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Virus-like particle vaccine induces cross-protection against human metapneumovirus infections in mice

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ARTICLE INFO

Article history:

Received 16 October 2012

Received in revised form 17 January 2013

Accepted 28 March 2013

Available online 11 April 2013

Keywords:

Metapneumovirus

Vaccine

Virus-like particle

Neutralization

Antibody

ABSTRACT

Human metapneumovirus (HMPV) is a paramyxovirus that causes acute respiratory-tract infections in children and adults worldwide. A safe and effective vaccine could decrease the burden of disease associated with this novel pathogen. We engineered HMPV viral-like particles (HMPV-VLPs) derived from retroviral core particles that mimic the properties of the viral surface of two HMPV viruses of either lineage A or B. These VLPs functionally display F and G HMPV surface glycoproteins. When injected in mice, HMPV-VLPs induce strong humoral immune response against both homologous and heterologous strains. Moreover, the induced neutralizing antibodies prevented mortality upon subsequent infection of the lungs with both homologous and heterologous viruses. Upon challenge, viral titers in the lungs of immunized animals were significantly reduced as compared to those of control animals. In conclusion, a HMPV-VLP vaccine that induces cross-protective immunity in mice is a promising approach to prevent HMPV infections.

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1. Introduction

Human metapneumovirus (HMPV) is a newly described member of the pneumovirinae subfamily [1]. HMPV is related to respiratory syncytial virus (RSV), which is the most significant respiratory pathogen of infancy and early childhood. Epidemiologic studies showed that HMPV is associated with significant morbidity in infants and other high-risk populations, such as immunocompromised patients [2–5]. Thus, HMPV is an important cause of hospitalizations, in the same way as the other

respiratory viruses, RSV, parainfluenza virus or influenza virus [6].

HMPV isolates can be separated into two major lineages (A and B). Nucleotide and amino-acid sequence identities between HMPV lineages A and B are 80 and 90%, respectively [7]. HMPV displays 3 surface glycoproteins (GP): F, responsible for membrane fusion, G involved in cell attachment and SH, a small hydrophobic protein. Importantly, antibodies induced against F correlate with protection in animal models [8–10].

As HMPV has a major impact on human health, safe and effective vaccines could decrease the disease burden associated with this new pathogen. Since the discovery of HMPV [1], several studies have focused on vaccine development. Different strategies have been investigated, such as inactivated virus, live-attenuated virus, recombinant proteins, or DNA encoding viral proteins. A major challenge is the difficulty to induce strong and long-lasting immune responses through the development of a sufficiently safe vaccine. Indeed, recombinant proteins do not always induce potent immune responses, live-attenuated viruses present reversion risks and inactivated vaccines often lead to enhanced diseases. Thus, alternative HMPV vaccine candidates are required to induce sustained immune responses and, specifically, high-titer neutralizing antibodies.

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incubated at 37 °C. Neutralization titers were defined as the highest dilution of antibody at which culture wells were negative for infection.

2.9. Virus titers in lungs

On day 5 post-infection, which is the time of maximal viral replication in BALB/c mouse lungs [21], animals were euthanized and their lungs were removed and snap frozen in liquid nitrogen. Subsequently, lungs were homogenized in PBS, and viral titers were determined as described previously [21]. Briefly, 10-fold serial dilutions of lung homogenates were performed in 24-well plates containing LLC-MK2 cells. Infected plates were incubated at 37 °C with 5% CO₂ and replenished with 1 µl fresh trypsin (0.0002%) every other day. Virus titers were reported as log₁₀(TCID₅₀)/ml lung homogenate. TCID₅₀ values were calculated by the method of Reed & Muench.

3. Results

3.1. Incorporation of F and G glycoproteins into retroviral VLPs

Incorporation of F and G glycoproteins of HMPV strain C-85473 could be detected on retrovirus-derived VLPs (Fig. 1). G protein was detected at a molecular weight of about 100 kDa for both digested and not digested VLPs. The uncleaved F0 form was detected at the expected molecular weight of 60 kDa, while the active F1 form was detected at a molecular weight of 47 kDa for trypsin-digested VLPs (Fig. 1). Thus, for all the subsequent experiments, HMPV-VLPs were activated by trypsin digestion. We noticed that F incorporation

