Adjuvant effect of the human metapneumovirus (HMPV) matrix protein in HMPV subunit vaccines

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The human metapneumovirus (HMPV) fusion (F) protein is the most immunodominant protein, yet subunit vaccines containing only this protein do not confer complete protection. The HMPV matrix (M) protein induces the maturation of antigen-presenting cells in vitro. The inclusion of the M protein into an F protein subunit vaccine might therefore provide an adjuvant effect. We administered the F protein twice intramuscularly, adjuvanted with alum, the M protein or both, to BALB/c mice at 3 week intervals. Three weeks after the boost, mice were infected with HMPV and monitored for 14 days. At day 5 post-challenge, pulmonary viral titres, histopathology and cytokine levels were analysed. Mice immunized with F + alum and F + M + alum generated significantly more neutralizing antibodies than mice immunized with F only [titres of 47 ± 7 (P<0.01) and 147 ± 13 (P<0.001) versus 17 ± 2]. Unlike F only [1.6 ± 0.5×105 TCID50 (g lung)−1], pulmonary viral titres in mice immunized with F + M and F + M + alum were undetectable. Mice immunized with F + M presented the most important reduction in pulmonary inflammation and the lowest T-helper T h 2/T h 1 cytokine ratio. In conclusion, addition of the HMPV-M protein to an F protein-based vaccine modulated both humoral and cellular immune responses to subsequent infection, thereby increasing the protection conferred by the vaccine.

INTRODUCTION

Human metapneumovirus (HMPV) is a leading cause of acute respiratory tract infections in children, the elderly and immunocompromised individuals, for which no vaccine is currently available (Feuillet et al., 2012). It is anticipated that these different target populations will require different immunization strategies. One recombinant live-attenuated HMPV vaccine (rHMPV-Pa) is currently in clinical trial (National Institute of Allergy and Infectious Diseases, clinicaltrials.gov). Notably, inactivated vaccines against paramyxoviruses have been shown to cause vaccine-enhanced disease upon infection in both humans and animals (Anderson, 2013; Fulginiti et al., 1967). This type of vaccine-enhanced disease was also observed in rodents when inactivated HMPV was used as an immunogen (Hamelin et al., 2007; Yin et al., 2007). However, HMPV subunit vaccines are unlikely to induce enhanced disease, especially in seropositive populations. Furthermore, they do not pose the transmission and reversion risk associated with live-attenuated vaccines.

Several groups have demonstrated that the viral fusion (F) protein is the most immunodominant HMPV protein (Biacchesi et al., 2004; Mok et al., 2008; Skiadopoulos et al., 2006). Although prophylactic passive antibody transfer has been shown to be protective (Ulbrandt et al., 2006; Williams et al., 2007), the F protein alone is not sufficient to confer complete and long-lasting protection (Cseke et al., 2007; Herfst et al., 2007). The use of commercially available adjuvants can improve the protection of F-based subunit vaccines (Cseke et al., 2007; Herfst et al., 2007). However, several studies have recently revealed that other viral proteins are implicated in immunity and pathogenesis of HMPV infections (Kolli et al., 2012; Le Nouèn et al., 2014; Ren et al., 2014). Of interest, one group showed that the HMPV matrix (M) protein induces the activation of antigen-presenting cells (APCs) in vitro, resulting in the maturation of these APCs and the secretion of a broad range of inflammatory cytokines. Furthermore, M-activated dendritic cells were shown to stimulate IL-2 and IFN-γ production by allogeneic T-lymphocytes (Bagnaud-Baule et al., 2011). The inclusion of other viral proteins, such as the M protein, into HMPV subunit vaccine formulations might therefore provide a more balanced and robust immune response.

