Design and Evaluation of a Multi-Epitope Peptide of Human Metapneumovirus

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Key Words

Human metapneumovirus · B epitope · Cytotoxic T lymphocyte epitope · T helper epitope · Multi-epitope peptide · Adjuvant

Abstract

Objectives: No licensed vaccines or therapeutic agents for human metapneumovirus (hMPV) infection exist to date. We aimed to construct a multi-epitope peptide (MEP) of hMPV to show promising results for epitope-based vaccine development. **Methods:** Six independent algorithms were screened to predict B-cell epitopes of hMPV, and three algorithms were used to predict cytotoxic T lymphocyte and T helper (Th) lymphocyte epitopes. Predicted epitopes were assembled in series with the spacers GP GPG and KK introduced, termed MEP. Recombinant *mep* genes were inserted into pET32a(+) plasmid and expressed in *Escherichia coli* strain BL21 (DE3). BALB/c mice were immunized with MEP with different adjuvants. Antibody titer, lymphocyte proliferation, cytotoxic T lymphocyte (CTL) activity and splenocyte cytokines were detected 2 weeks later after the last immunization. Microneutralization assay was used to detect neutralizing antibodies. **Results:** Six B-cell epitopes, four CTL epitopes and two Th epitopes were screened to construct the *mep* gene. Expressed MEP induced $>10^4$ antibodies in BALB/c mice, and produced anti-MEP antibody reacting with

hMPV strains specifically as detected in indirect fluorescent assay (the titer was 160). The lymphocyte proliferation index, CTL activity and splenocyte cytokines of the MEP immunization groups were higher than in the control group ($p < 0.05$). Both IgG1 and IgG2a antibodies could be detected in the different groups, and balanced Th1/Th2 cytokines were secreted by splenocytes in these groups. The mean neutralizing titers of the MEP+CpG ODN, MEP+Alum and MEP+Alum+CpG ODN groups were 87 (95% CI 50–126), 93 (95% CI 67–121) and 96 (95% CI 69–147), respectively. **Conclusion:** MEP of hMPV elicited both strong humoral immunity and cell-mediated immunity in mice. The anti-MEP serum could neutralize hMPV infection in vitro. Joint use of CpG ODN and aluminum hydroxide adjuvants obtained the best immune effects. This study may contribute to hMPV epitope-based vaccine development.

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Introduction

Acute respiratory tract infections (ARTIs) are significant causes of human mortality and morbidity, and are also the leading cause of morbidity in children under 5

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years of age worldwide [1]. Human metapneumovirus (hMPV) is a relatively newly described virus. It was first isolated in 2001 and currently appears to be one of the major respiratory viruses affecting children, the elderly [2] and immune-compromised individuals [3]. hMPV is classified as the first human member of the *Metapneumovirus* genus in the *Pneumovirinae* subfamily within the *Paramyxoviridae* family [4]. It is the second most important pathogen for young children following respiratory syncytial virus (RSV). Serologic studies have confirmed that worldwide hMPV circulation has been present for at least 50 years [5]. hMPV is responsible for 5–15% of pediatric hospitalizations for ARTIs [6] and it can be reinfected throughout an individual's lifetime [7–9]. To date, no effective medicines and licensed vaccines are available [10].

Epitope-based vaccines have emerged as a new kind of vaccine and are attracting increasing attention. The recent development of bioinformatics tools provides the opportunity for epitope-based vaccine research, and many bioinformatic tools are freely available online [11–19]. This study aimed to predict the hMPV B- and T-cell epitope using various online epitope prediction software tools, and assemble the putative epitopes together to construct a multi-epitope peptide (*mep*) gene consisting of both B and T epitopes. This would then be expressed in *Escherichia coli* BL21 (DE3) and the immune response of the recombinant *E. coli*-expressed MEP peptide evaluated with different adjuvants.

