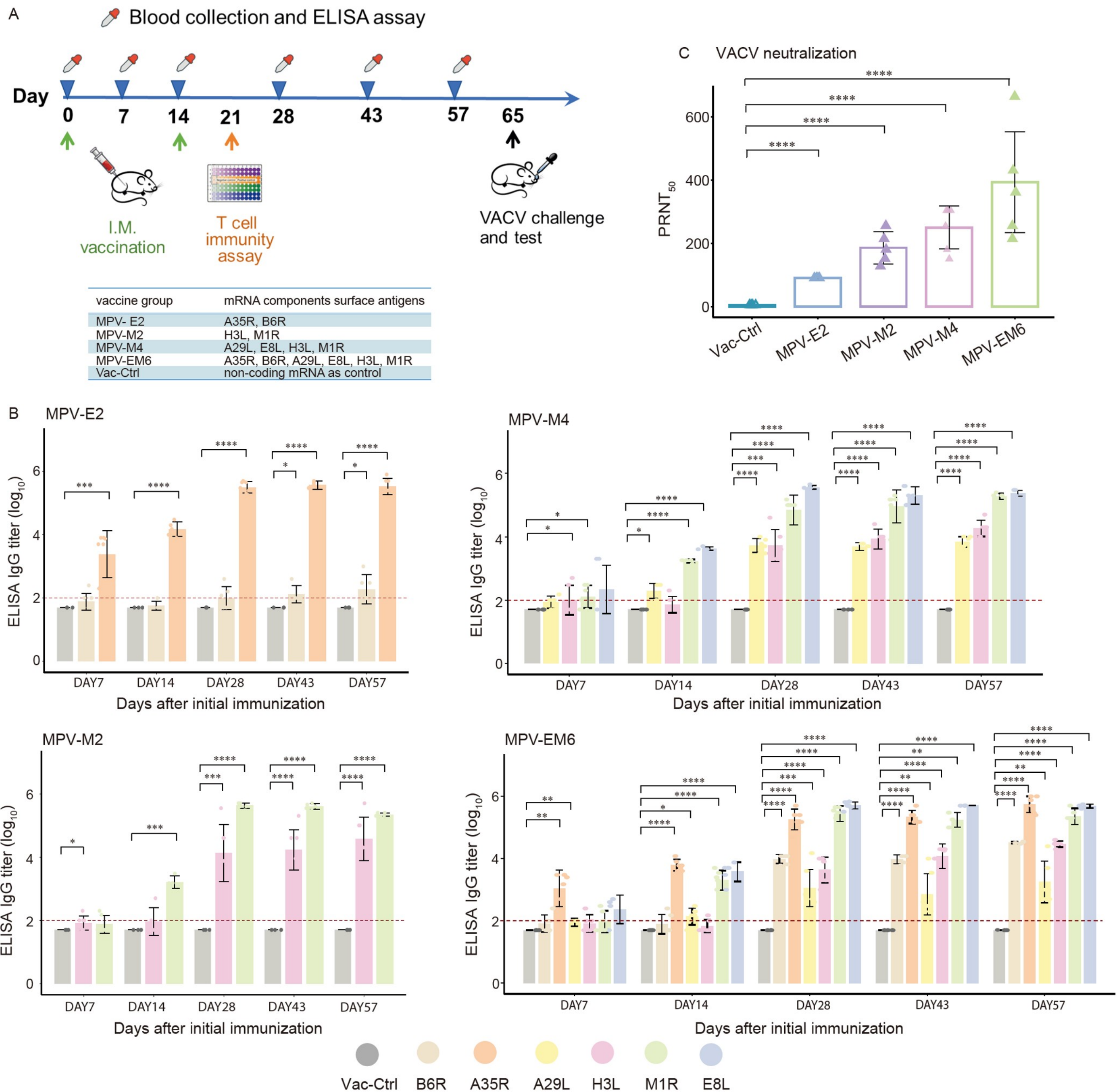


Figure 2 MPV mRNA vaccines induced robust IgG and neutralizing antibody responses in mice. A, Schematic diagram of immunization, serum collection and challenge in mice. Groups of BALB/c mice (n=7) received MPV-E2, MPV-M2, MPV-M4, MPV-EM6, or Vac-Ctrl via the i.m. route. Sera were collected on days 0, 7, 14, 28, 43, and 57 after the initial immunization and assessed for MPV antigen-specific IgG by ELISA. Eight weeks post initial vaccination, mice were challenged with a lethal dose (1×10^6 PFU) of VACV. B, The MPV antigens specific IgG antibody titers determined by ELISA. Data are shown as mean±SEM. Comparisons were performed by Student's t-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$). Source data are provided in Table S2 in Supporting Information. C, The MPV neutralizing antibody titers of mice sera (57 days post-vaccination) measured by PRNT assay. Data are shown as mean±SEM. Comparisons were performed by Student's t-test (****, $P<0.0001$). Source data are provided in Table S3 in Supporting Information.