In the current study, we expressed the HMPV-F ectodomain (rHMPV-F) into a mammalian expression system to preserve its native conformation and glycosylation, as well as the full-length rHMPV-M protein into a prokaryotic
expression system. We then immunized BALB/c mice twice intramuscularly with the rHMPV-F protein, adjuvanted with alum, the most widely used commercially available adjuvant, the rHMPV-M protein or both, at 3 week intervals. Protection from HMPV infection was evaluated 3 weeks after the boost when mice were challenged with HMPV. The addition of the rHMPV-M protein to the rHMPV-F-based subunit vaccine reduced viral titres below the limit of detection. Importantly, the rHMPV-M protein also modulated the immune response towards a more balanced T-helper Th2/Th1 cellular response.

RESULTS

Successful expression of recombinant HMPV-F and -M proteins

Analyses of the F protein ectodomain and the full-length M protein by SDS-PAGE followed by Coomassie blue staining or Western blotting revealed that the purified proteins were >90% pure, with predicted molecular masses of 55 and 32 kDa, respectively (Fig. 1a, b). A small second band was observed at ~48 kDa for the HMPV-F protein. Incubation of the protein with increasing concentrations of trypsin increased the secondary band, indicating that this band represented the cleaved F1 form of the protein. In addition, Western blot analysis of the F protein ectodomain under native conditions revealed a band at 165 kDa, demonstrating that the protein was present in its trimeric form (Fig. 1c).

Non-adjuvanted and adjuvanted rHMPV-F vaccines are immunogenic in BALB/c mice

Other teams have developed and examined HMPV-F subunit vaccines in rodent models. Herfst et al. (2007) immunized Syrian golden hamsters with a HMPV-F subunit vaccine adjuvanted with alum, Specol or Iscom matrix, and Cseke et al. (2007) immunized cotton rats with a HMPV-F subunit vaccine adjuvanted with TiterMax Gold. As different animal models and immunization strategies were used by these groups, we decided to perform a preliminary small-scale experiment, in which BALB/c mice were immunized twice at a 3 week interval with 10 or 30 µg rHMPV-F or 30 µg rHMPV-F adjuvanted with alum. Following viral challenge with a lethal dose of virus (1.4 × 10^6 TCID50), control mice immunized with supernatant of non-transfected 293F cells, with or without alum, showed >80% mortality. Fifty per cent mortality was observed in mice immunized with 10 µg rHMPV-F, whereas mice immunized with 30 µg rHMPV-F, with or without alum, only showed 17% mortality (data not shown). Based on these observations, 30 µg rHMPV-F was chosen as the dose for subsequent intramuscular immunizations.

BALB/c mice immunized twice intramuscularly at a 3 week interval with 30 µg rHMPV-F adjuvanted with alum produced significantly more neutralizing antibodies than mice immunized with 30 µg rHMPV-F alone (47 ± 6.7 versus 17 ± 1.7, respectively; P < 0.05) (Fig. 2a). Mice immunized with rHMPV-M only did not produce neutralizing antibodies and the addition of rHMPV-M to rHMPV-F did not significantly increase the production of neutralizing antibodies (13 ± 1.7 and 27 ± 3.3 versus 17 ± 1.7 for rHMPV-F + 30 µg rHMPV-M and rHMPV-F + 60 µg rHMPV-M versus rHMPV-F, respectively). However, BALB/c mice immunized twice intramuscularly at a 3 week interval with 30 µg rHMPV-F adjuvanted with alum and 30 µg rHMPV-M produced significantly more neutralizing antibodies than mice immunized with 30 µg rHMPV-F only (147 ± 13.3 versus 17 ± 1.7, respectively; P < 0.001) or mice immunized with rHMPV-F + alum (147 ± 13.3 versus 47 ± 6.7, respectively; P < 0.01).

![Fig. 1. Expression of rHMPV-F and rHMPV-M proteins. (a) SDS-PAGE followed by Coomassie blue staining and (b) Western blot under denaturing conditions. (c) Confirmation of the trimeric confirmation of HMPV-FΔTm by Western blot under native conditions.](https://www.microbiologyresearch.org/content/journals/10.1099/jgv.0.080251-0/fig1.png)
Immunization of BALB/c mice with adjuvanted rHMPV-F vaccines decreases pulmonary viral titres

