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Hepatitis E vaccine candidate harboring a non-particulate immunogen of E2 fused with CRM197 fragment A

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ABSTRACT

The Hepatitis E vaccine (Hecolin, licensed in China) harbors a potent particulate immunogen, p239, designed from a 26-aa N-terminal extension of its poorly immunogenic parental protein, E2. Although an effective vaccine, we sought to design a fusion protein in a non-particulate form that could improve the delivery and immunogenicity of E2 epitopes. The non-toxic mutant of diphtheria toxin, CRM197 (Cross-Reacting Material 197) has been successfully used as a carrier protein for conjugated vaccines to enhance the immunogenicity of polysaccharides. Here, we designed a fusion non-particulate protein of E2 and the catalytic domain (fragment A) of CRM197 and evaluated its antigenicity, immunogenicity and disease prevention efficacy in primates. This fusion protein, named CRM197(A)-E2, was bacterially expressed and purified by chromatography. CRM197(A)-E2 presented as a homodimer in solution, similar to its parental E2 protein, and exhibited excellent antigenicity against representative neutralizing monoclonal antibodies, like E2 and p239. However, CRM197(A)-E2 manifested higher immunogenicity in mice compared with that achieved by the particulate p239, as indicated by the 10-times lower ED_{50} value and 2-log higher HEV-specific antibody level that could persist for at least 28 weeks. In addition, both the 1 μg and 10 μg doses of CRM197(A)-E2 adjuvanted with aluminum could protect vaccinated monkeys against HEV challenge, matching that achieved with only the higher (10 μg) dose of the p239 vaccine. These results suggest that the CRM197 fragment A alone serves as an intra-molecular adjuvant to remarkably enhance the immunogenicity of the target of interest in a non-particulate form. These findings may pave the way for rational vaccine design, especially in cases where particulates are not accessible.

1. Introduction

Vaccination through inoculation is an efficient route for defending against pathogenic infection and the spread of infectious disease. Whereas traditional vaccines use whole-attenuated or killed pathogens, subunit vaccines differ in that they contain only part of the microbial components (Moyle, 2015; Moyle and Toth, 2013). Although subunit vaccines offer improved safety, they are generally less immunogenic compared with whole pathogens. How to enhance the immunogenicity of antigens from viruses or pathogenic bacteria is an important consideration in subunit vaccine design. In general, a subunit antigen candidate with excellent immune properties is selected and incorporated along with a potent immunostimulant(s), which improves its recognition, processing and delivery to the immune system to elicit an effective immune response (Bachmann and Jennings, 2010; Chauhan et al., 2017; Rehm, 2017).

Currently, two approaches can be used individually or combinedly to overcome weak immunogenicity of subunit antigens (Bookstaver

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et al., 2018; Moyle, 2017; Vartak and Sucheck, 2016). The first is to design particulate antigens. Virus-like particles (VLPs), for example—mainly derived from the capsid protein of pathogens—offer good immunogenicity owing to their relatively larger size (tens of nm) and repetitively arrayed epitopes. VLP-based vaccines are used against virus infection, such as from Hepatitis B virus, human papillomavirus, and Hepatitis E virus (Diaz-Arnold and Leary, 1989; Hanna and Bachmann, 2006; Li et al., 2005). The second approach, which is more often used for smaller antigens without the capacity for self-assembly into particles, incorporates potent immunostimulatory components called adjuvants, which are licensed for vaccine formulation, such as traditional aluminum salts, monophosphoryl lipid A (MPL), and squalene (Bonam et al., 2017; Shah et al., 2017). Subunit antigens can also be fused to carrier proteins, as they exhibit intra-molecular adjuvanticity and thereby increase the immunogenicity of the antigen; Diphtheria and Tetanus toxoids, meningococcal outer membrane protein complex (OMPC), and *H. influenza* protein D (HiD) have been used as carrier proteins in licensed vaccines (Pichichero, 2013).

Diphtheria toxin (DT) and its non-toxic mutant, CRM197 (Cross-Reacting Material 197) are well studied (Broker et al., 2011). DT consists of two fragments: fragment A (aa1–190), which contains the catalytic C domain, and fragment B, which consists of the transmembrane (T) and receptor-binding (R) domains. CRM197 harbors a mutation at position 52 on fragment A, which eliminates its cytotoxicity (Malito et al., 2012). However, CRM197 still retains strong immunogenicity and binds the DT-specific receptor, and therefore has been successfully employed in conjugated vaccines. Prevnar 13, for example, used for the prevention of pneumonia, is a conjugated vaccine composed of 13 serotypes of pneumococcal polysaccharide covalently conjugated to CRM197 (Gruber et al., 2012). Coupling to CRM197 (Dagan et al., 2010) aids in the rapid increase in Th1- and Th2-secreting T cells, and their subsequent production of cytokines, which, in turn, induces B cell proliferation and antigen-specific antibody secretion, thereby enhancing the immunogenicity of the polysaccharide to which it is conjugated. The benefit of CRM197 as an intra-molecular adjuvant has been shown with other proteins: Tobias and his group (Tobias et al., 2017) recently showed that a hybrid peptide located on human epidermal growth factor receptor 2 can induce an effective immune response and antitumor activity when grafted to CRM197. Thus, CRM197 has strong immunogenicity, non-toxicity, and can be potentially used as carrier protein.