virus strain (VACV; Tian Tan strain) to assess the multivalent mRNA vaccines in protecting mice from a lethal dose of VACV infection. Both VACV and MPV belonged to the *Orthopoxvirus* genus of *Poxviridae* family, which are highly conserved in their protein coding genes. Smallpox vaccine was previously shown to protect host animals from infection by MPV and VACV (Hatch et al., 2013; Hooper et al., 2004; Wyatt et al., 2004). The five groups of mice vaccinated with MPV-E2, MPV-M2, MPV-M4, MPV-EM6, or Vac-Ctrl, respectively, were challenged intranasally with a lethal dose (10^6 PFU) of VACV on day 65 after the initial immunization (Figure 2A). The body weight of each group was recorded continuously for 19 days post VACV administration. In all the groups, no mice showed any sign of rash, which was

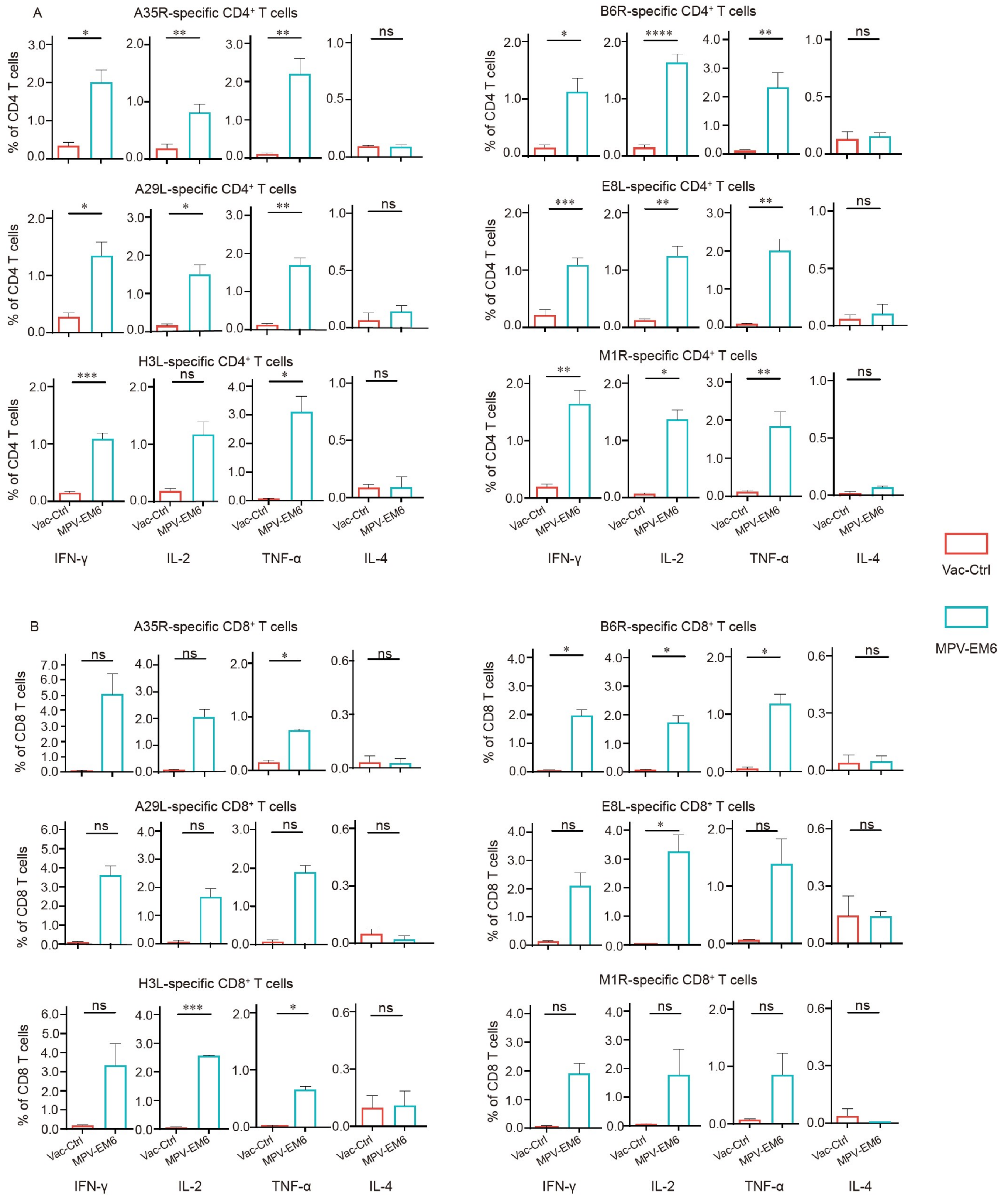


Figure 3 Antigen-specific T cell immune response in mice immunized with MPV mRNA vaccines. IFN-γ, IL-2, TNF-α, and IL-4 in $CD4^+$ (A) and $CD8^+$ (B) T cells were detected with ICS assay and analyzed using flow cytometry assay. The splenocytes were isolated and stimulated with each of the recombinant antigens. Data are presented as mean±SEM. *P* values were analyzed with unpaired *t*-test ($n \geqslant 3$); ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

consistent with the previous report (Fang et al., 2005). While all groups started losing weight 2 days post infection, the MPV-EM6 and MPV-M4 vaccinated groups had the best protection with losing no more than an average 7% body weight, and almost fully recovering 5 days post infection (Figure 4A). The control group mice continued to lose body weight until 6 days post infection when they were sacrificed due to excess weight loss. Both MPV-E2 and MPV-M2 offered significant protection with no more than an average 14% body weight loss, and full recovery 9 days after the VACV challenge. Importantly, all the combinational mRNA vaccines enabled a complete survival of immunized mice while no mice in the control group survived. We further examined the viral load in different groups of infected mice. On day 6 post-challenge when the control group mice were sacrificed, we measured viral abundance by qPCR on samples from lung, nose, throat swab, and spleen (Figure 4B). While VACV DNA was detected with high abundance in all the tissue samples of the control mouse group using qPCR, it was significantly reduced in lung, throat swab, and spleen, or below detectable level in nose in all groups of vaccinated mice. Small differences in the titer of lung, throat swab, and spleen tissues were detected among the 2-, 4-, and 6-valency vaccine groups, but did not reach significance. For example, for the viral titer of the throat swab samples, the adjusted *P*-values for comparisons between MPV-E2 and MPV-M4, between MPV-M2 and MPV-M4, between MPV-E2 and MPV-EM6, and between MPV-M2 and MPV-EM6, were 0.1187, 0.0545, 0.1082, and 0.0501, respectively.

DISCUSSION

