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Antibody-mediated protection against MERS-CoV in the murine model ☆

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ABSTRACT

Murine antisera with neutralising activity for the coronavirus causative of Middle East respiratory syndrome (MERS) were induced by immunisation of Balb/c mice with the receptor binding domain (RBD) of the viral Spike protein. The murine antisera induced were fully-neutralising *in vitro* for two separate clinical strains of the MERS coronavirus (MERS-CoV). To test the neutralising capacity of these antisera *in vivo*, susceptibility to MERS-CoV was induced in naive recipient Balb/c mice by the administration of an adenovirus vector expressing the human DPP4 receptor (Ad5-hDPP4) for MERS-CoV, prior to the passive transfer of the RBD-specific murine antisera to the transduced mice. Subsequent challenge of the recipient transduced mice by the intra-nasal route with a clinical isolate of the MERS-CoV resulted in a significantly reduced viral load in their lungs, compared with transduced mice receiving a negative control antibody. The murine antisera used were derived from mice which had been primed subcutaneously with a recombinant fusion of RBD with a human IgG Fc tag (RBD-Fc), adsorbed to calcium phosphate microcrystals and then boosted by the oral route with the same fusion protein in reverse micelles. The data gained indicate that this dual-route vaccination with novel formulations of the RBD-Fc, induced systemic and mucosal anti-viral immunity with demonstrated *in vitro* and *in vivo* neutralisation capacity for clinical strains of MERS-CoV.

1. Introduction

The Middle East respiratory disease syndrome (MERS) first emerged in 2012 in Saudi Arabia [1,2]. Since then, there have been an estimated 2311 laboratory-confirmed cases with 811 deaths, reported from a total of 27 countries in the eastern Mediterranean region and from 12 countries elsewhere [1]. Saudi Arabia, however, remains the main focus of infection and a disease outbreak in South Korea involving 186 cases was traced back to an index case who had travelled from Saudi Arabia. Whilst the incidence of MERS cases in Saudi Arabia peaked in 2014, there are still a significant number of cases reported from the country and in the period September 2012–May 2018, there were 1844 cases including 716 deaths with a case fatality rate of 38.8% [1]. MERS coronavirus (MERS-CoV) is a member of the betacoronavirus genus [3] and as for other betacoronaviruses, bats may provide a natural reservoir for the virus [3,4], but high levels of antibodies to MERS-CoV in dromedary camels [5] suggest that the dromedary camel is the principal source for animal-to-human transmission of MERS-CoV [6]. However, evidence of human-to-human transmission comes from the reporting of outbreaks in countries remote from Saudi Arabia such as the UK, Europe, USA, and China where small outbreaks have also occurred [1].

MERS-CoV is an enveloped, positive-sense, single-stranded RNA virus [3]. The virus possesses an envelope-anchored trimeric spike protein which binds to the human receptor dipeptidyl peptidase 4 (DPP4 or CD26) and gains host cell entry by the fusion of viral and host membranes [7]. The spike protein comprises an S1 sub-unit and a membrane fusion S2 sub-unit. In the coronaviruses, the S1

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sub-units are further divided into N-terminal and C-terminal sub-domains and for MERS-CoV, it is the C-terminal sub-domain that comprises the receptor-binding domain (RBD) [8]. The RBD also incorporates a receptor-binding motif at its C-terminal and the crystal structures of MERS-CoV RBD [8] and of the RBD bound to the extracellular domain of human DPP4 have been reported [9].

The RBDs of the coronaviruses represent vaccine and therapeutic targets and the RBD of MERS-CoV as a vaccine antigen has been demonstrated to induce neutralising antibody [10] and to protect mice transduced with a viral vector expressing hDPP4, or non-human primates from viral challenge [11–15]. There are significant ongoing efforts to develop vaccines for MERS-CoV infection, predominantly involving live attenuated viral vectors such as adenovirus, modified vaccinia Ankara or measles [16] to induce anti-viral immunity and some of these vaccines are already in clinical trials.