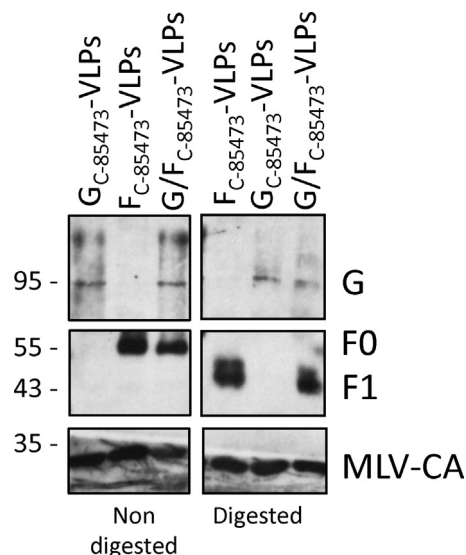


Fig. 1. Production and characterization of human metapneumovirus virus-like particles (HMPV-VLPs). 293T cells were co-transfected with four expression vectors encoding the murine-leukemia virus (MLV) Gag-Pol proteins, a packaging-competent MLV-derived genome encoding the green fluorescent protein (GFP) and the F envelope glycoprotein and/or G envelope glycoprotein from the C-8547. VLPs were harvested from the supernatant of transfected cells and were digested with trypsin. Biochemical analysis of HMPV-VLPs purified by ultracentrifugation over a sucrose cushion. Upon separation by SDS-PAGE, the incorporation levels of F and G on MLV core particles were determined by Western blot analysis using polyclonal sera against HMPV C-85473 strain and MLV capsid (CA) protein. HMPV-VLPs were analyzed before and after trypsin digestion, as indicated.