In the current study, we developed an array of mRNA vaccines that target MPV, with the objectives of preventing future outbreaks, examining their efficacy and mechanisms of action, and alleviating safety concerns associated with their use. We assessed the potential of six monkeypox surface proteins as immunogens, two from EV and four from MV, using four different combinations that were designed to contain EV-only, MV-only, and EV+MV surface antigens. The study also investigated the neutralizing activity of the vaccine-induced antisera against VACV and their ability to activate antigen-specific T cells in mice. Our study demonstrated that the multi-valent mRNA vaccines, containing various combinations of surface antigens, were highly effective in protecting mice from a lethal dose of VACV challenge. These findings shed light on the immunogenicity potential of each antigen and provide valuable insights into the development of effective and safe multi-valent mRNA vaccines against monkeypox virus.

Among the six surface antigens, our results showed that each of them can induce a robust and potent immune response in mice. We used a two-dose immunization scheme and a seven-day schedule for blood testing after the first vaccination, which gave a closer look on the initial immune response to the immunization with different antigen combinations. Significant effects were detected for four antigens, i.e., A35R, M1R, E8L, and H3L as soon as seven days after initial immunization, which continued to increase to day 14 when a second boost immunization was applied. The immune response for three antigens, i.e., A35R, E8L, and M1R, was found to reach a peak on 14 days after the 2nd immunization, whereas for others, i.e., H3L, B6R, and A29L, would continue posting increase albeit at very small scales. The different response dynamics of each antigen reflect the difference in immunogenicity of each molecule, but may also be contributed by the way each antigen molecule was constructed for expression *in vivo*. Surprisingly, B6R mRNA did not induce a strong immune response with two administrations of the MPV-E2 vaccine, but produced a much stronger IgG response with two immunizations of MPV-EM6 (Figure 2B). Although the same amount of B6R mRNA was applied for MPV-E2 and MPV-EM6, the higher quantity of total RNA in MPV-EM6 may help boosting the response to B6R antigen. Since the same amount of mRNA for each antigen was used in the combinational mRNA vaccines, it is worth considering to adjust their ratios to optimize the effects based on the immunogenicity data and the response dynamics analysis. In comparison to two smallpox vaccines developed using traditional methods, Dryvax and MVA-BN, which are either modified live or live attenuated vaccines, our mRNA vaccines peaked in mice at 2 weeks post-second immunization, having a comparable response time and a higher neutralization titer, 660 *vs*. ~100, in PRNT assay (Meseda et al., 2005). Due to the different conditions used in these studies, the comparison can only serve as a reference to show that our mRNA vaccines, which are still in their early development phase, are good candidates with promising potential. Our current work provides a good basis for future optimization of mRNA vaccines by combining different EV/MV surface antigens to achieve the best protection against MPV.