Materials and Methods

Virus and Cell Lines

Vero-E6 cells were grown in MEM supplemented with 8% fetal calf sera, 100 U/ml penicillin and 0.1 g/l streptomycin (all from Gibco, Life Technologies Corp., Carlsbad, Calif., USA). P815, kept in our laboratory, were used as target cells for the cytotoxic T lymphocyte (CTL) assay. Monolayers of Vero-E6 cells were infected with either TJ-hMPV-2 strain (type A) or TJ-hMPV-3 strain (type B), which were isolated in-house with Vero-E6 cells from throat swabs of young children with ARTIs conducted in 2009 in our laboratory [6]. After adsorption for 1 h at 37°C, virus growth medium (VGM) containing 2.5 µg/ml TPCK-trypsin was added to the flasks. Fresh VGM was replaced every 3–4 days. The cytopathic effect was observed every day until 75–100% of the cells were infected. Virus-infected cells were harvested and prepared as hMPV antigen slides to proceed to indirect fluorescent assay (IFA).

Epitope Prediction

Three bioinformatics tools – Bepipred (threshold 0.35) [11], Bcepred (threshold 2.38) [12] and LEPS [13] – were used for B-cell epitope prediction from hMPV F protein, which utilized the physicochemical properties of the protein, such as hydrophilicity, flexibility, surface probability, solvent accessibility, polarity, antigenic-

ity and turns. Another two tools were also used to predict the B epitope – ABCpred (threshold 0.51) [14], based on artificial neural networks, and LBTOPE (probability threshold 50%) [15], based on a larger database using support vector machines. The identification of candidate H-2Kd-binding CTL epitopes from hMPV F, G, N, M, SH, M2-1 and M2-2 proteins was conducted using two predictive algorithms: NetMHCpan [16] and NetMHC [17]. NetMHCII [18, 19] was used to predict H2-IA (MHC-II)-restricted T helper (Th) epitopes from the same proteins as the CTL epitope prediction. A binding score >0.426 was considered as the threshold for CTL or Th epitopes. The hMPV L and P proteins were not mapped for epitopes due to their large sizes. Three published CTL epitopes were also used in this study [20].

Design and Synthesis of the *mep* Gene

In order to construct gene coding for the MEP that retained the functions of the epitopes and decreased the interactions among them, we selected a theoretically optimal combination of predicted epitopes using DNASTar software. The *mep* gene fragment was designed by arranging the predicted epitopes in tandem in the order of B, CTL and Th epitopes. The linkers GPGPG and KK were used to separate the epitopes from each other. The MEP codons were optimized for the *E. coli* host and synthesized by Invitrogen (Regensburg, Germany).

Molecular Cloning, Expression and Purification of Recombinant *mep*

The *mep* gene was double-digested with *Bam*HI and *Hind*III, followed by ligation into the corresponding restriction sites of the pET32a(+) vector (Novagen, Darmstadt, Germany) to yield the recombinant plasmid pET32a(+)-*mep*. The plasmid was transformed in *E. coli* strain BL21 (DE3). The recombinant bacteria were grown until the OD₆₀₀ of the culture reached 0.6, and protein expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The expression was optimized by growing the cells at 37 and 30°C. One milliliter of cell culture was harvested every hour up to 5 h and centrifuged at 6,000 g for 20 min at 4°C. The cell lysate was sonicated and proceeded with SDS-PAGE as well as supernatant to determine the expressed MEP presenting in the inclusion bodies or in soluble form. The supernatant was purified on an Ni-affinity chromatography column (Ni-NTA His Bind Resin 5 ml, 1 × column; Novagen). The purification steps were performed according to the manufacturer's instructions. The purified MEP was analyzed by SDS-PAGE and tested by Western blot analysis, as described by Ding et al. [21], and the concentration was tested by Bradford assay.

Immunization of Mice

Four- to six-week-old female BALB/c mice were purchased from the Academy of Military Medical Sciences animal center and housed in a pathogen-free facility at Tianjin Medical University. All work was carried out in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee at Tianjin Centers for Disease Control and Prevention, China.