Hepatitis E is a major public health issue, especially in developing countries, and is caused by infection with the Hepatitis E virus (HEV) (Aggarwal and Krawczynski, 2000; Sridhar et al., 2015). The HEV genome consists of three open reading frames (ORFs), of which the ORF2 encodes the virus capsid protein (Debing and Neyts, 2014). The truncated HEV capsid protein, E2 (aa394–606), naturally forms homodimers through hydrophobic interactions (Zhang et al., 2001) but is poorly immunogenic in mice and monkeys (Li et al., 2005). The p239 peptide from the ORF2 (aa368–606) is a recombinant vaccine against HEV derived from E2 through an N-terminal extension of 26 aa (Li et al., 2015; Zhang et al., 2015). Licensed in China in 2012, this vaccine is a potent particulate antigen. p239 and E2 share a common region (aa459–606) that encompasses numerous immune-dominant neutralizing epitopes and is responsible for virus-host interactions. However, comparably, the E2 protein has poor immunogenicity (Li et al., 2005).

Although the particulate p239 vaccine is effective, we sought to design a fusion protein in a non-particulate form to improve the delivery and immunogenicity of E2 epitopes. In this study, we constructed a fusion protein comprising E2 and the fragment A of CRM197, hereafter referred to as CRM197(A)-E2. We explored the biochemical nature, antigenicity and immunogenicity of the fusion protein in both mice and monkeys as compared with its parental E2 and the particulate p239 immunogen. We further evaluated the protection offered against the development of Hepatitis E disease in monkeys, using the HEV vaccine, Hecolin, for comparison. Our results showed that fragment A of CRM197 can serve as an intra-molecular adjuvant, and paves the way for vaccine design using a high-immunogenic, non-particulate immunogen.

2. Materials and methods

2.1. Construction, expression and purification of fusion protein CRM197(A)-E2

The CRM197(A)-E2 gene was cloned into the pTO-T7 expression plasmid, which was transferred into ER2566 *E. coli* strain for expression of CRM197(A)-E2 protein. The bacteria were centrifuged and lysed, and the CRM197(A)-E2 protein was purified by a three-step purification process following by dialyzing into neutral buffer for renaturation.

2.2. Biochemical and biophysical analysis of CRM197(A)-E2

The sedimentation coefficient of CRM197(A)-E2 was determined by sedimentation velocity experiments using a Beckman XL-A analytical ultracentrifuge. The purity and molecular weight of CRM197(A)-E2 were assessed by SDS-PAGE and gel filtration column using high performance size-exclusion chromatography (HPSEC). Protein morphology was assessed using the Tecnai G2 Spirit transmission electron microscope (TEM). Protein thermal stability was estimated by differential scanning calorimetry (DSC) using a MicroCal VP-DSC instrument.

2.3. Antigenicity of CRM197(A)-E2

The antigenicity of the CRM197(A)-E2 was analyzed by western blotting (WB) and enzyme-linked immunosorbent (ELISA) using a panel of 11 monoclonal antibodies (mAbs), and alkaline phosphate-conjugated goat anti-mouse (GAM-AP) and horseradish peroxidase-conjugated goat anti-mouse (GAM-HRP) were used as secondary antibodies, respectively.

2.4. Immunogenicity of CRM197(A)-E2 and protection study in animals

Half-effective dose (ED_{50}) of CRM197(A)-E2, as well as the persistence of an immune response against the p239 particles, were estimated in Balb/c mice. The immunogenicity and protection efficacy were tested in Cynomolgus monkeys.

Please refer to a detail description on the methods in supplementary materials and methods.

3. Results

3.1. Design and characterization of fusion protein CRM197(A)-E2

The E2 protein of the HEV ORF2 (aa394–606) harbors various neutralizing epitopes but is poorly immunogenic in mice and monkeys (Li et al., 2005). However, p239 created through a 26-aa N-terminal extension of its parental E2 (Fig. 1A), is a potent particulate immunogen. We sought to design a fusion protein in a non-particulate form to improve the delivery and immunogenicity of E2 epitopes.

A fusion protein, CRM197(A)-E2, was constructed by fusing fragment A of CRM197 and E2 using a flexible peptide linker. CRM197(A)-E2, E2 and p239, were expressed in *E. coli* and purified by chromatography. Under mildly reducing SDS-PAGE (Fig. 1B), all three proteins presented predominantly as homodimers, with molecular masses of ~90 kDa, 37 kDa, and 40 kDa for CRM197(A)-E2, E2, and p239, respectively. All three proteins became monomeric after heating at 100 °C for 5 min (Fig. 1B lane H).