The neutralizing activities of immune sera from mice vaccinated with different combinations of antigens showed a good correlation to their total IgG antibody titers (Figure 2B and C). Due to the limited number of immunogen combinations that can be studied, with the six MPV-surface antigens that we chose, we designed a five-group study: two 2-valent, one 4-valent, and one 6-valent vaccines, and one non-coding RNA control. In addition, we chose to use the same amount of mRNA for each antigen in the multi-valency study. In this way, when more antigens were combined, there would be equal stimulus from each single antigen; and the additive effect of multi-antigens may be analyzed and separated based on the effect of each single one at the same amount. The sera collected from mice vaccinated with MPV-E2, MPV-M2,

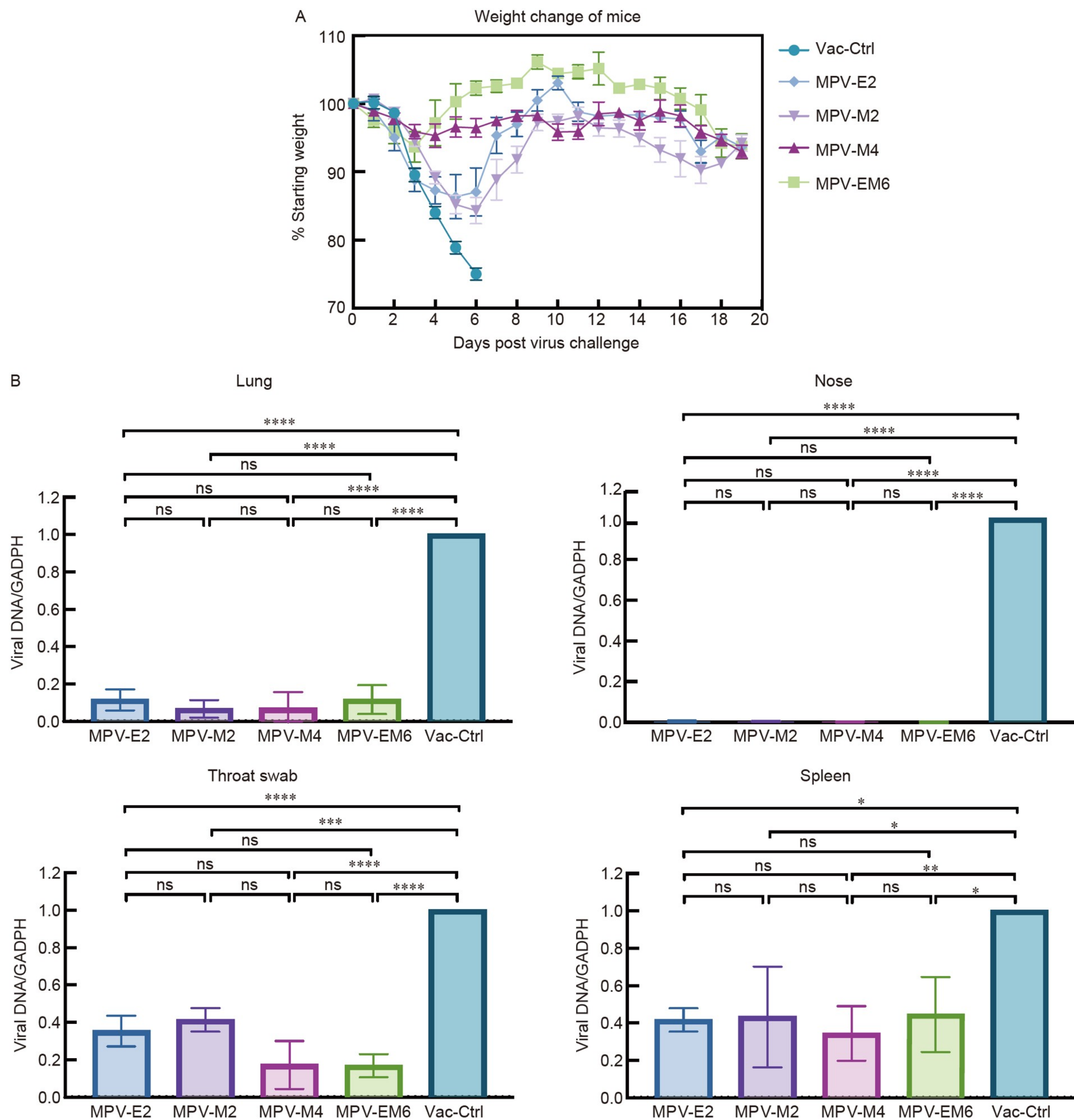


Figure 4 MPV mRNA vaccines protected mice from lethal dose of VACV challenge. Five groups of BALB/c mice (n=7) received different mRNA vaccines or placebo via the i.m. route. 65 days post initial vaccination, mice were challenged intranasally with a lethal dose (1×10^6 PFU) of VACV Tian Tan strain. Weight loss was monitored daily, and mice were sacrificed when weight loss reaching 25% of original weight. Lung, nose, throat swab, and spleen were collected for detection of virus load. A, Body weight of each mouse and survival were recorded every day after challenge. B, Viral loads in lung, nose, throat swab and spleen were determined by qPCR. The viral DNA levels are normalized relative to that of GAPDH. Data are shown as mean±SEM ($n\geqslant3$). Significance was analyzed by two-way ANOVA with Tukey's multiple comparisons tests (ns, not significant; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$).

MPV-M4, or MPV-EM6 increased in their ability to neutralize VACV in PRNT assay. Interestingly, using equivalent immune doses, MPV-M2 and MPV-E2, the two 2-valent vaccines, induced different IgG titers in vaccinated mice, and induced different neutralizing activities against VACV (Figure 2B and C). The two 2-valent vaccines had somewhat different protection, indicating the same dose of mRNA vaccines can have different protection depending on the immunogens (Figure 4A). In experiments when adding more immunogens (4- and 6-valency) with the "old" immunogens staying at the amounts same as in the 2-valency, we observed that, while the "old" immunogens in 2-valent vaccines induced comparable response in mice vaccinated with 4-valent or 6-valent vaccines, the added "new" immunogens induced independent IgG responses, and the effect of the "new" immunogens are comparable between the 4-valent and 6-valent vaccines (Figure 2B). The best-performing group protections are those with the highest total doses, whereas the protection