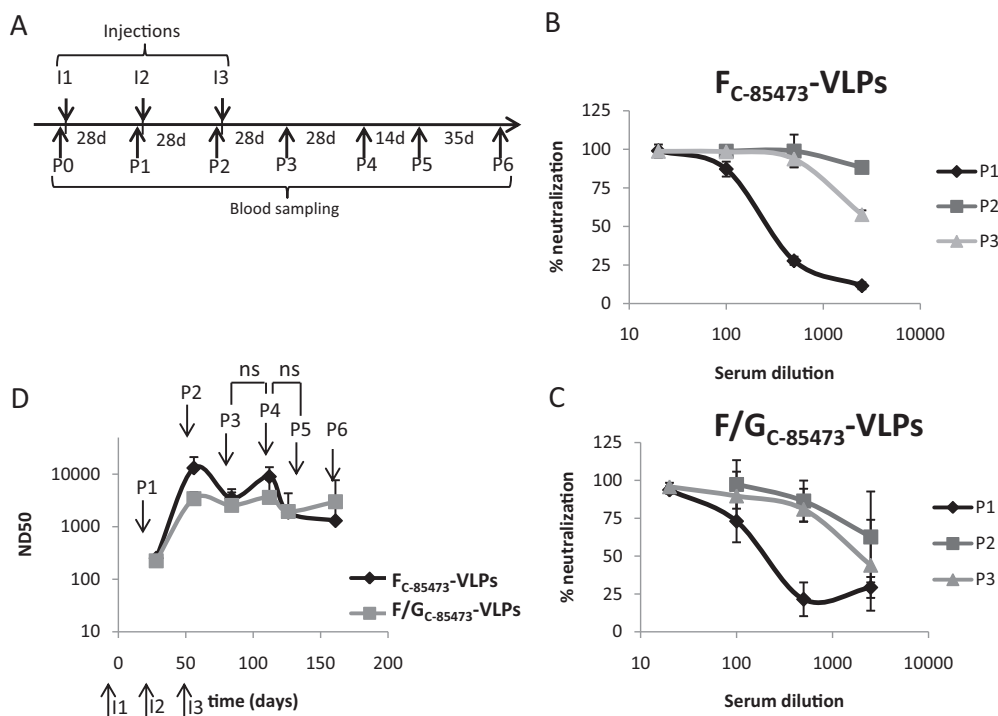


Fig. 2. Humoral immune responses induced by HMPV-VLPs. (A) Outline of the immunization protocol. BALB/c mice were inoculated 3 times (I1 to I3), at 28-days intervals, by IP injection HMPV-VLPs displaying F, G, or F/G HMPV glycoproteins from HMPV strain C-85473. At different time points following immunization, blood samples were harvested (P1 to P6) to determine the production of neutralizing antibodies. The neutralization curves of sera from mice immunized with F-VLPs (B) and F/G-VLPs (C), harvested 28 days after each injection (P1, P2 and P3), were determined for different serum dilutions (1/20, 1/100, 1/500 and 1/2500). The results are expressed as the neutralization levels (mean $\pm$ SD; $n = 4$) determined in the immune sera relative to P0 pre-immune sera. Curves in graph B were compared with corresponding curves in graph C using a paired Student's *t*-test and did not show any significant difference. (D) Dilution allowing 50% neutralization (ND50) obtained via immunization with F-VLPs and F/G-VLPs were deduced from the neutralization curves that were determined until P6, i.e. 161 days after the first immunization (A). (ns), no statistically significant difference ($p \geq 0.05$).