Next, AUC and HPSEC were used to analyze the sedimentation coefficients and molecular weights of the purified proteins (Fig. 1C and D). For CRM197(A)-E2, a predominant sedimentation peak was

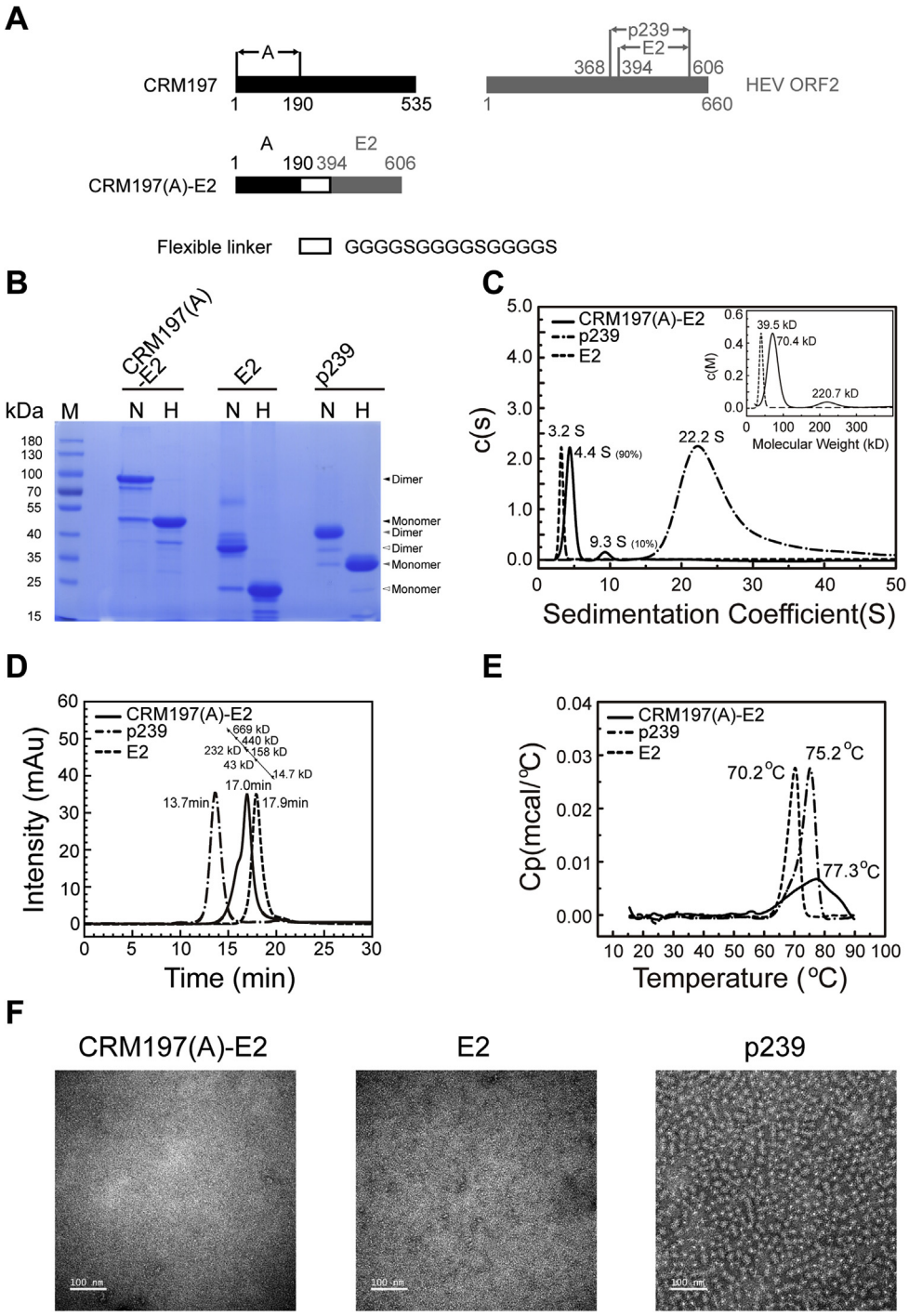

Fig. 1. (A) Schematic map of the CRM197(A)-E2 construct design. Fragment A of CRM197 was fused to the N-terminus of E2 (ORF2, aa394–606) via a flexible linker. **(B)** SDS-PAGE analysis of CRM197(A)-E2, E2, and p239 after purification. Lanes marked with "N" indicate the sample under non-reducing conditions (in the absence of β-mercaptoethanol and not heated). Lanes marked with "H" indicate the sample under reducing conditions (mixed with β-mercaptoethanol and heated at 100 °C for 5 min). The monomer and dimer of CRM197(A)-E2, p239 and E2 were indicated by black arrowheads, gray arrowheads and hollow arrowheads, respectively. **(C)** Purified CRM197(A)-E2, E2, and p239 analyzed by analytical ultracentrifugation. Sedimentation coefficients of CRM197(A)-E2, E2, and p239 particles were determined by sedimentation velocity (SV) tests; molecular weights of CRM197(A)-E2 and E2 in solution were evaluated by the c(M) method. **(D)** The molecular sizes of CRM197(A)-E2, E2, and p239 were evaluated by high-performance size-exclusion chromatography (HPSEC). **(E)** Differential scanning calorimetry (DSC) profiles of CRM197(A)-E2, E2, and p239. The melting transition of CRM197(A)-E2, E2, and p239 were estimated at peak T_m of 77.3 °C, 70.2 °C, and 75.2 °C, respectively. **(F)** The morphology of CRM197(A)-E2, E2, and p239 observed by transmission electron microscopy (TEM).

observed at 4.4 S, which corresponded to the major component. Sedimentation coefficients for E2 and p239 particles were 3.2 S and 22.2 S, respectively. The molecular weights of CRM197(A)-E2 and E2 were 70.4 kD and 39.5 kD, respectively, indicating that both proteins presented as dimers in solution. The retention times for CRM197(A)-E2, E2, and p239 were 17.0 min, 17.9 min, and 13.7 min, respectively (Fig. 1D); this reflects the much smaller molecular size of CRM197(A)-E2 and E2 compared with p239 particles in solution.