can be analyzed and separated based on the effect of each single one. These results suggested that in general the effective immune responses not only require a higher number of antigens, but also are positively correlated with the combined dosage of all immunogens.

VACV and MPV are highly conserved in their protein-coding genes, which belong to the *Orthopoxvirus* genus of *Poxviridae* family. Thus, it is no surprise that mRNA vaccine against MPV can protect mice from VACV infection. Our results showed that all the mRNA vaccines with different combinations of MPV-antigens can protect a mouse model from a lethal dose of VACV infection (Figure 4A). Their degree of protection is in general agreement with the results of serum neutralization experiments (Figure 2C). As expected, the MPV-EM6 vaccine provided the best protection with the least body weight loss and swift recovery. That the MPV-M4 vaccine with MV-only antigens also offered strong protection against VACV is a little surprise to us. Furthermore, it is unexpected both MPV-E2 and MPV-M2 vaccines offered protection against challenge of lethal VACV dosage, albeit less effective compared with MPV-M4 and MPV-EM6 (Figure 2C). These results are in contrast to the earlier studies of subunit smallpox vaccine that showed EV antigens-induced immunity did not protect mice from lethal viral challenge (strain VACV-WR), but both EV- and MV-antigens were required for complete protection (Paran et al., 2013; Xiao et al., 2007; Xiao et al., 2020). It is possible that vaccines with EV- or MV-antigens, i.e., MPV-E2, MPV-M2, or MPV-M4, generated an immune response with strong enough cross-reaction to counter VACV infection by inhibiting viral propagation *in vivo*. It is also likely that mRNA-based vaccines generated a stronger hormonal and cellular immune response than the subunit smallpox vaccines. No EV- or MV-only antigen vaccine was designed in the recently reported study (Fang et al., 2023), thus it is warranted for further study to understand the possible mechanism of action.

The mRNA vaccines developed against SARS-CoV-2 and its variants showed great ability to induce broad and durable T cell immune response (Hurme et al., 2022; Pegu et al., 2021; Wherry and Barouch, 2022). We have shown in the current study that the mRNA vaccine MPV-EM6 induced antigens-specific T-cell response in immunized mice (Figure 3). Evidently, each of the six antigens was able to induce the production of Th1 cytokines, i.e., IFN-γ, IL-2, TNF-α, but not IL-4 in $CD4^+$ T cells, which indicated the mRNA vaccines against MPV induced a strong antigen-specific $CD4^+$ T cell response that is Th1-biased. However, it remains a big question mark that how long the immunity induced by mRNA vaccines encoding the MPV-surface antigens can sustain, in comparison to the long durability of immunity that was generated by regular smallpox vaccines (Josefson, 2003; Taub et al., 2008; Viner and Isaacs, 2005).