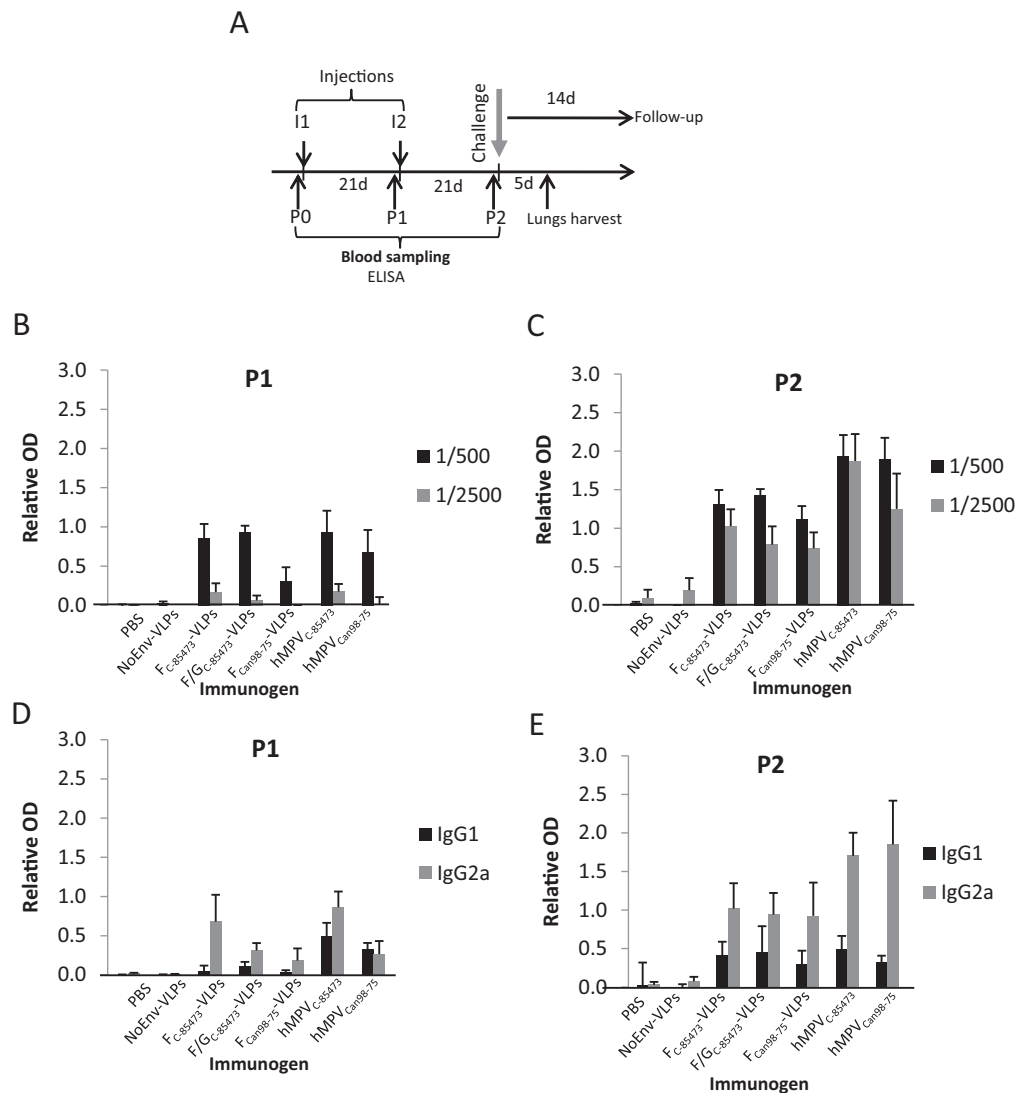


Fig. 3. Induction of IgG in the serum of VLP-vaccinated mice. (A) Outline of the immunization protocol. BALB/c mice were vaccinated twice (I1 and I2), at a 21-days interval, with HMPV-VLPs displaying F, G, or F/G HMPV glycoproteins from either C-85473 or Can98-75 strains. Control mice were vaccinated with PBS only, with VLPs devoid of glycoproteins (NoEnv-VLPs) or were infected intranasally with live C-85473 or Can98-75. Twenty-one days after each inoculation, blood samples were harvested (P1 and P2) to determine the production of antibodies. Twenty-one days after the last immunization, mice were intranasally infected with C-85473 or Can98-75 HMPV (challenge). An HMPV-F_{C85473} ELISA was performed on the blood samples to detect F antibodies using F_{C85473}-VLPs as antigen, and anti-IgG (B and C), anti-IgG1 and anti-IgG2a (D and E) as secondary antibody.

was reduced by 25–40% when G was co-incorporated. An identical strategy was used for F and G from HMPV strain Can98-75 (B lineage).

Moreover, we demonstrated that F incorporated on VLPs was correctly folded and functional (Supplemental Figure 1). Indeed, when the HMPV-VLPs were generated with replication-defective viral components encoding a GFP marker, their cell entry capacity could precisely [23] be determined by FACS analysis. Specifically, we found that HMPV-VLPs harboring F and G proteins (F/G-VLPs) or F alone (F-VLPs) from either C-85473 or Can98-75 strains were functional and mediated robust entry into cells.

3.2. HMPV-VLPs induce humoral immune responses

To analyze the humoral immune responses of immunized mice, we developed here a VLP-micro-neutralization assay. Indeed, such VLP-based assays are particularly sensitive, flexible, robust and reproducible and allow a large screen of multiple serum samples [15,24,25]. We found that P1 sera, harvested 4 weeks after the first injection of F_{C85473}-VLPs (Fig. 2A and B), had

significant neutralizing activity, with over 75% neutralization obtained at 1/100 dilution. The P2 and P3 sera had much higher neutralizing activities, allowing over 95% neutralization even at 1/500 dilution. Of note, the third injections did not increase neutralization. The neutralization curves obtained for sera of mice injected with F/G-VLPs exhibited a similar neutralizing activity as compared to those obtained with F-VLPs (Fig. 2C), while sera of mice injected with G-VLPs did not show any neutralizing activity (Supplemental Figure 2). In order to evaluate the persistence of neutralizing antibodies over time, we harvested sera at 3 other time points (P4, P5, P6), to up to 161 days after the first immunization. The dilutions allowing 50% neutralization (ND50) were calculated and showed. The apparent increase of ND50 at P4 compared to P3 was not statistically significant and, despite a slight decrease, high-level neutralizing antibodies persisted for up to 5 months (Fig. 2D). Immunization with F_{Can98-75}-VLPs similarly induced neutralizing antibodies (Supplemental Figure 3).

Altogether, these results indicated that retroviral-derived VLPs incorporating F alone or in combination with G induced