In DSC thermograms, we found three major endothermic transitions corresponding to different melting temperatures (Tm) for the three proteins: the first transition was at ~70.2 °C for E2, the second at ~75.2 °C for p239, and the third at ~77.3 °C for CRM197(A)-E2 (Fig. 1E). Similar thermal stabilities were seen for CRM197(A)-E2 and p239, which were slightly better than that for E2. Finally, in negative-stain transmission electron microscopy, only p239 presented as particles, with a diameter of 20–30 nm; no particles were observed in the TEM images for CRM197(A)-E2 or E2 (Fig. 1F).

3.2. Antigenicity of CRM197(A)-E2

To investigate the conformational integrity of the E2 peptide after fusion to the C-terminal CRM197-A fragment, we analyzed the antigenicity of the fusion protein using WB and ELISA, with E2 and p239 serving as controls. Eleven mAbs, recognizing conformational epitopes (mAb 8C11, 8G12, 12A7, 1E8, 9B2 and 12F12) and linear epitopes (16D7, 12A10, 9H7, 3A11 and 1B7), were used. These mAbs were

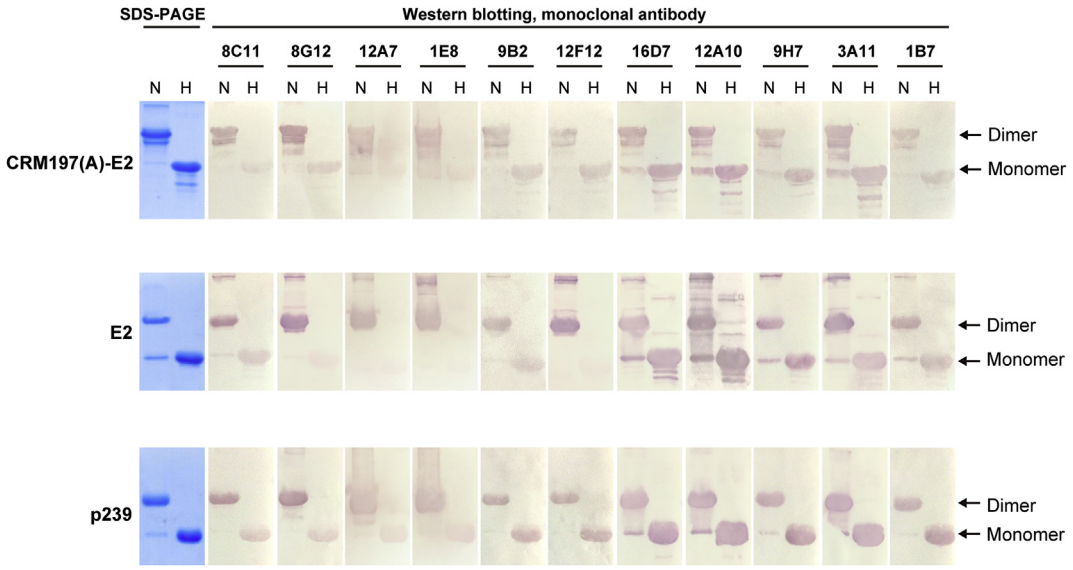

Fig. 2. Western blot analysis of CRM197(A)-E2, E2, and p239 against a mAb panel. Among these mAbs, 8C11, 8G12, 12A7, 1E8, 9B2 and 12F12 recognize conformational epitopes, whereas 16D7, 12A10, 9H7, 3A11 and 1B7 recognize linear epitopes. Lanes marked with "N" indicate samples under non-reducing conditions (in the absence of β-mercaptoethanol and not heated), whereas lanes marked with "H" indicate samples under reducing conditions (mixed with β-mercaptoethanol and heated at 100 °C for 5 min).

previously used to neutralize HEV infection in primates (8C11 and 8G12) and capture hepatitis E viruses in an immune capture assay in vitro (12A10 and 16D7) (Gu et al., 2015; He et al., 2008; Zhang et al., 2005).

In WB analysis, all three proteins showed a similar reactivity pattern with the mAb panel: mAbs recognizing conformational epitopes predominantly interacted with the dimeric form whereas mAbs recognizing linear epitopes reacted with both dimers and monomers (Fig. 2). The antigenicity of the CRM197(A)-E2 protein was further evaluated by ELISA. We found similar EC_{50} values for all three proteins (Fig. 3), suggesting a similar interaction activity. Collectively, these results suggest that key epitopes on the E2 domain are well preserved in the CRM197(A)-E2 fusion protein.