Twenty-four BALB/c mice were divided into four groups (6 mice in each group) as follows: MEP 20 µg + CpG ODN 10 µg; MEP 20 µg + Alum 100 µl; MEP 20 µg + CpG ODN 5 µg + Alum 100 µl, and an untreated group. The first three groups were immunized intraperitoneally on weeks 0, 2 and 4, respectively, whilst the

untreated mice were used as controls. Two weeks after immunization, the mice were sacrificed and their sera and spleens were collected for the following tests. Adjuvant Imject Alum was from Thermo Fisher (Pierce, Rockford, Ill., USA) and CpG ODN 1826 (phosphorothioate modified 5'-TCCATGACGTTTCCT-GACGTT-3') was synthesized by Invitrogen (Shanghai, China).

Determination of Antibody Titers and Isotypes by ELISA

MEP-specific IgG, IgG1, IgG2a and IgA levels in the serum were determined by ELISA. Ninety-six-well microtiter plates (Costar, USA) were coated with 100 μ l of MEP (2 μ g/ml) at 4°C overnight. After washing and blocking with 10% fetal bovine serum, serial 10-fold dilutions ($10^2 \sim 10^6$) of antisera were added to the wells and incubated at 37°C for 1 h, then HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a and IgA antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were added subsequently. After incubation at 37°C for 1 h, TMB substrate (Thermo Scientific, Waltham, Mass., USA) was used to develop color. A 2-mol/l H_2SO_4 preparation was used as the stop solution. Antibody titers were assessed as the reciprocal of the highest dilution of sera producing an OD_{450}/OD_{630} nm ratio value of 2.1 to that of the preimmunized mice sera.

Indirect Immunofluorescent Assay

Vero-E6 cells were infected with hMPV virus which was isolated at our laboratory [6]. Every day the cytopathic effect was observed until up to 75~100% of the cells were infected. The cells were harvested and centrifuged at 2,000 g for 10 min. After washing 3 times with phosphate-buffered saline (PBS), the cells were transferred onto a slide and fixed with acetone. The slide was covered with 2-fold diluted anti-MEP serum at 37°C for 1 h, FITC-conjugated goat anti-mouse IgG was subsequently added and incubated at 37°C for 1 h. Fluorescence was observed after sealing the slide. IFA antibody titer was assessed as the reciprocal of the highest dilution of sera producing specific fluorescence.

Lymphocyte Proliferation Assay

Two weeks after the last immunization, mice were sacrificed and the spleens were aseptically removed. Splenocytes were separated and washed twice with fresh culture medium (RPMI 1640). Lysis buffer (0.1% ammonium chloride) was added to the pellet to remove the red blood cells and the cells were counted. The single-cell suspension was cultured in triplicate in 96-well plates at 5×10^5 cells/well in RPMI 1640 medium (Sigma, St. Louis, Mo., USA). The cells were then stimulated in vitro with 50 μ g/ml MEP. Cells with medium alone were used as unstimulated controls. The cells were incubated at 37°C for 72 h, and 20 μ l of MTS working solution (Promega, Madison, Wis., USA) containing 2 mg/ml MTS and 0.92 mg/ml PMS was added to each well. After incubation at 37°C for 2 h, the $OD_{490\text{ nm}}$ was read. The stimulation index was calculated as: $OD_{490\text{ nm}}$ of the stimulated group/ $OD_{490\text{ nm}}$ of the control group. All cultures were taken in triplicate and the results expressed as the mean stimulation index $\pm$ SD. The confidence intervals were also calculated.

CTL Assay

The CTL assay was performed as described previously [22]. Briefly, 5×10^5 splenocytes per well in RPMI 1640 medium with 10% fetal calf serum were used as effector cells, and 2×10^4 P815 cells were used as target cells (the ratio of effector cells to target

cells was 25:1). The target cells were distributed into triplicate wells of a 96-well plate. The effector and target cells were mixed and incubated at 37°C for 4 h before the supernatant was collected. Lactate dehydrogenase activity released into the culture medium was measured with a cytotoxicity assay kit (Promega) according to the manufacturer's instructions.