Here, we were interested to determine the relative importance of inducing systemic and/or mucosal immunity in vaccination to protect against MERS-CoV, an infection predominantly of the respiratory tract and lungs. To this end, we have used a dual route immunisation regimen in Balb/c mice to induce both systemic and mucosal immunity, to generate RBD-specific murine antisera. Initially, we immunised Balb/c mice sub-cutaneously (s.c.) with RBD-Fc in the MF59 adjuvant to induce RBD-specific IgG. Subsequently, we have immunised further groups of Balb/c mice by s.c. priming and per oral (p.o.) boosting with the RBD-Fc, to induce both systemic IgG and mucosal IgA responses. To do this, we have used novel formulations of RBD-Fc coated onto microcrystals formed from histidine or glutamine and also incorporating calcium phosphate for sub-cutaneous priming [17], whilst the formulation for oral boosting comprised RBD-Fc in reverse micelles dispersed in a self-emulsifying oil phase, which has been optimised from previous formulations [18,19]. The advantages of these formulations are that they are very stable under extremes of temperature [20]. Furthermore, on translation to the clinic, only one injected priming dose would be required, followed by a p.o. booster dose; the latter could be self-administered in capsule form. We have compared the relative abilities of the two sources of antiserum to neutralise clinical isolates of MERS-CoV *in vitro*. To do this, we have used two clinical strains of MERS-CoV (Erasmus Medical Center 2012 or EMC2012 and London1-2012:), each of which were derived from severely-ill individuals who had contracted the virus in the Middle East in 2012. Subsequent sequencing of the polymerase gene from these isolates indicated them to be newly-emerged members of the betacoronavirus genus with a close sequence homology and phylogenetic relationship to the bat coronaviruses HKU4 and HKU-5 [21,22].

Mice are not naturally susceptible to MERS-CoV infection, but susceptibility can be induced by the administration of an adenovirus vector which induces expression of the human receptor (hDPP4/CD26) for the virus *in vivo* for a limited time, providing a non-lethal murine model of the disease [23]. We have used this transduced mouse model to test the capacity of the antiserum derived from the dual route immunisation to neutralise MERS-CoV *in vivo*, by passive transfer prior to challenge with the EMC2012 strain and we have demonstrated a significant reduction in viral load in lung tissue in transduced mice.

2. Materials and methods

2.1. Expression and purification of RBD

The RBD was synthesised and expressed according to methods adapted from Du et al. [10]. In brief, a single DNA fragment containing an in-frame fusion of the coding sequences for the human IL2 signal peptide, the RBD and human IgG1-Fc was synthesised. This was transferred into the plasmid pEF-DEST51 (Invitrogen) so that the target sequence was expressed as a secreted protein with a C-terminal human IgG1 Fc tag. This construct was transfected by cationic transfection into human embryo kidney (HEK) cells in suspension (FSHEK) or adherent HEK cells stably expressing the Sv40 large T antigen (293FT), using serum-free media and incubated for 4–7 days. Small scale purifications of RBD-Fc were performed using Protein A chromatography. For this, medium from the transfected cells was treated with ammonium sulphate to precipitate the protein, prior to dialysis and resuspension in buffers for binding to Protein A beads. The latter were washed and eluted with buffer containing 1 M urea. Protein concentration was determined by UV absorbance spectroscopy and purity was estimated by SDS-PAGE with Coomassie staining and subsequent optical densitometry using a Syngene G:Box imaging system.

2.2. Formulation of RBD-Fc for injected and oral immunisation

The RBD-Fc was incorporated on glutamine calcium phosphate (CaP) microcrystals for s.c. immunisation, using methodology adapted from [17]. Briefly, aqueous mixtures of RBD-Fc with sodium orthophosphate and glutamine were precipitated as CaP protein-coated microcrystals (CaP-PCMC), by addition to of a 19-fold excess of isopropanol containing dissolved calcium chloride. The resultant suspension contained self-assembled microcrystals comprising a glutamine core with the RBD-Fc protein embedded in a thin surface layer of CaP (now termed RBD-Fc-PCMC). The PCMC were isolated by vacuum filtration and dried to a powder. Protein content and integrity was determined by ELISA and SDS-PAGE.