Viral replication in lungs of unadjuvanted and alum-adjuvanted rHMPV-F-immunized mice was significantly reduced (P<0.01) compared with mock-immunized mice (14.6 ± 3.6 versus 1.6 ± 0.5 and 2.6 ± 1.3 × 10³ TCID₅₀ (g lung)⁻¹ for mock-immunized versus rHMPV-F-immunized with and without alum, respectively) and between the two immunized groups (P=0.05) (Fig. 2b). Viral replication in the lungs of rHMPV-M-immunized mice did not differ significantly from mock-immunized mice (37.9 ± 13.2 and 14.6 ± 3.6 × 10³ TCID₅₀ (g lung)⁻¹ for rHMPV-M-immunized and mock-immunized mice, respectively). However, the addition of rHMPV-M to rHMPV-F did significantly reduce lung viral titres in a dose-dependent manner (1.3 ± 0.4 and 0.35 ± 0.17 versus 2.6 ± 1.3 × 10³ TCID₅₀ (g lung)⁻¹ for rHMPV-F + 30 µg rHMPV-M and rHMPV-F + 60 µg rHMPV-M versus rHMPV-F alone; P<0.05 and P<0.001, respectively). In addition, the viral replication in lungs of mice immunized with the combination of rHMPV-F, rHMPV-M and alum was also reduced below the limit of detection (0.26 ± 0.15 × 10³ TCID₅₀ (g lung)⁻¹; P<0.001) compared with rHMPV-F only and rHMPV-F + alum.

Immunization with adjuvanted rHMPV-F vaccines reduces HMPV disease severity in challenged mice

Upon viral challenge, the mean maximum weight loss of mice immunized with rHMPV-F with and without alum was significantly reduced compared with mock-immunized mice (20 % weight loss), but did not significantly differ between the two groups (10.4 ± 1.8 versus 10.4 ± 0.4 %, rHMPV-F with and without alum, respectively) (Fig. 2c). However, the mean maximum weight loss of mice immunized with rHMPV-M...
only was significantly reduced compared with rHMPV-F-immunized mice (6.5 ± 1.0 versus 10.4 ± 0.4%; P<0.01). Addition of rHMPV-M to rHMPV-F also significantly reduced the mean maximum weight loss (7.2 ± 1.0 and 6.9 ± 1.2% for rHMPV-F + 30 μg rHMPV-M and rHMPV-F + 60 μg rHMPV-M, respectively; P<0.05). Furthermore, the mean maximum weight loss of mice immunized with the combination of rHMPV-F + rHMPV-M + alum was significantly reduced compared with rHMPV-F-immunized mice (8.4 ± 0.4 versus 10.4 ± 0.4% for rHMPV-F + rHMPV-M + alum versus rHMPV-F only; P<0.01). Fig. 3 shows complete weight loss curves of the different groups over 14 days post-challenge. Notably, mortality (87.5%) was only observed in mock-immunized HMPV-challenged mice.

Although no statistically significant differences in histopathology scores were observed between any of the groups, pulmonary inflammation did appear to correlate with weight loss. We observed a trend towards higher pulmonary inflammation in mock-immunized mice (mean histopathology score of 11.8 ± 0.5) and pulmonary inflammation remained similar when alum was added to rHMPV-F (11.0 ± 0.5 versus 10.3 ± 0.4 for rHMPV-F with and without alum, respectively) (Fig. 2d). A trend towards lower pulmonary inflammation was observed in rHMPV-M-immunized mice (mean histopathology score of 7.1 ± 0.2) and when rHMPV-M was added to rHMPV-F (8.8 ± 0.5 and 7.4 ± 1.9 versus 10.3 ± 0.4 for rHMPV-F + 30 μg rHMPV-M and rHMPV-F + 60 μg rHMPV-M, respectively, versus rHMPV-F alone). Finally, a trend towards lower pulmonary inflammation was also observed for rHMPV-F + rHMPV-M + alum-immunized mice (mean histopathology score of 7.1 ± 1.6) compared with both rHMPV-F and rHMPV-F + alum (10.3 ± 0.4 and 11.0 ± 0.5, respectively).