Cytokine Secretion by Splenocytes

The splenocytes were incubated in RPMI 1640 medium with 10% fetal calf serum. MEP protein was added at a final concentration of 50 μ g/ml. After incubation for 72 h, the splenocyte culture supernatants were collected for cytokine detection. Eight cytokines, IL-2, IL-12, IFN- γ (Th1 type), IL-4, IL-5, IL-10 (Th2 type), TNF- α and GM-CSF, in supernatant from the splenocytes cultured from vaccinated mice and control mice were examined using a liquid chip assay (Bio-Plex Pro™ Mouse Cytokine Th1/Th2 Assay; Bio-Rad, Hercules, Calif., USA).

Microneutralization Assay

Serum virus-neutralizing antibody titers were determined by a microneutralization assay as described elsewhere, with slight modifications [23]. Briefly, the sera were incubated at 56°C for 30 min to inactivate nonspecific inhibitors. Positive-control wells without serum and negative-control wells without virus or serum were included in triplicate in each plate. Two-fold dilutions of serum were performed in VGM, and then mixed with an equal volume of infection medium containing 50 PFU of TJ-hMPV-2 strains. The antibody-virus mixtures were incubated at 37°C for 2 h and then transferred onto 24-well plates containing Vero-E6 cells. Following incubation for 3 h, the mixtures were removed and 200 μ l of fresh VGM (supplemented with 0.75% hydroxymethyl cellulose, 2-fold dilutions of sera, as well as 2.5 μ g/ml TPCK-treated trypsin) were added to each well. The plates were incubated at 37°C. Five days later, the cells were fixed in 10% formaldehyde in PBS for 15 min at 4°C, air dried and permeabilized by 0.2% Triton X-100 in PBS. A monoclonal antibody against hMPV N protein (clone 507, Chemicon) was diluted to 1:500 in PBS containing 5% fetal calf sera and 0.5% Tween 20 and added to the cells, followed by incubation at 37°C for 2 h. Cells were washed and treated with HRP-conjugated goat anti-mouse IgG (1:5,000, Santa Cruz) in PBS with 5% fetal calf serum and 0.5% Tween 20 (50 μ l/well) for 2 h at room temperature. After washing, True Blue peroxidase (KPL, Gaithersburg, Md., USA) was added to each well, followed by incubation at 37°C for 30 min. Neutralizing titer was defined as the reciprocal of the dilution of serum that reduced color development by 50% normalized by that of the positive-control wells.

Statistical Analysis

One-way ANOVA and independent sample t tests were conducted for statistical analysis using SPSS 18.0 software.

Results

Predicted B, CTL and Th Epitopes, and the Design and Synthesis of a mep Gene

We selected a theoretically optimal combination of 6 B epitopes, 4 CTL epitopes and 2 Th epitopes (table 1) to

PSCSGKKGNYGPGPGQLAREEQIENPRQSRGPGPGCQNAGSTVYYPNEKDCETRGDHVGPGPGCNINISTT
 NYPCKVSTGRHPGPGPGSKVEGEQHVIKGPGPGYITNQDADTVTIKKSPKAGLLSLGPGPGCYLENIEIIGPG
 PGGYIDDNQSIGPGPGNYIKVENNLKKARAVSNMPTSAGQIKGPGPGFPLFQANTPPAVLLD

Fig. 1. The MEP amino acid sequence. Boxes indicate linker sequences.