3.3. Immunogenicity of CRM197(A)-E2 and persistence of an induced immune response in mice

The immunogenicity of CRM197(A)-E2 was further evaluated by measuring the half-effective dosage (ED_{50}) in mice as a measure of seroconversion, with a lower ED_{50} indicative of better immunogenicity. In previous studies, we compared the immunogenicity of the aluminum-adjuvanted p239 particle and E2 protein in mice, and found that p239 was 240-times higher than that of the E2 protein (Li et al., 2005). Therefore, here we compared only the fusion protein with p239. Balb/c mice were intraperitoneally immunized with a single dose of aluminum-formulated CRM197(A)-E2 or control Hecolin (containing p239 particles) in serial dilution dosages. Seroconversion was tested using ELISA at 4 weeks post-inoculation. We found that mice in the p239 group had seroconversion rates of 93%, 69%, 50%, 17%, 0% and 0% for 1.6, 0.4, 0.1, 0.025, 0.006 and 0.002 μg dosage, respectively. In contrast, mice in the CRM197(A)-E2 group had seroconversion rates of 100%, 100%, 100%, 100%, 17% and 0% for the same serial dosages, respectively. Using the Reed-Muench method, the ED_{50} values were 0.1 μg for p239 and 0.01 μg for CRM197(A)-E2 (Table 1).

We next estimated the persistence of an induced immune response in mice by monitoring HEV-specific antibody titers over 28 weeks (Fig. 4). The dosages were set as 0.05 μg, 0.5 μg, and 2 μg for both CRM197(A)-E2 and Hecolin (containing p239 particles), and 0.5 μg and 2 μg for E2, because of its poor immunogenicity (Li et al., 2005). Each group of mice (n = 5 per group) was vaccinated thrice at 0, 2, and 4 weeks. As expected, E2 proteins presented with low immunogenicity, with antibody titers of not more than 2 log for both 0.5 μg and 2 μg dosages up to 11 weeks. In comparison, CRM197(A)-E2 and p239 vaccines were highly immunogenic in a dose-dependent manner. CRM197(A)-E2 and p239 vaccines shared similar antibody titers in three dosage immunizations at 1 week after the primary inoculation ($\sim 10^4$ for 2 μg and 0.5 μg, and $\sim 10^3$ for 0.05 μg). The antibody titers in all groups increased sustainably following the two booster immunizations at weeks 2 and 4, except for the group inoculated with 0.05 μg of the p239 vaccine, which remained below $\sim 10^3$ until week 7 and then slowly dropped to less than 10^2 throughout the monitoring period. CRM197(A)-E2 and p239 groups shared similar antibody response profiles; albeit, with different antibody levels: antibody titers reached a maximum level at week 5 after the two booster immunizations and remained high for about 6 weeks, with some decline over time; at week 12, antibody titers decreased and remained stable until week 28.

As to the antibody titers, CRM197(A)-E2 showed a 1-log higher titer than p239 at its maximum value ($\sim 10^9$ vs. $\sim 10^8$) for the 2 μg dosage, followed by comparable titers of $\sim 10^6$ at weeks 14–28. At the lower dosage (0.5 μg), the titer shortfall between CRM197(A)-E2 and p239 increased significantly, with a 3-log higher titer for the maximum value (10^9 vs. 10^6) and a 2-log higher titer at the plateau phase at weeks 14–28 (10^6 vs. 10^4). At the lowest dosage (0.05 μg), CRM197(A)-E2 showed excellent immunogenicity with $\sim 10^8$ titer at the peak value and $\sim 10^6$ at the plateau value versus 10^3 and 10 for p239, respectively, which demonstrated a significant difference between CRM197(A)-E2 and p239 (Fig. 4 and Fig. S1A). In general, mice that were immunized with the CRM197(A)-E2 or p239 elicited an efficient HEV-specific antibody response lasting for at least 28 weeks. The antibody titers induced by CRM197(A)-E2 were 2- to 5-log higher than those induced by p239, with the lower dosage demonstrating the significant advantage of the fusion protein, CRM197(A)-E2. Taken together, the immunogenicity of E2 is significantly enhanced after fusion to fragment A of CRM197, even more so than its particulate form p239 bearing the 26-aa N-terminal extension of E2.

3.4. Disease protection efficacy in monkey model

Finally, we tested the efficacy of CRM197(A)-E2 for hepatitis E prevention in a monkey disease model (Li et al., 2005). Four groups of