The West African lineage of MPV is responsible for the current MPV outbreak among the two main clades of MPV. There are a limited number of mutations/polymorphisms that were observed among the different MPV lineages (Isidro et al., 2022; Luna et al., 2023). The next question is whether our current MPV mRNA vaccines will protect people from infection by new MPV variants or other MPV lineages. Unlike SARS-CoV-2 mRNA vaccines that concentrated on the membrane-bound spike protein as a main target, which has mutated to many variants to evade neutralizing antibodies, the current design of MPV mRNA vaccines has targeted multiple surface proteins. Notably, our results of cross-neutralization and protection of animal models from VACV infection point to a great potential for it to offer broad protection against outbreaks of MPV variants or different MPV lineages in the future, although much work remains to be done to test our mRNA vaccines in other animal models like non-human primates for the preparation of human trials.

In summary, the swift responsible time and flexibility of mRNA vaccines provide an un-paralleled platform for rapid development and deployment of vaccines against any threat of pathogenic outbreaks. We developed an array of multi-valent mRNA vaccines that target monkeypox virus, and assessed their efficacy, response dynamics, mechanisms of action, and ability to protect an animal model against the lethal challenge of VACV. Our results revealed that dynamic immune response and robust protection induced by our MPV mRNA vaccines, using the design of combinations of EV-, MV- or both antigens. These findings provide insight into the protective mechanism of multi-valent mRNA vaccines and the foundation for further development and deployment of MPV mRNA vaccines for enhanced protection against monkeypox virus outbreak.

MATERIALS AND METHODS

Cells, viruses and animals

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% penicillin and streptomycin (P/S, Thermo Fisher Scientific, USA). All cells were grown at 37°C, 5% CO_2 atmosphere as described (Chen et al., 2022). The vaccinia virus Tian Tan strain (GenBank: AF095689.1) was obtained from the Institute of Virology at the Chinese Center for Disease Control and Prevention and propagated in BHK-21 cells.

Female BALB/c-mice (5 weeks old, female) were purchased from VitalRiver (Pinghu, China). All animal experiments in this study were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai. The mice were bred and maintained in a specific pathogen-free (SPF) environment in good health.

Sequence sources and cloning construction

For mRNA vaccine design, the sequences of six MPV surface antigens, A35R, B6R, A29L, E8L, H3L, and M1R, were obtained from the monkeypox virus reference sequence at NCBI (accession: GCF_014621545.1). The reference sequence was from the strain isolated from Rivers State, Nigeria, which was the most recent common ancestor strain from the African continent (Mauldin et al., 2022). Three MPV proteins, E8L, H3L, and M1R, do not have signal peptides, for which the human "tissue plasminogen activator signal peptide" (tPA signal peptide, GenBank: E04506) sequence was fused to their N-termini. tPA is a commonly used heterologous signal peptide for increasing expression levels of recombinant proteins in mammalian hosts (Costa et al., 2006). The other proteins, A35R, B6R, and A29R, have their own signal peptide, and were not changed. The six antigen sequences were synthesized (AZENTA, Suzhou, China) and cloned into vector pCDNA3.1(+), with an upstream T7 RNA polymerase promoter, 5′ UTR region, downstream 3′ β-globin UTR region, and poly(A) tail as flanking sequences.

In vitro mRNA synthesis and transfection analysis

The MPV-surface antigens cloning constructs were amplified by PCR using KOD DNA polymerase (TOYOBO, Japan) and digested with *Aar*I enzyme (Thermo Fisher Scientific) to prepare templates for mRNA synthesis. The linearized DNA templates were transcribed *in vitro* using the HiScribe T7 High Yield RNA Synthesis Kit (Yeasen, Shanghai, China). UTP was replaced with N1-Me-pseudo-UTP in the reactions. mRNA was capped using Cap 1-GAG m7G (5′) ppp (5′) (2′OMeA) pG (Yeasen), following the manufacturer's instructions. The mRNA products were purified by phenol/chloroform extraction and ethanol precipitation before resuspended in RNase-free water. The integrity of mRNA was analyzed by agarose gel electrophoresis.

mRNA transfection was performed using Lipofectamine MessengerMAX (Thermo Fisher Scientific). HEK 293T cells were plated in 6-well plates in Opti-MEM and transfected with 2 μg mRNA per well. 16 h after transfection, cells were collected and lysed in RIPA buffer (Beyotime, Shanghai, China). The samples were treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled at 100°C for 10 min, before they were separated with SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane for Western blot analysis. The membrane was incubated with the anti-His Tag mouse monoclonal Ab (Beyotime, 1:2,000 dilution) for 1 h at room temperature, and subsequently with the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Beyotime, 1:5,000 dilution) for 1 h at room temperature.