Fig. 2. **a** SDS-PAGE analysis of MEP expressed in *E. coli*. Lane 1: protein marker (kDa; Transgene, Beijing, China); lane 2: noninduced pET32a(+); lane 3: supernatant from cell lysis of pET32a(+)-*mep* induced with IPTG; lane 4: pellet from cell lysis of pET32a(+)-*mep* induced with IPTG. **b** Western blot of purified MEP with anti-His antibody. Lane 1: protein marker (kDa; Transgene); lane 2: noninduced pET32a(+); lane 3: Western blot of purified MEP. **c** Western blot of purified MEP with anti-MEP serum. Lane 1: protein marker (kDa; Transgene); lane 2: noninduced pET32a(+); lane 3: Western blot of purified MEP. **d** SDS-PAGE analysis of purified MEP. Lane 1: purified MEP; lane 2: protein marker (kDa; Transgene).

design an MEP. The *mep* gene was designed by arranging the 12 epitopes in tandem in the amino acid sequences, as shown in table 1. The epitopes were separated from each other by GPGPG or KK. The designed *mep* codon was optimized as the *E. coli* host, and the amino acid sequence is shown in figure 1.

Table 1. Predicted epitopes of hMPV

Epitope type	Protein	Amino acid sequence	Position
B-cell epitope	F	PSCSGKKGNY	290–299
	F	QLAREEQIENPRQSR	88–102
	F	CQNAGSTVYYPNEKDCETRGDHV	311–333
	F	CNINISTTNYPCKVSTGRHP	350–369
	F	SKVEGEQHVIK	428–438
	F	YITNQDADTVTI	409–420
CTL epitope	N	SPKAGLLSL	307–315 ^a
	M2-2	CYLENIEII	56–64 ^a
	M2-1	GYIDDNQSI	81–89 ^a
	SH	NYIKVENNL	51–59
Th epitope	F	ARAVSNMPTSAGQIK	228–242
	M	FPLFQANTPPAVLLD	36–50

^a Published epitopes [20].

MEP Protein Expression in *E. coli*

The MEP protein was highly expressed in *E. coli* and had the expected size of 40.55 kDa, as verified by SDS-PAGE after induction with IPTG (fig. 2a). The MEP was expressed in both the inclusion body and soluble form (fig. 2a), and the soluble form was purified by passing the crude preparation through an Ni-affinity chromatography column. The purified MEP had a purity of 95% and was analyzed by Western blotting (fig. 2b, c).

Anti-MEP Antibody Titers in the Serum of Immunized Mice

The anti-MEP antibody IgG, IgG1, IgG2a and IgA titers are shown in table 2. All groups elicited potent humoral responses except for the controls. IgG titers were all above 10^4 , and both IgG1 and IgG2a were found to be significant. The IgG1 concentration was a little bit higher than that of IgG2a (IgG1/IgG2a was 1.15–1.37). IgA could be detected in the MEP+Alum and MEP+Alum+CpG ODN groups.

Fig. 3. IFA of the Vero-E6 cells infected with type A hMPV strain (TJ-hMPV-2 stain). **a** Anti-MEP serum reacted with hMPV strain. **b** hMPV monoclonal antibody against hMPV N protein (clone 507, Chemicon) reacted with hMPV strain (positive control). **c** Serum-free PBS reacted with hMPV strain (negative control). Original magnification $\times 200$.

Table 2. Mean titers of anti-MEP antibody in the different groups

Group	IgG	IgG1	IgG2a	IgG1/IgG2a	IgA
MEP+CpG ODN	1.47×10^4	6.8×10^3	2.15×10^3	1.15	<100
MEP+Alum	3.17×10^5	4.64×10^5	1.47×10^4	1.37	147
MEP+Alum+CpG ODN	1.48×10^5	3.16×10^5	4.64×10^4	1.18	2.15×10^3

Reactivity of Anti-MEP Serum with hMPV Strains

IFA showed that anti-MEP serum can react with both type A and type B hMPV strains. The mean IFA titers were both 160 (fig. 3).

Lymphocyte Proliferation and CTL Activity Assay

To evaluate the presence of possible T epitopes recognized in BALB/c mice, splenocyte proliferation and CTL activity assays were carried out. Compared with the controls, the MEP+CpG ODN, MEP+Alum and MEP+Alum+CpG ODN groups showed a significantly ($p < 0.05$) higher proliferation and higher CTL activity (fig. 4, 5).