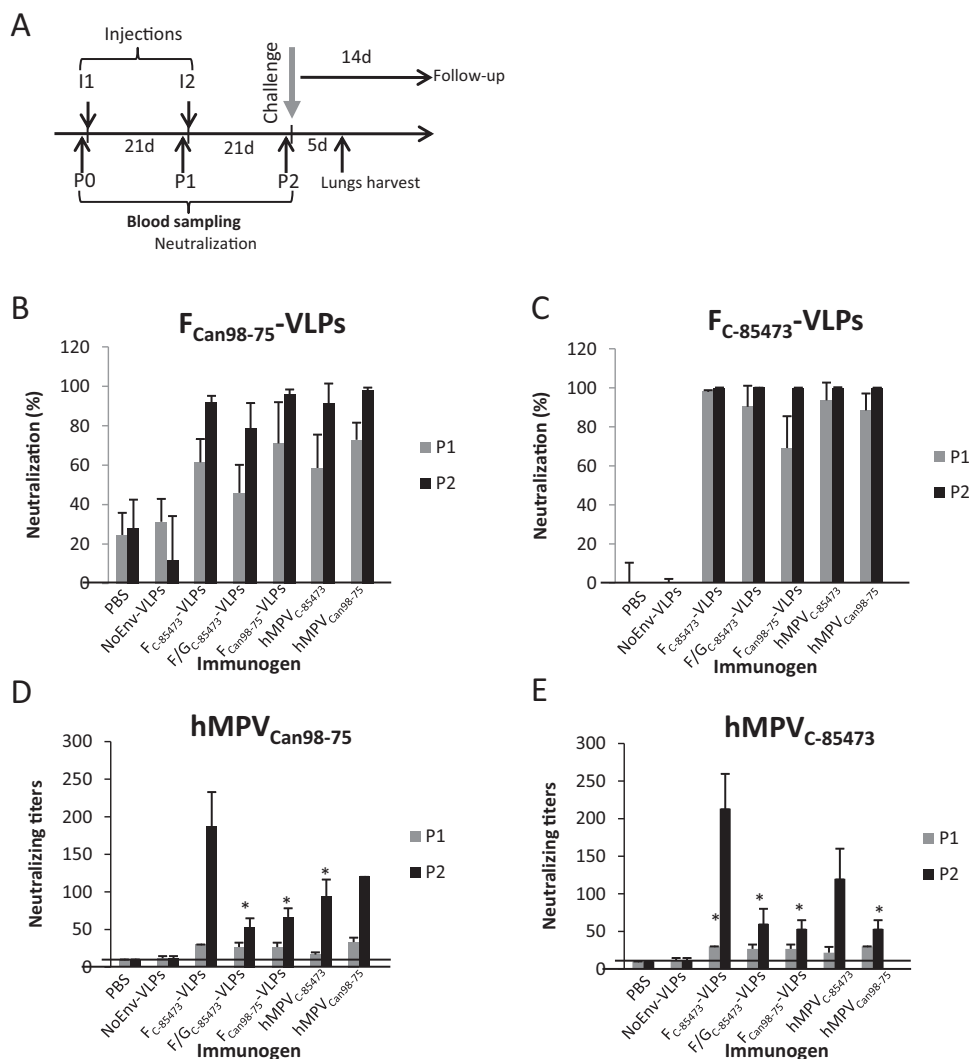


Fig. 4. Induction of neutralizing antibodies in the serum of VLP-vaccinated mice. (A) Outline of the immunization protocol. In a VLP microneutralization assay, the neutralization efficiency was determined for 1/100 dilution of serum samples using F_{Can98-75}-VLPs (B) or F_{C-85473}-VLPs (C) incorporating a GFP marker gene. Results are expressed as percentages of neutralization (mean standard deviation; $n=6$) of the transduction titers relative to P0 pre-immune serum samples. Neutralizing antibodies present in P1 and P2 serum samples were evaluated by an HMPV neutralization assay against Can98-75 (D) and C-85473 (E). Neutralization titers were defined as the highest dilution of antibody at which culture wells were negative for infection. (*) Statistically significant difference ($p < 0.05$) between F_{C-85473}-VLPs mice and other groups of mice (Student's t -test).

neutralizing antibody responses in mice. Maximal levels of neutralization could be reached after 2 consecutive injections.

3.3. HMPV-VLPs induce a IgG2a-dominant antibody response

Serum samples (P1 and P2) of mice inoculated with F_{C-85473}-VLPs, F/G_{C-85473}-VLPs or F_{Can98-75}-VLPs, or infected with HMPV 85473 or Can98-75 were harvested 21 days after each immunization to evaluate IgG, IgG2a and IgG1 antibody production, using an ELISA with F_{C-85473}-VLP-coated microplates (Fig. 3A). High-level total anti-F IgGs were induced after boost in all mouse groups, with relative ODs 40–60 fold higher for sera from the immunized animals compared to controls (Fig. 3B and C). Low IgG1 antibody responses were observed both after prime and boost injections in contrast to the high levels of IgG2a antibody that increased significantly (Fig. 3D and E).

These results confirmed that HMPV-VLPs are immunogenic and induce IgG2a-dominant responses.