MPV-mRNA LNP encapsulation and characterization

LNP encapsulation for MPV-mRNA was prepared using a microfluidic mixer as described (Ramachandran et al., 2022) Briefly, the prepared mRNA was first diluted in 50 mmol L^{-1} sodium acetate buffer, before mixed with a lipid mixture in ethanol at a volume ratio of 3:1. The lipid mixture containing cationic ionizable lipid (SM102), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and DMG-PEG2000 was dissolved in ethanol with a molar ratio of 50:10:38.5:1.5. Subsequently, the MPV-mRNA LNP was exchanged to PBS buffer (pH 7.4) using a 30 kD MWCO Amicon filter (Millipore, Merck, USA) and filtrated through a 0.22 μm filter before storage at 4°C until use.

The encapsulation efficiency, mRNA concentration, and particle size distribution of MPV-mRNA LNP were measured. In brief, encapsulated and non-encapsulated samples of LNP-mRNA were prepared by 1:100 dilution in 2% Triton X-100 and 1× TE buffer, respectively. To quantify encapsulation efficiency, encapsulated and non-encapsulated samples were detected using the Quant-iT™ RiboGreen™ RNA Reagent and RNA standards provided in the kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The sample fluorescence was measured using a fluorescence microplate reader (excitation ~480 nm, emission ~520 nm). The mRNA encapsulation efficiency (EE) was determined using the following equation:

$$\%\text{mRNA EE}=\left(\frac{\text{encapsulated mRNA}}{\text{encapsulated+non-encapsulated mRNA}}\right)\times 100.$$

The particle size distribution and polydispersity of MPV mRNA LNPs were measured by dynamic light scattering using DynaPro NanoStar according to the manufacturer's instructions (Wyatt, USA).

Mice vaccination

BALB/c mice (5 weeks old, female) were divided into five groups (n=7) and immunized intramuscularly with two doses of 7.5 μg of each antigen-encoding mRNA: MPV-E2, MPV-M2, MPV-M4, MPV-EM6, or Vac-Ctrl containing noncoding mRNA as a placebo. 7.5 μg antigens for A35R, B6R, A29L, E8L, H3L, and M1R were equal to 19.9, 14.6, 24.7, 14.7, 15.2, and 18.4 pmol, respectively. The initial immunization was given on day 0, followed by a booster dose on day 14. Blood samples were collected at days 0, 7, 14, 28, 43, and 57 after the initial immunization to measure antibody levels. The T cell immunity response was measured using splenocytes isolated 7 days after the booster immunization.

MPV-antigens expression in *E. coli* and purification

The signal peptides and transmembrane regions of the six

MPV-antigens, A35R, B6R, A29L, E8L, H3L, and M1R, were removed by PCR amplifications before they were cloned into the pGEX-4T-1 vector for expression in *E. coli*. The cloning construction was carried out using the in-fusion cloning protocol as performed previously (Zhang et al., 2020). These plasmids were transformed into Rosetta (DE3) strain (WEIDI, Shanghai, China). Expression of MPV-antigens was induced using 300 μmol L^{-1} isopropyl-β-D-thiogalactoside (IPTG, Beyotime) at 16°C for 18 h. The recombinant proteins were purified using the Ni-NTA agarose resin (Yeasen) or GST-tag purification resin (Beyotime) following the manufacturers' protocol.

Enzyme-linked immunosorbent assay

IgG antibody titers against MPV-antigens, A35R, B6R, A29L, E8L, H3L, and M1R, were determined by ELISA using the purified recombinant proteins. 96-well plates (Corning, USA) were coated with 5 μg mL^{-1} recombinant protein and incubated overnight at 4°C. Plates were washed with TBST (20 mmol L^{-1} Tris, 137 mmol L^{-1} NaCl, 0.1% Tween, pH 7.4) and blocked with 5% non-fat dry milk for 1 h at 37°C. Serial dilutions of heat-inactivated mouse serum were added to the wells and incubated for 1 h at 37°C, followed by incubation with HRP-conjugated goat anti-mouse IgG (Yeasen, diluted 1:5,000 in 1% milk/TBST) for 1 h at 37°C. Next, the plates were treated with the 3,3′,5,5′-tetramethylbenzidine (MesGen, Shanghai, China) for 15 min before reactions were terminated with 2 mol L^{-1} hydrochloric acid. Absorbance at 450 nm was recorded using a Varioskan Flash microplate reader (Thermo Fisher Scientific).