Splenocyte Cytokine Secretion

The cytokine secretions by mice splenocytes are shown in figure 6. The MEP+CpG ODN and MEP+Alum+CpG ODN groups all showed substantially higher IFN- γ secretion (Th1-type; $p < 0.05$) compared with the controls. In the MEP+CpG ODN, MEP+Alum and MEP+Alum+CpG ODN groups, the secretion of Th2-type cytokines (IL-4, IL-5, IL-10) increased significantly ($p < 0.05$), with IL-10 in the MEP+Alum+CpG ODN showing the greatest increase. GM-CSF (both Th1/Th2) secretions in the

MEP+CpG ODN group increased most significantly ($p < 0.05$). These results indicated that MEP immunized with different adjuvants could elicit both Th1-type and Th2-type immune responses, which influenced the Th1/Th2 response balance.

Microneutralization Assay

The mean anti-MEP serum neutralization titers of MEP+CpG ODN, MEP+Alum and MEP+Alum+CpG ODN are shown in figure 7. The results indicated that anti-MEP sera could neutralize hMPV infection in vitro.

Discussion

hMPV is identical in gene order to the avian pneumovirus (AMPV), which also belongs to the *Metapneumovirus* genus [4]. As hMPV has a major impact on human health, safe and effective vaccines could decrease the disease burden associated with this new pathogen. Due to the complicated immune mechanism of *Paramyxoviridae* family viruses, great difficulties have been encountered in hMPV vaccine development. Virus-inactivated vaccines against paramyxoviruses have been shown to

Fig. 4. Lymphocyte proliferation assay in the different groups. The plots indicate 95% CI (a) and the mean $\pm$ SD (b).

Fig. 5. CTL activity in the different groups. The plots indicate 95% CI (a) and the mean $\pm$ SD (b).

cause vaccine-enhanced disease upon infection in both humans and animals [24, 25].

An epitope-based vaccine has several advantages in that it is safe, toxic-free, stable, easy to produce and offers the opportunity to engineer the epitopes rationally for potency and breadth. Epitope-based vaccines have been

widely reported to show promising results for a range of viral, bacterial and parasitic diseases [26–28]. Recent epitope studies of hMPV have mainly focused on CTL epitopes, which were important to viral clearance. Herd et al. [29] developed a CTL epitope vaccine, and demonstrated that it could elicit CTL- and Th1-type immune responses,

Fig. 6. Cytokine secretion by mice splenocytes. The plots indicate 95% CI (a) and the mean $\pm$ SD (b).

Fig. 7. Serum neutralization antibody titers of the different groups. The plots indicate 95% CI (a) and the mean $\pm$ SD (b).

and that the viral titer was decreased significantly in mice lungs. Later the same team predicted 7 human CTL epitopes, 5 of which were conservative in both A- and B-type hMPV [30]. Rock et al. [31] conducted an extensive prediction of hMPV T-cell epitopes and confirmed these epitopes with verification tests.

Although some CTL epitopes have been identified, many other epitopes are still unknown. Traditional epitope identification methods include chemical or biological methods, peptide probe scanning technology, random peptide library and artificial prediction [32]. The disadvantages of the first three methods, such as being cumbersome, necessitating a heavy workload, being time-consuming and having a high cost, limit their application. The development of bioinformatic technology makes epitope prediction possible. Nowadays, many algorithms and software are developed for T- and B-cell epitope prediction, increasing both efficiency and accuracy significantly [11–19]. In this study, we combined several website tools based on different principles to predict T and B epitopes in order to increase accuracy as much as possible.

For an ideal humoral and cellular immune response, epitope vaccines should contain both B, CTL and Th epitopes. Th cells activated by a vaccine that contains CD4+ Th epitopes may secrete several CTL-inducing or antiviral cytokines, induce CTL responses and maintain CTL memory [33]. Th epitopes may enhance antibody production against the B cell determinant [34], and the linking of a Th epitope to a CTL determinant is effective in the generation of antiviral CTLs [35].