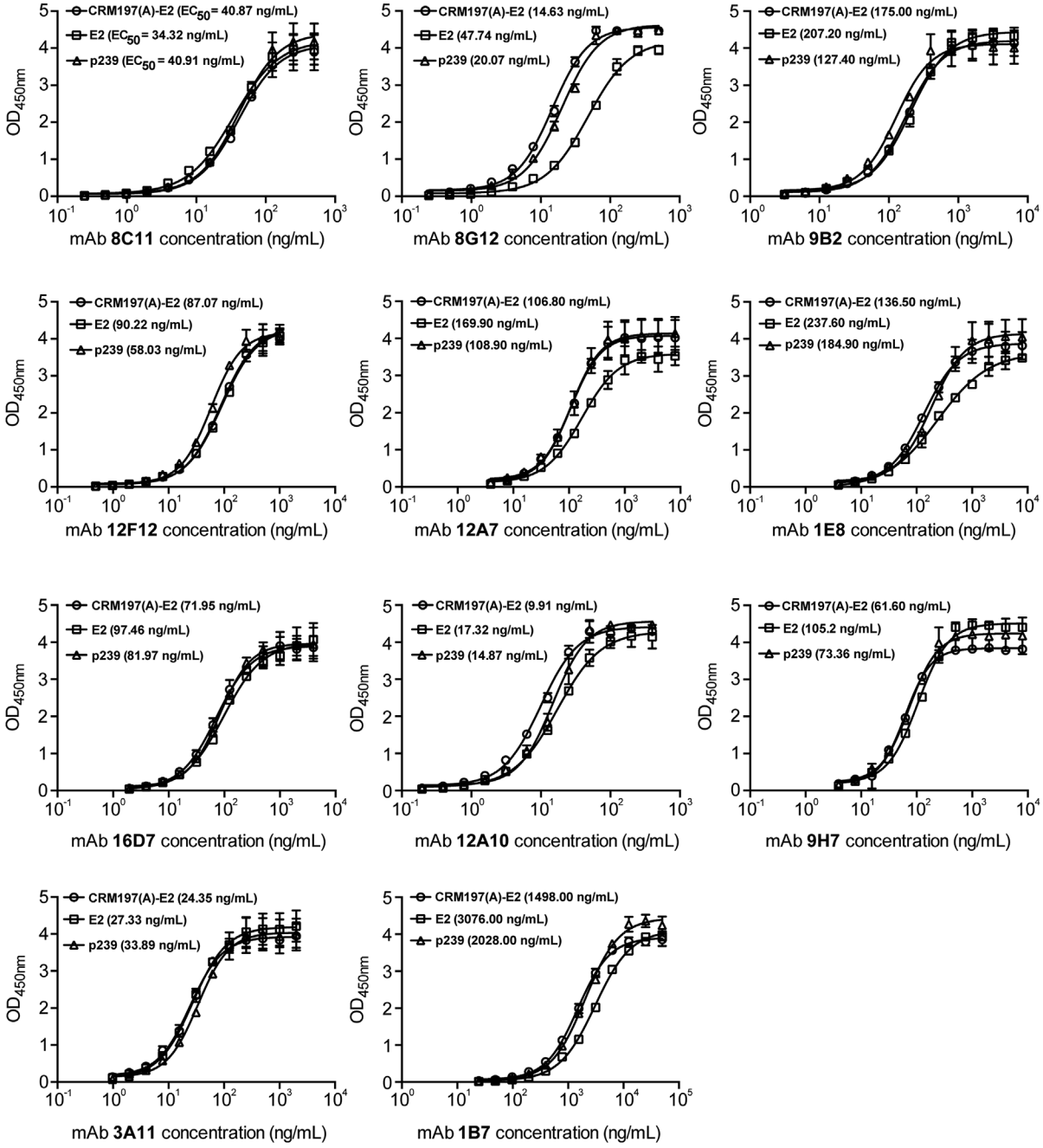

Fig. 3. CRM197(A)-E2 reactivity with 11 mAbs was tested by enzyme-linked immunosorbent assay. EC_{50} values were calculated by sigmoid trend fitting for CRM197(A)-E2, E2 and p239.

Cynomolgus monkeys (3 per group) were intramuscularly immunized: CRM197(A)-E2 (1 μg and 10 μg), p239 (10 μg) and placebo. Monkeys received a prime-boost immunization at weeks 0 and 4, and were then challenged with a 4.2×10^7 genome-equivalent dose of HEV genotype 1 at week 7. Changes in serum ALT levels, virus excretion in stools and anti-HEV antibody responses were measured for 23 weeks.

At week 7, before virus challenge, the production and elicitation of both IgM and IgG were comparable among the three experimental groups, with a peak IgG titer of $\sim 10^4$ and a decreased IgM titer of $\sim 10^2$. After virus challenge, IgM titers further decreased to an average value below 100, whereas IgG titers persisted at a relatively higher level ($\sim 10^4$ to 10^5) for 15–16 weeks (Fig. 5A and Fig. S1B). Upon HEV challenge, the monkeys in the placebo group acquired antibody responses of IgM (10^4) and IgG (10^3) at weeks 9.5 and 10, respectively, and then declined over time. Abnormal ALT levels and virus shedding in stools were measured at 9.5 weeks and persisted for 7 weeks; in this period, peak serum ALT and shedding virus copy numbers were $\sim 152 \pm 12$ IU/L and $10^{6.27 \pm 0.58}$ copies, respectively. In comparison, throughout the entire virus incubation, infection and convalescence stages, none of the monkeys in the experimental groups developed symptoms related to hepatitis E disease, with normal ALT levels (Fig. 5B), and no virus shedding in stools (Fig. 5C). Thus, vaccination of CRM197(A)-E2 in both the high (10 μg) and low (1 μg) dosage groups as well as those in the p239 (high dosage) group could prevent hepatitis E disease and HEV infection in monkeys (Fig. 5B and C).

4. Discussion

Previous studies have shown that CRM197 increases the production of Th1- and Th2-secreting T cells during the immune response and

Table 1
Half-Effective Doses (ED_{50}) in Mice for Aluminum-Formulated CRM197(A)-E2 and p239 Vaccines.

Dose (μg)	CRM197(A)-E2		p239	
	Seroconversion no./ inoculated no.	ED_{50} (μg)[a]	Seroconversion no./ inoculated no.	ED_{50} (μg)[a]
1.600	6/6	0.01	5/6	0.10
0.400	6/6		3/6	
0.100	6/6		4/6	
0.025	6/6		2/6	
0.006	1/6		0/6	
0.002	0/6		0/6	

BALB/c mice were intraperitoneally inoculated once with the indicated doses of aluminum-formulated CRM197(A)-E2 or p239 vaccine and bled 4 weeks later for antibody response.