ICS and flow cytometry analysis

The ICS assay was performed as previously described (Tan et al., 2017; Xu et al., 2021), with some modifications to measure MPV-antigen-specific T cells in immunized mice. Briefly, spleens were collected from mice on day 7 post 2nd vaccination and grinded with filters. Cells were suspended in RPMI-1640 medium containing 10% FBS followed by the removal of red blood cells. Totally 2×10^6 splenocytes were placed in each well of 96 U-plates and stimulated with each MPV-antigen, i.e., A35R, B6R, A29L, E8L, H3L, or M1R (10 μg mL^{-1} for each protein), respectively at 37°C for 16 h, or with the positive control, 10 ng mL^{-1} PMA (Phorbol 12-myristate 13-acetate) and 1 μg mL^{-1} ionomycin. Cytokine secretion inhibitor (Brefeldin A, BD Biosciences, USA) was added 2 h before cell collection and washed with cell staining buffer (BD Biosciences). The cells were stained with a cocktail of fluorescently conjugated antibodies for mouse cell surface markers (anti-CD4/FITC, anti-CD8/APC, and anti-CD44/Brilliant Violet 605; BD Biosciences). The Fc receptors of cells were blocked using CD16/CD32 antibodies (Mouse BD Fc Block; BD Biosciences) for 30 min at 4°C in dark. Following washing with cell staining buffer (BD Biosciences), cells were stained with Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific) for 10 min in dark. After fixation and permeabilization, the cells were washed twice and incubated with intracellular cytokine staining reagent (anti-IFN-γ/PerCP-Cy5.5, anti-IL-2/PE, anti-TNF-α/PE-Cy7; anti-IL-4/PE-Cy7; BD Biosciences). All samples were analyzed with the LSRFortessa flow cytometer (BD Biosciences). FlowJo software was used for flow cytometry data analysis.

Virus challenge

On day 65 after the initial vaccination, mice were challenged with 10^6 PFU of VACV (strain Tian Tan, GenBank accession NO. AF095689.1) via the i.n. route. Weight loss was monitored every day. Mice were sacrificed on day 6 post-challenge and the lung, nose, spleen, and throat swab tissues were collected for analysis of viral loads.

Viral loads in tissues

The viral loads in lung, nose, spleen, and throat swab tissues of mice on day 6 after VACV challenge were determined by qPCR. Viral DNA was extracted using the FastPure Viral DNA/RNA Mini Kits (Vazyme, Nanjing, China). The Real-time PCR reactions were carried out using Hieff® qPCR SYBR Green Master Mix (Yeasen) and run on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA). The PCR primers that targeted MPV E9L gene were used to assay viral loads as follows:

forward primer: 5′-TCAACTGAAAAGGCCATCTATGA-3′;

reverse primer: 5′-GAGTATAGAGCACTATTTCTAAATCCCA-3′.

The qPCR was performed under the following conditions: 95°C for 3 min, 40 cycles of amplification at 95°C for 10 s and 60°C for 30 s.

Plaque reduction neutralization test

The neutralizing antibody titers for blood samples collected on day 57 after vaccination were determined by PRNT. BHK-21 cells were seeded in 12-well plates, and twofold serial dilutions of serum samples were mixed with an equal volume of VACV Tian Tan strain containing ~100 PFU of virus per milliliter, the virus/serum mixtures were added to wells of the 12-well plates containing BHK-21 cell culture monolayers. The plates were then incubated at 37°C for 90 min. Cells were overlaid with 0.5% agarose in DMEM (Gibco) with 2.5% inactivated FBS. After incubated at 37°C for 72 h, cells were fixed by 4% formaldehyde and stained by

0.2% crystal violet, before the Plaque numbers were recorded. The $PRNT_{50}$ values were calculated as described (Reed and Muench, 1938).

Statistical analysis

Statistical analyses were performed using R (R 4.0.5) and Prism 9.4.1 software (GraphPad). Data are presented as means ±standard errors in all experiments. Statistical significance was obtained from Student's *t*-test in Figures 2, 3 and S3 (in Supporting Information). Two-way ANOVA with Tukey's multiple comparisons test was used to assess statistical significance for grouped datasets in Figure 4B, and the *P*-value was adjusted using statistical hypothesis testing in Prism.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

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