3.4. HMPV-VLPs induce cross-neutralizing antibodies

To compare the immunogenicity of both F proteins, cross-neutralizing antibodies against F_{C-85473}-VLPs or F_{Can98-75}-VLPs were determined using our VLP micro-neutralization assay (Fig. 4A–C). After the first injection, all vaccinated mice developed neutralizing antibodies against VLPs carrying the homologous and heterologous F proteins. After the boost injection, the neutralization levels reached almost 100%, in agreement with above results (Fig. 2).

Then, we determined if these antibodies could cross-neutralize live virus using a HMPV-neutralization assay with C-85473 and Can98-75 HMPV strains (Fig. 4D and E). HMPV neutralizing-antibody titers were determined by end-point dilution assays. Similar to the VLP-micro-neutralization assay, the results showed that sera from all mice immunized twice with VLPs displaying C-85473 or Can98-75 glycoproteins neutralized both HMPV strains and therefore validated our micro-neutralization assay. Neutralization levels were higher in the case of F_{C-85473}-VLP-immunized mice suggesting an higher immunogenicity of F_{C-85473}, perhaps owing to a better incorporation of this F GP on the VLPs.

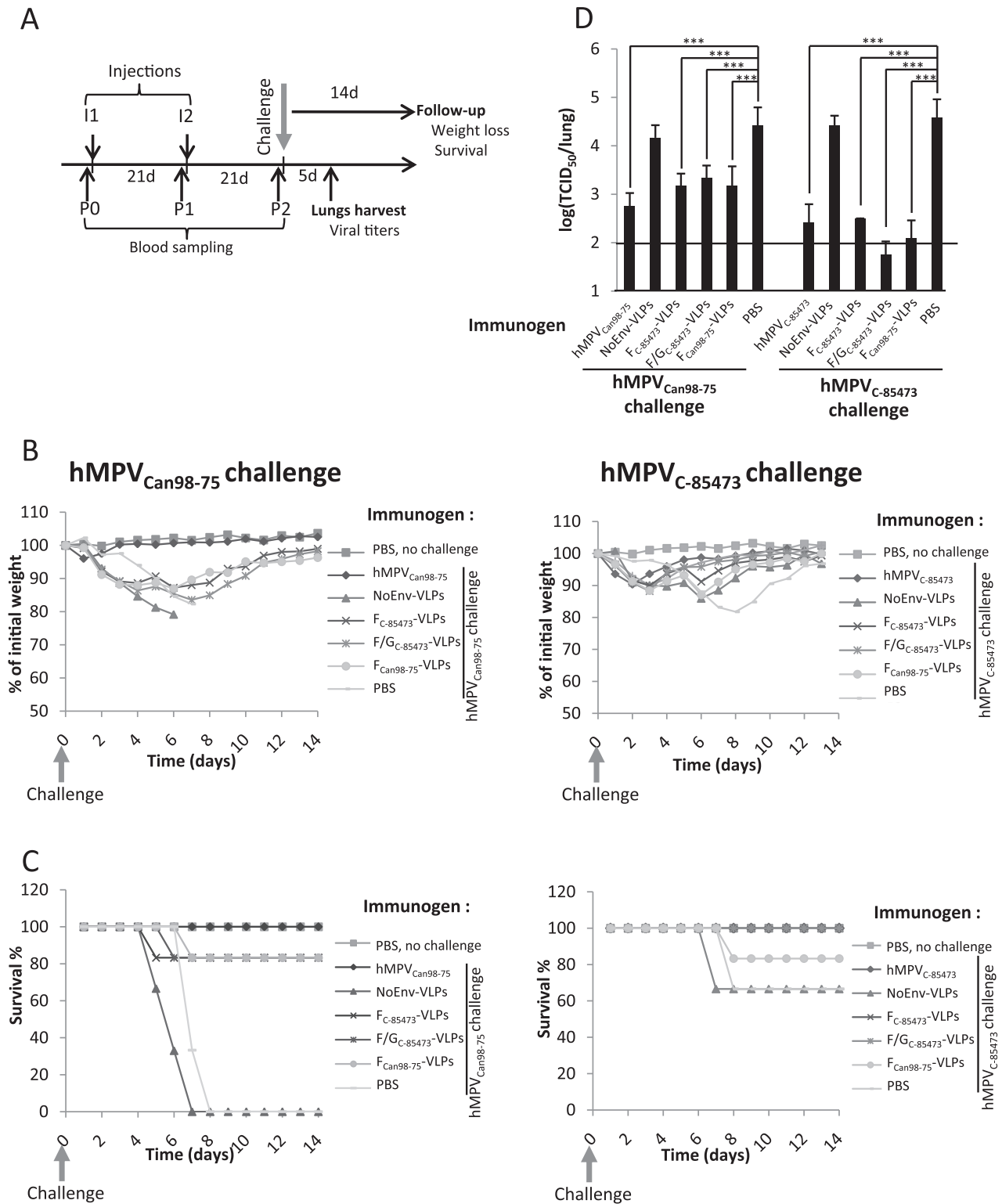


Fig. 5. Protection of HMPV-VLP-vaccinated mice against HMPV challenge. (A) Outline of the immunization protocol. Average weight losses (B) and survival rates (C) of immunized mice after challenge. The results were obtained from 6 mice per group, monitored for 14 days post-challenge with HMPV Can98-75 (left panels) or C-85473 (right panels). Naive mice, inoculated with PBS and not challenged, were included as a control group. (D) Infectious virus titers in the lungs of mice challenged with HMPV Can98-75 or C-85473, as indicated. Lungs were collected on day 5 post-infection and virus present in tissues was quantified by serial dilutions on LLC-MK2 cells. Virus titers were reported as $\log_{10}$ TCID₅₀ (ml lung homogenate)⁻¹. (****) Statistically significant differences ($p < 0.001$) between indicated groups (Student's t -test).

Altogether, these results showed that injection of VLPs displaying HMPV glycoproteins from either lineage A or B induces cross-reactive antibody responses that can efficiently neutralize infectious HMPV.

3.5. Immunization with HMPV-VLP protects mice against HMPV infection