**rHMPV-M protein modulates Th2/Th1 ratios**

IL-4 levels in the lungs were significantly increased when alum was added to rHMPV-F (1598.5 ± 158.1 versus 1051.2 ± 80.6 pg g⁻¹ for rHMPV-F with and without alum, respectively; P<0.05) (Fig. 4a). Conversely, rHMPV-M induced significantly reduced IL-4 levels compared with rHMPV-F only (389.7 ± 41.0 pg g⁻¹ for rHMPV-M; P<0.001). Also, the addition of rHMPV-M to the rHMPV-F + alum vaccine significantly reduced IL-4 levels in challenged mice (720.3 ± 147.8 versus 1598.5 ± 158.1 pg g⁻¹ for rHMPV-F + rHMPV-M + alum and rHMPV-F + alum, respectively; P<0.01)

Mock-immunized mice showed higher levels of IFN-γ in the lungs than rHMPV-F-immunized mice (137.0 ± 9.0 versus 99.7 ± 11.0 pg g⁻¹, respectively; P<0.05) (Fig. 4b). IFN-γ levels were also significantly increased when alum was added to rHMPV-F (132.1 ± 2.6 versus 99.7 ± 11.0 pg g⁻¹ for rHMPV-F with and without alum, respectively; P<0.05) and when rHMPV-M (60 μg) and rHMPV-F were combined (141.4 ± 10.2 versus 99.7 ± 11.0 pg g⁻¹, respectively; P<0.05). Finally, IFN-γ levels were reduced in the rHMPV-F + rHMPV-M + alum group compared with the rHMPV-F + alum group (68.8 ± 7.0 versus 132.1 ± 2.6 pg g⁻¹; P<0.001).

The IL-4/IFN-γ ratio was significantly higher in rHMPV-F-immunized mice than in mock-immunized mice (5.3 ± 1.0 versus 12.9 ± 1.2 and 11.6 ± 0.8 for mock-immunized versus rHMPV-F with and without alum, respectively; P<0.01) (Fig. 4c). However, the IL-4/IFN-γ ratio was significantly reduced in rHMPV-M-immunized mice compared with rHMPV-F-immunized mice (3.9 ± 0.4 versus 11.6 ± 0.8; P<0.001). The addition of the greater dose of rHMPV-M (60 μg) to rHMPV-F showed a trend towards a reduced IL-4/IFN-γ ratio compared with unadjuvanted F, although the difference was not significant (8.5 ± 1.5 versus 11.6 ± 0.8 for rHMPV-F + 60 μg rHMPV-M versus rHMPV-F alone). There was also a trend towards reduced IL-4/IFN-γ ratios for rHMPV-F + rHMPV-M + alum-immunized mice compared with rHMPV-F + alum (10.3 ± 1.3 versus 12.9 ± 1.2, for rHMPV-F + rHMPV-M + alum and rHMPV-F + alum, respectively).

![Fig. 3. Weight loss curves of immunized mice (n=8) infected with 1.0x10⁵ TCID₅₀ HMPV strain C-85473 and monitored for 14 days. By day 7 post-infection, seven out of eight mice in the mock-immunized group had reached the end point of 20% weight loss and hence the curve was stopped on day 7 for this group.](image-url)
**Fig. 4.** (a) IL-4 levels, (b) IFN-γ levels and (c) IL-4/IFN-γ ratios of immunized mice (n=6) infected with 1.0×10^6 TCID<sub>50</sub> HMPV strain C-85473 on day 5 post-challenge. *P<0.05; **P<0.01; ***P<0.001 compared with rHMPV-F only and ¤¤P<0.01; ¤¤¤P<0.001 compares rHMPV-F+alum to rHMPV-F+rHMPV-M+alum as determined by Student's t-test.
DISCUSSION