[a] The ED_{50} values for aluminum-formulated CRM197(A)-E2 and p239 vaccine were calculated according to the method by Reed and Muench.

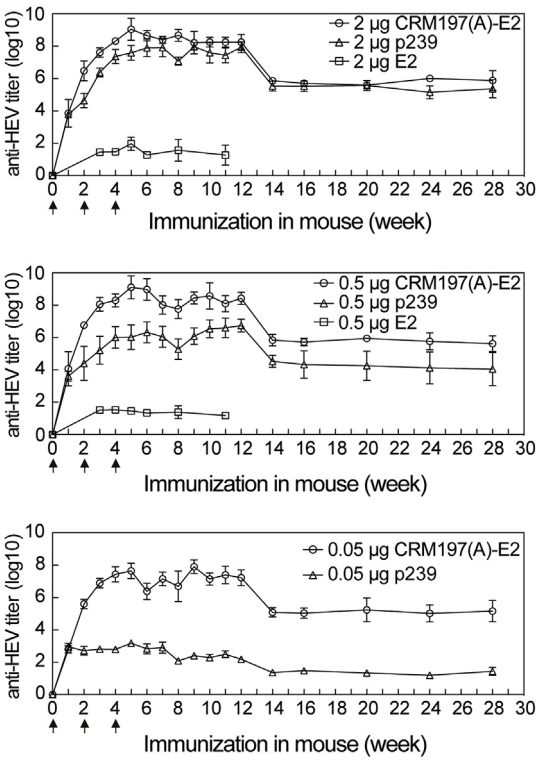

Fig. 4. Antibody production and persistence of an immune response in Balb/c mice. Different dosages of CRM197(A)-E2, E2 and p239 were administered in mice, and the antibody titers were monitored up to 28 weeks. The immunization program was set as weeks 0, 2, and 4, as indicated by black arrows underneath the horizontal axis.

subsequently induces the differentiation and maturation of B cells by heterogeneous cytokines. Therefore, a stronger antibody response and immunological memory are elicited when the CRM197 carrier protein is combined with a capsular polysaccharide (Guttormsen et al., 1999; Kamboj et al., 2001). CRM197 has been used as a carrier protein in several licensed conjugated vaccines, including PREVNAR7, PREVNAR13 and HibTITER. In these vaccines, the polysaccharide is chemically covalently linked to the CRM197 protein. However, the immunogenic properties associated with combining CRM197 or the CRM197 functional domain with another protein via genetic fusion have not been completely evaluated. Fragment A, as the catalytic domain of DT, plays a pivotal role in cytotoxicity. Although the amino acid mutation at position 52 renders it enzymatically inactive, fragment A of CRM197 still retains the ability to bind the DT-specific receptor and stimulate a humoral and cellular immune response in the human body. Thus, we sought to determine whether the functional domain of CRM197 alone (i.e., fragment A) could function as a carrier protein.

The HEV vaccine, developed based on p239 particles, is of good efficacy, immunogenicity and safety, and was licensed in China in 2012. p239 (aa 368–606) and E2 (aa 394–606) share a common region of ORF2, referred to as E2s (aa 459–606), which harbors the major antigenic determinants of the HEV vaccine and is responsible for virus-host interactions. Due to its intrinsic self-assembly property, p239 can form particles, making it an excellent immunogen for immune recognition and antibody elicitation. In this study, we attempted to design a non-particulate immunogen by fusing E2 to the minimized functional fragment of CRM197, and evaluated whether this immunogen maintained the good antigenicity and immunogenicity of p239. As expected, CRM197(A)-E2 mainly associated as a homodimer in solution and conferred high immunogenicity in mice. However, CRM197(A)-E2 showed 10-times stronger immunogenicity than that of p239 in the ED_{50} assay, and elicited a 10- to 1000-fold higher antibody titer at the same dosage (2 μg and 0.5 μg) as p239. Even at lower dosages (0.05 μg), CRM197(A)-E2 still induced an antibody titer as high as 10^8 that persisted at 10^6 for at least 28 weeks, compared with the relatively much lower immunogenicity of p239 at no more than 10^3 titer and less than 10^2 over the long term, coming to levels as low as that of E2, which is known to have poor immunogenicity. Taken together, the CRM197 A-fragment can enhance the immunogenicity of the E2 dimer better than p239, which particulates E2 via the N-terminal hydrophobic tail (Zhang et al., 2016).