Infection by the Can98-75 strain led to 100% mortality by day 8 after challenge in control mice inoculated with PBS as well as with NoEnv-VLPs (Fig. 5A–C, left panels). In contrast, mice previously infected with the homologous virus, Can98-75, recovered and survived the challenge. Similarly, most mice immunized with HMPV-VLPs survived the challenge, indicating a cross-protection induced by immunization by VLPs harboring non-homologous F or F/G glycoproteins. On the other hand, challenge with the C-85473 strain of HMPV was sub-lethal due to a slightly lower inoculum than that of Can98-75 virus (as determined by back titration studies), since mice immunized with PBS only or with NoEnv-VLPs exhibited profound weight loss yet with 67% survival by day 8 post-challenge (Fig. 5B and C, right panels). In contrast, mice previously immunized with the homologous virus, C-85473, or with homologous or heterologous HMPV-VLPs exhibited partial weight loss and survived the challenge.

At day 5 post-challenge, HMPV present in the lungs of 6 mice per group was quantified. For mice challenged with the C-85473 virus, almost no virus was detected in lungs from animals previously infected with homologous virus, or immunized with homologous F_{C-85473}-VLPs and F/G_{C-85473}-VLPs or with heterologous F_{Can98-75}-VLPs, in contrast to naïve mice or to mice injected with NoEnv-VLPs (Fig. 5D). As for Can98-75 challenge, residual viruses were detected among all groups of immunized mice, but the amount of virus was significantly lower than that present in control mice (Fig. 5D).

Altogether, these results demonstrated that HMPV-VLPs injection induce cross-protection in mice.

4. Discussion

The development of vaccines that prevent serious HMPV respiratory tract disease in young children, immuno-compromised individuals and the elderly is of utmost importance. In this study, we used a new approach to develop an HMPV vaccine based on HMPV-VLPs that rely on the incorporation of HMPV glycoproteins from either of the two main lineages onto retroviral core particles. The immunogenicity of VLPs displaying the F and/or G HMPV glycoproteins was evaluated in mice. We showed that HMPV-VLPs stimulated cross-protective humoral immune responses. More importantly, immunization of mice with HMPV-VLPs resulted in protection of animals against lethal infection.