Owing to the detrimental results obtained with formalin-inactivated respiratory syncytial virus and measles vaccines (Anderson, 2013; Fulginiti et al., 1967), enhanced disease continues to be an important concern for all types of non-replicating paramyxovirus vaccines. This type of vaccine-enhanced disease might, at least in part, be due to poor induction of neutralizing antibodies and an imbalanced Th2/Th1 response (Anderson, 2013; Schickli et al., 2009). Moreover, vaccine-enhanced disease accompanied by a Th2 cellular bias has been observed in animals following formalin-inactivated HMPV immunization (de Swart et al., 2007; Hamelin et al., 2007; Yim et al., 2007). However, in a seropositive population, a subunit vaccine could be sufficient to boost existing immunity without inducing enhanced disease. In addition, adjuvants can be used to guide and modulate the immune response to a more balanced response and an increased production of neutralizing antibodies. Bagnaud-Baule et al. (2011) demonstrated that the HMPV-M protein is capable of activating APCs in vitro as well as increasing IFN-γ production by autologous T-cells when co-cultured with HMPV-M-activated dendritic cells. These observations led us to hypothesize that the HMPV-M protein could have an immunomodulatory effect in vivo and that the addition of the HMPV-M protein to a HMPV-F protein-based subunit vaccine could increase the protection against viral infection in BALB/c mice, even though the HMPV-M protein is not an immunodominant protein and does not induce the production of neutralizing antibodies on its own.

As described in other previous studies (Cseke et al., 2007; Herfst et al., 2007), immunization with rHMPV-F induced the production of neutralizing antibodies in our study. Although Herfst et al. (2007) did not observe an increase in neutralizing titres in animals immunized with rHMPV-F adjuvanted with alum, we observed almost a threefold increase in neutralizing antibodies when rHMPV-F was adjuvanted with alum. This difference might be due to the higher concentration of rHMPV-F (30 versus 10 µg), different source of Alhydrogel or the animal model used (hamsters versus mice).

Cox et al. (2014) recently investigated the immunogenicity of virus-like particles (VLPs) composed of full-length rHMPV-M and rHMPV-F proteins in C57BL/6 mice. When the rHMPV-M VLPs were not supplemented with rHMPV-F and not adjuvanted, no neutralizing antibodies were induced in twice-immunized mice. We were also unable to demonstrate the induction of neutralizing antibodies in mice immunized with rHMPV-M only. Immunization with VLPs containing both F and M did induce the production of neutralizing antibodies in the Cox et al. (2014) study, but the greatest increase in neutralization titres was observed when these VLPs were adjuvanted with commercially available adjuvants. We observed that the addition of rHMPV-M to the rHMPV-F subunit vaccine did not significantly increase neutralization titres, but when this mixture was adjuvanted with alum another threefold increase in neutralizing antibodies was observed.

Interestingly, despite the lack of neutralizing antibody production in mice immunized with rHMPV-M, we observed a significant reduction in weight loss after viral challenge in all groups of mice receiving rHMPV-M. None of the above-mentioned studies reported changes in weight loss in HMPV-challenged immunized mice, but our observations indicate an important role for the HMPV-M protein in the immune response following HMPV infection. Similarly to the results reported by Cox et al. (2014) with HMPV VLPs, no difference in pulmonary viral titres was found in mice immunized with rHMPV-M only, suggesting that the reduction in disease severity was not due to a reduction in viral replication. However, Cox et al. (2014) did observe a reduction in pulmonary viral titres when the rHMPV-M VLPs were adjuvanted. We did not investigate the effect of adjuvanting the rHMPV-M protein, but this might be of interest in future studies. Nevertheless, when we combined rHMPV-M at high dose with rHMPV-F with or without alum, pulmonary viral titres were reduced below the limit of detection. These results are again in concordance with the observations that VLPs composed of rHMPV-M and rHMPV-F adjuvanted or not reduced lung viral titres below the limit of detection.