In vaccine manufacturing, the immunogen is generally produced along with a proper adjuvant to enhance the immunogenicity of the vaccine for long-term protection efficacy. The aluminum-based adjuvant is most frequently used in licensed human vaccines that are required for a long duration, because the aluminum salt functions as an antigen delivery system and promotes a strong humoral immune response. However, there are limitations in using aluminum-based adjuvants in vaccines that need to stimulate a robust cellular immune response. In contrast, liposome-based adjuvants accompanied by immunostimulatory molecules, such as the AS01 adjuvant system, not only promote an antigen-specific B cell response but also activate T cells for the production of various cytokines. It is well-known that virus-like particles possess ideal immunogenicity in terms of their nano-scale and multiple repetitive epitope dosage. However, numerous immunogens are incapable of self-assembly and are poorly immunogenic, as exemplified by the HIV ENV and Flu HA proteins. In this study, we propose fragment A of CRM197 as an intra-molecular adjuvant carrier protein that can be fused to non-particulate antigens in regular genetic manipulation to substantially enhance the immunogenicity of the E2 protein. Our findings may provide a paradigm for non-particulate subunit vaccine design.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

S.L. and Q. Zhao designed the study. K.W., L.Z., X.Z., C.S., T.C., J.L., and M.Z. performed experiments. K.W., L.Z., X.Z., Y.W., Q. Zheng, Z.Z., H.Y., T.W., Y.G., J.Z., Q. Zhao, S.L. and N.X. analyzed data. K.W. and S.L. wrote the manuscript, S.L. and N.X. approved the manuscript. All authors contributed to experimental design.

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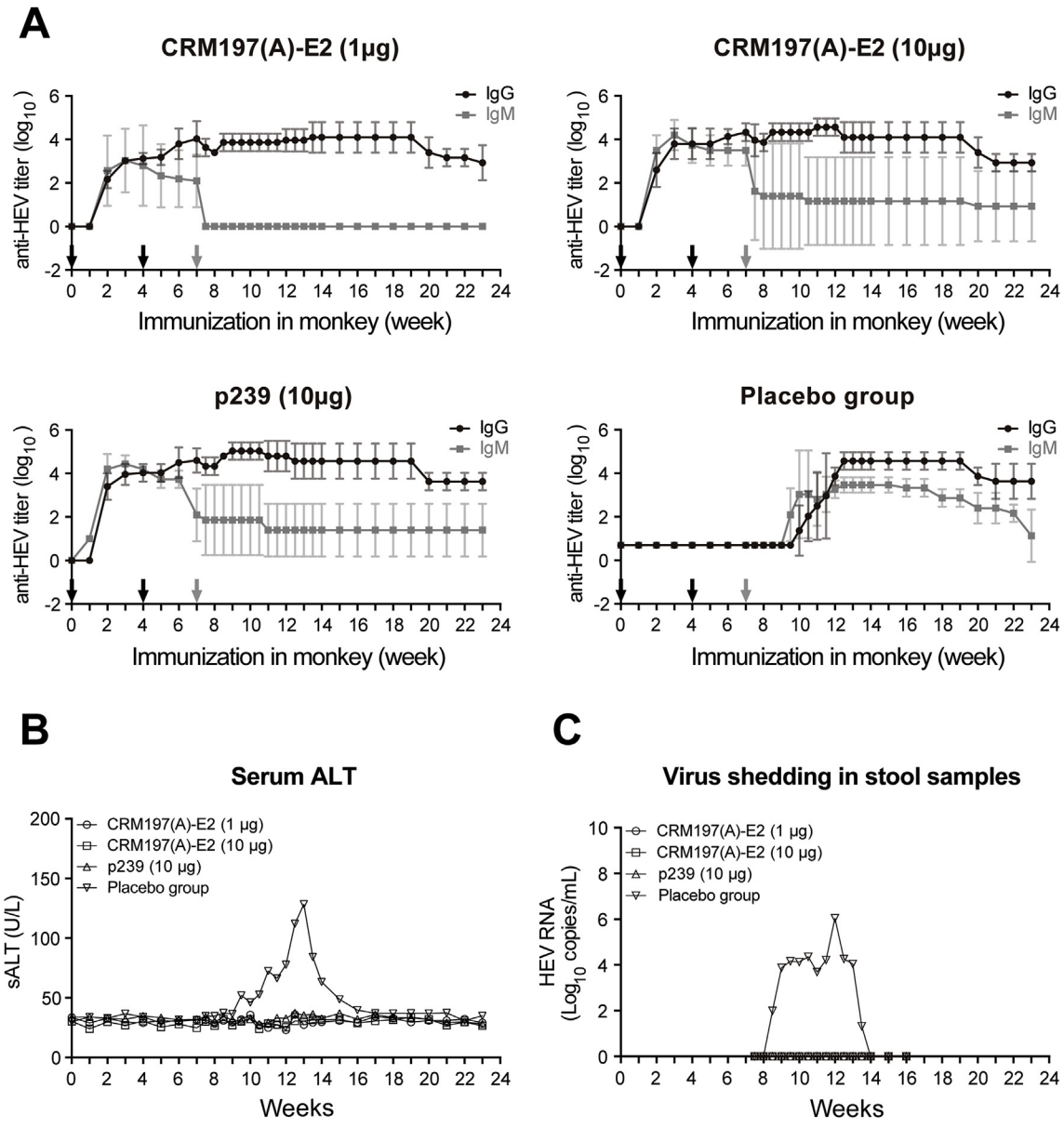

Fig. 5. The prevention efficacy of CRM197(A)-E2 against hepatitis E disease and HEV infection in a monkey model. p239 and placebo served as positive and negative controls, respectively. The time points of inoculation and virus challenge are indicated by black and gray arrows, respectively. **(A)** The IgG/IgM antibody production profiles. **(B)** Serum alanine aminotransferase (ALT) levels and **(C)** virus shedding in stool samples plotted against immunization time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.02.013.

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