The hMPV genome consists of a single negative strand of RNA of approximately 13 kb, containing eight genes that are presumed to encode nine different proteins [5]. Epitopes from the hMPV N and SH proteins were administered to mice as a peptide vaccine to evaluate the generation of effector and memory cytotoxic lymphocyte and Th1-type cytokine responses, which seem linked to the protection against viral disease [29]. As it is too large (about 2,000 aa), the L protein of hMPV is not suitable for epitope prediction, and without any CTL epitopes in the P protein of human RSV for reference, we selected N, M, F, M2-1, M2-2, SH and G proteins to predict the CTL and Th epitopes. Four CTL and 2 Th epitopes were selected.

Several groups have demonstrated that the viral fusion (F) protein is the most immunodominant hMPV protein [36], and the F protein is highly conserved among hMPV subgroups, with a >95% amino acid similarity between A and B groups and >97% similarity within a single group, suggesting that a broadly neutralizing antibody response

could be elicited if a shared epitope was targeted [37]. In this study, we focused on the F protein to predict B-cell epitopes. In combination, six software applications predicted the results, and 6 peptides were selected as B-cell epitopes.

Spacer introduction is a simple way to design a tandem MEP. KK is an important proteasome for MHC-II-restricted antigen processing [38], and Livingston et al. [39] designed a universal spacer GPGPG. In this study, 6 B-cell epitopes, 4 CTL epitopes and 2 Th epitopes were predicted and connected in series as an MEP. To our knowledge, our study is the first report of tandem assembling of a B, CTL and Th MEP for hMPV. GPGPG and KK residues were introduced as a spacer between adjacent epitopes, which not only minimized junctional epitopes, but also simultaneously augmented proteasome processing, whilst also increasing the immunogenicity [38]. The designed *mep* gene was expressed in *E. coli*. Expressed peptide could stimulate strong and specific antibodies (titer >10⁴), and these antibodies could react specifically with hMPV stains (as shown in fig. 3).

Previous research indicated that the Th2 response could lead to an enhanced severity of infection; however, other research using the human RSV G1F/M2 vaccine induced a balanced Th1/Th2 response that could protect mice from virus challenge [40]. IgG1/IgG2a and Th1/Th2 cytokine secretion results showed that the MEP designed in this study induced a balanced Th1/Th2 response, and microneutralization assay also showed that it could neutralize the hMPV strain in vitro.

Adjuvants can be used to guide and modulate the immune response to a more balanced response and an increased production of neutralizing antibodies. Traditional adjuvants include aluminum hydroxide and Freund's adjuvant, amongst others. More and newer adjuvants have recently been reported. Traditional adjuvants can induce side effects at the injection site, and usually elicit Th2-biased responses. CpG ODN is a newly developed adjuvant that can stimulate a strong immune response and promote a Th1-biased response. Preclinical studies have shown that CpG ODN can increase the immune response effect of bacterial, viral and parasitic vaccines [41]. Meanwhile, CpG ODN can induce high-titer IgA in mucosa, which plays an important role in the prevention and control of viral respiratory infection. In this study, we evaluated the effects of CpG ODN and aluminum hydroxide, and found that their joint use obtained the best effects (more IgG and IgA were produced). As the cytokine results showed, aluminum hydroxide induced a Th2-biased response, while CpG ODN elicits both Th1

and Th2 responses, and the joint use of CpG ODN and aluminum hydroxide produced the highest concentration of Th1 and Th2 cytokines, and also induced more IgA.

In conclusion, we predicted 6 B epitopes, 4 CTL epitopes and 2 Th epitopes of hMPV, and developed an MEP that can elicit strong humoral immunity, a CTL response and balanced Th1/Th2 immunity. MEP can also induce neutralizing antibodies in mice. The joint use of CpG ODN and aluminum hydroxide obtained the best immune effects, and this may lay a foundation for hMPV epitope-based vaccine development.

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Disclosure Statement

No conflicts of interest are declared.

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