VLPs may represent effective and highly safe vaccines, although this strong potential remains to be fully exploited. While the production of VLPs based on the expression of RSV structural proteins alone was inefficient [26], VLPs based on assembly of RSV glycoproteins onto heterologous systems, such as viral cores derived from Newcastle disease virus [26,27] or from influenza virus [28] were efficiently produced. Such VLPs displaying RSV-F and/or RSV-G induced neutralizing antibodies and protected mice against RSV infection. Alternatively, recent studies have underscored the successful use of retrovirus-derived VLPs as a vaccine platform that can specifically induce neutralizing antibody responses against HCV [16] or influenza virus [14]. Thus, the further development of such VLPs represents a promising approach for pneumovirinae vaccines. Here, we have developed a novel HMPV vaccine candidate based on retroviral cores. These HMPV-VLPs are efficient immunogens and

elicit robust and cross-reactive humoral responses. They exhibit attractive features for their further development as HMPV vaccine candidate.

First, the incorporation of F alone on VLPs was sufficient to induce neutralizing antibodies whereas co-incorporation of F and G proteins did not improve the yield of neutralizing antibodies. This confirms previous studies showing that anti-G antibodies do not neutralize HMPV [29,30] whereas F is a major protective antigen [8,9]. Moreover, immune responses that we observed were IgG2a-dominant, suggesting a Th1-oriented response that could circumvent issues of enhanced disease associated with inactivated vaccine [31].

Second, HMPV-specific neutralizing antibody titers induced by our F-VLPs or F/G-VLPs persisted at high levels for several months. Whether this persistence can induce long-term protection will be the scope of our further studies in large animal models. Indeed, HMPV-infected patients exhibit a transient production of HMPV specific antibodies [32], which is reproduced in experimentally-infected macaques that showed a decrease over time of the protective immunity induced by HMPV infection [33].

Third, we showed that HMPV-VLPs displaying F from either lineage A (C-85473) or B (Can98-75) of HMPV induced cross-neutralizing antibodies and cross-protection. This result is consistent with the fact that cross-protection was detected when animals were immunized with HMPV particles from one lineage and challenged with HMPV of another lineage [8,33,34]. In contrast, soluble F protein used as immunogen induced only partial cross-neutralization between the two HMPV lineages in hamsters [35] and did not protect macaques [36]. Our VLPs displaying HMPV-F protein most probably induced potent cross-neutralizing antibody responses because the incorporated F glycoprotein was expressed in its native and functional conformation.

Finally, when mice vaccinated with HMPV-VLPs were challenged with HMPV, they survived the challenge and virus replication in the lungs was strongly reduced. Moreover, although HMPV Can98-75 was very virulent (due in part to a higher inoculum) and although residual virus was still detected in the lungs after 5 days post-challenge, most vaccinated animals survived this challenge in contrast to naïve mice. Importantly, in agreement with the results of neutralization assays, no difference was observed for HMPV-VLPs incorporating F protein of either lineage A or B, which demonstrated cross-protection. Similar to the results of neutralization assays, co-presentation of G and F proteins on the VLPs did not influence the yield of protection *in vivo*. Thus, VLPs incorporating F from any lineage seemed sufficient to confer cross-protection against HMPV challenge. Of note, high-level neutralizing antibodies were induced by HMPV-VLPs that were injected without adjuvant. This contrasted with the finding that soluble F, used in several previous vaccine studies, was only protective in the presence of strong adjuvants [35–37].

In conclusion, using VLPs displaying HMPV-F glycoproteins, we were able to induce high-titer neutralizing antibodies that protected animals against a lethal HMPV challenge. This study underlines the potential of VLPs as a vaccination strategy against HMPV. Besides, HMPV-VLPs could easily be adapted to take advantage of the promising approach of DNA immunization. Indeed, genetic information necessary to produce HMPV-VLPs (glycoproteins and structural Gag protein) can be encoded on a single minimal expression vector [15,38]. Resulting vaccines would be inexpensive, easy to produce and easy to store.

Acknowledgements

We acknowledge the contribution of the AniRA platform (PBES animal facility and cytometry) of SFR BioSciences Gerland-Lyon

Sud (UMS3444/US8). This work was supported by the European Research Council [ERC-2008-AdG-233130-HEPCENT to F.-L.C.] and by the LABEX ECOFACT (ANR-11-LABX-0042) of Université de Lyon. C.L. was supported by a pre-doctoral fellowship from the French Ministry of Research. This work was also supported by a grant to G.B. from the Canadian Institutes of Health Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.03.051>.

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