In our study, as in previous reports (Cseke et al., 2007), there was a discordance between persisting pulmonary inflammation but significantly reduced viral titres on day 5 post-infection. However, all groups receiving rHMPV-M showed a trend towards reduced cumulative histopathology scores compared with mock-immunized or rHMPV-F- and rHMPV-F+-alum-immunized mice. Importantly, our results do not include a time-course of pulmonary inflammation, so any difference in kinetics of resolution of pulmonary inflammation could not be evaluated. Furthermore, IL-4 levels were significantly increased in rHMPV-F-immunized mice compared with mock-immunized mice, highlighting once more the Th2-bias induced by HMPV immunization compared with HMPV infection (Hamelin et al., 2005). Similarly to the results obtained by Cseke et al. (2007), a significant increase in the IL-4/IFN-γ ratio was observed in mice immunized with rHMPV-F compared with mock-immunized mice and this ratio further increased when rHMPV-F was adjuvanted with alum. Conversely, HMPV-M immunization yielded very low IL-4/IFN-γ ratios and the addition of rHMPV-M to rHMPV-F reduced the IL-4/IFN-γ ratio, although the difference was not statistically significant. Finally, the addition of alum to rHMPV-F increased both IL-4 and IFN-γ levels, whereas the addition of rHMPV-M to rHMPV-F + alum reduced both cytokines.

The mechanism by which rHMPV-M modulates the immune response following immunization requires further investigation. It is likely that rHMPV-M influences the cellular immune response, as suggested by the Th2/Th1
cytokine ratios. Although Cox et al. (2014) only observed increased recruitment of lymphocytes to the lungs when rHMPV-M VLPs were adjuvanted (Cox et al., 2014), the effect of the M protein on T-cell effector function has not yet been investigated and will be the object of future studies.

Despite the fact that rHMPV-M does not induce neutralizing antibodies on its own, we have demonstrated that the addition of rHMPV-M to a rHMPV-F-based subunit vaccine not only modulates the immune response following viral challenge, but also acts as an adjuvant increasing neutralizing antibodies induced by rHMPV-F and improving the protection following challenge. Additional studies are warranted to complete our evaluation, such as the investigation of cross-protection against other HMPV clades and the long-term benefits of these promising vaccines.

**METHODS**

**Virus strains and cells.** LLC-MK2 cells (ATCC CCL-7) were maintained in minimal essential medium (Life Technologies) supplemented with 10% FBS (Wisent). The HMPV group A strain C-85473, a clinical isolate that was passaged 10 times, was grown on LLC-MK2 cells in infection medium consisting of OptiMEM (Life Technologies) supplemented with 0.0002% trypsin (Sigma). Virus stocks were concentrated on Amicon columns (Fisher Scientific) as recommended by the manufacturer (Freestyle 293 was performed by transfecting 293-F cells with pcDNA5- rHMPV-F vector using TOPO TA cloning (pcDNA5; Life Technologies) and subsequently transformed into One Shot TOP10 chemically competent Escherichia coli (Life Technologies). Plasmids were purified with a HiSpeed Miniprep Kit (Qiagen). Reverse transcription was performed on the extracted material, using random primers (Amersham) and the viral RNA Mini kit (Qiagen). Reverse transcription was performed on the extracted material, using random primers (Amersham) and the SuperScript II RT kit (Life Technologies). cDNA was used as template to generate a PCR product encoding the F protein ectodomain (rHMPV-F; GenBank accession number KM408077.1) using the following primers: 5'-ACAACTCGAGATGGAGTCCTAC-3' and 5'-GACGATCTTTTCTTCTTCTG-3'. The C-85473 PCR product was cloned into a commercial vector using TOPO TA cloning (pcDNA5; Life Technologies) and subsequently transformed into One Shot BL21 Star (DE3) chemically competent E. coli (Life Technologies). Plasmids were purified with a HiSpeed Miniprep Kit (Qiagen). The concentration of the rHMPV-F protein was determined by a spectrophotometric assay (Bio-Rad). Purified rHMPV-F was further analysed using SDS-PAGE and Western blot by using mAb 1017 (a gift from MedImmune).

**Construction and production of the M protein.** The cDNA from HMPV C-85473 was used as a template to generate the PCR product encoding the M protein (GenBank accession number KX408077.1) using the following primers: 5'-ACAACCTCGAGAGTGGAGTCCTAC-3' and 5'-CAGCGATCTTTTCTTCTTCTG-3'. The PCR product was digested with restriction endonucleases XhoI and BamHI, and ligated into the pet19b vector (EMD Millipore), containing an N-terminal polyhistidine (His6) tag. Plasmids were transformed into One Shot BL21 Star (DE3) chemically competent E. coli (Life Technologies). Protein expression was induced using IPTG (Sigma) for 4 h at 37 °C. rHMPV-M was purified using Ni-NTA affinity chromatography under denaturing conditions and dialysed against endotoxin-free PBS (Sigma). The absence of endotoxin contamination was confirmed using the Limulus amebocyte assay (Cambrex) and protein concentrations were determined by a Bradford protein assay (Bio-Rad). Purified rHMPV-M was further analysed using SDS-PAGE and Western blot by using mAb 4831 (ViroStat).

**BALB/c mouse studies.** BALB/c mice (4–6 weeks old; Charles River Laboratories) were housed in groups of three to five mice in microisolator cages. The mice (20 per group) were immunized twice intramuscularly at 3 week intervals, with 60 μg rHMPV-M alone, 30 μg rHMPV-F alone, 30 μg rHMPV-F adjuvanted with Alhydrogel (alum) (1:1; Cederlane), 30 μg rHMPV-F adjuvanted with 30 or 60 μg rHMPV-M, or 30 μg rHMPV-F adjuvanted with Alhydrogel and 30 μg rHMPV-M. As a control group, mice were immunized with purified and concentrated supernatant of untransfected 293F cells. At 3 weeks post-boost, the mice were infected intranasally with 1×10^6 TCID₅₀ HMPV strain C-85473 in 25 μl OptiMEM. The day before immunization or infection, blood samples were taken to evaluate humoral responses. Eight animals were monitored on a daily basis for 14 days for weight loss and the presence of clinical signs, such as reduced activity and ruffled fur. The end point was determined at 20% weight loss. On day 5 post-infection, six mice per group were euthanized using sodium pentobarbital and lungs were removed for the evaluation of viral titres by cell culture and cytokine levels using a bead-based multiplex immunoassay, and six other mice were evaluated for histopathological changes using haematoxylin/eosin staining. The animal studies were approved by the Animal Protection Committee of the Centre Hospitalier Universitaire de Québec according to the guidelines of the Canadian Council on Animal Care.

**Pulmonary viral titres.** Lungs were removed on day 5 post-infection and snap frozen in liquid nitrogen. The lungs were subsequently weighed, homogenized in 1 ml PBS and then centrifuged at 2000 r.p.m. for 10 min. The supernatant was used to determine viral titres reported as TCID₅₀ (g lung)^{-1}.

**Pulmonary cytokine levels.** An aliquot of 250 μl lung homogenates was added to 250 μl 50 mM KPO₄ pH 6.0 buffer containing 0.2% CHAPS (Sigma) and 0.2% protease inhibitor cocktail (Sigma) and then stored at −20 °C. On the day of the experiment, samples were centrifuged at 13,000 g for 10 min at 4 °C and then 50 μl supernatant was used for cytokine quantification using a commercial multiplex Mouse Cytokine T₃/1(T),2 Assay (Bio-Rad) according to the manufacturer’s instructions. Experiments were performed in flat bottom 96-well plate and results were analysed with a Luminex system (Qiagen).
Histopathology. Lungs were removed on day 5 post-infection and fixed with 4% buffered formalin. Fixed lungs were subsequently embedded in paraffin, sectioned in slices of 5 μm and stained with haematoxylin/eosin. The histopathological scores were determined by two independent researchers who were blinded to experimental data. A semiquantitative scale was used to score bronchial/endobronchial, peribronchial, perivascular, interstitial, pleural and intra-alveolar inflammation as described previously (Hamelin et al., 2005).

Statistical analysis. Statistical analyses were performed using Prism 5 software (GraphPad). All groups were compared with the unadjuvanted RHMVP-F group using Student’s t-test. An additional Student’s t-test compared alum-adjuvanted RHMVP-F with RHMVP-F adjuvanted with both alum and RHMVP-M.

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