For oral dosing, the RBD-Fc was incorporated in mineral oil with added excipients using methodology adapted from [18,19]. The oral formulation comprised RBD-Fc with the mucosal adjuvant cholera toxin B sub-unit (CTB), retinoic acid (RA), vitamin D, E and trehalose debehenate (TDB), a synthetic analog of the mycobacterial trehalose dimycolate [25] and imiquimod, a TLR 7/8 agonist [26].

2.3. Immunisation and sampling of mice

Specific pathogen-free female Balb/c mice (6–8 weeks of age) were obtained from a commercial breeder and used throughout this study. On receipt, mice were randomised for allocation to cages and given free access to food, water and environmental enrichment. Mice were fully acclimatised to the animal housing facility for at least five days prior to any procedure. All animal procedures were performed in accordance with UK legislation as stated in the UK Animal (Scientific Procedures) Act 1986. The Institutional Animal Care and Use Committee approved the relevant Project licence.

Naïve mice were randomised for allocation to a treatment group (typically 5 per group) and immunised in one of two regimens: either with a s.c. priming dose followed by two s.c. doses, given at 10 and 31 days after the prime; alternatively, mice received a s.c. priming dose followed by an oral or s.c. booster dose 21 days after the prime (Table 1). For s.c. immunisation, mice received 2.5 μg of RBD-Fc-PCMC in 0.1 ml PBS injection volume, whereas for all per oral (p.o.) dosing, mice received 25 μg of RBD-Fc in a total volume of 0.1 ml mineral oil (MO), by oral gavage. Where RBD-Fc was administered s.c. in the conventional adjuvants MF59 or alhydrogel, MF59 (Novartis, US) was used in a 1:1 ratio by volume with RBD-Fc in PBS, whilst alhydrogel (Brenntag Biosector, Denmark) was used in a 1:5 ratio with RBD-Fc by volume in PBS.

At selected intervals after dosing, mice were blood-sampled from the tail vein for assay of specific antibody titre. At the end

Table 1
Murine Immunisation regimens.

Regimen 1	Day 0	Day 10	Day 31
Treatment group 1	Prime s.cut with 2.5 μg RBD-Fc in alhydrogel	Boost s.cut with 2.5 μg RBD-Fc in alhydrogel	Boost s.cut with 2.5 μg RBD-Fc in alhydrogel
Treatment group 2	Prime s.cut with 2.5 μg RBD-Fc in MF59	Boost s.cut with 2.5 μg RBD-Fc in MF59	Boost s.cut with 2.5 μg RBD-Fc in MF59
Regimen 2	Day 0	Day 21	
Treatment group 1	Prime s.cut with 2.5 μg RBD-Fc on PCMC	Boost s.cut with 2.5 μg RBD-Fc on PCMC	
Treatment group 2	Prime s.cut with 2.5 μg RBD-Fc on PCMC	Boost p.o. with 25 μg RBD-Fc in oil vehicle	
Treatment group 3	Prime p.o. with 25 μg RBD-Fc in oil vehicle	Boost p.o. with 25 μg RBD-Fc in oil vehicle	
Treatment group 4	Prime s.cut with 2.5 μg RBD-Fc in MF59	Boost s.cut with 2.5 μg RBD-Fc in MF59	
Treatment group 5	Empty microcrystals	Oral vehicle	

of the immunisation schedule, individual mice were terminally anaesthetised for collection of blood by cardiac puncture, then culled prior to removal of small and large intestines for collection of faecal pellets for extraction of IgA.

2.4. Serological assays for titre and neutralising antibody

Titres of RBD-Fc-specific antibody in serum samples were determined by ELISA. In brief, test sera were bound to microtitre plates pre-coated with RBD-Fc and antibody binding was detected with an HRPO-labelled secondary antibody to mouse IgG, IgG1, IgG2a or IgA (Bio-Rad). A standard curve for calibration comprising the relevant murine Ig isotype (Sigma) captured with an anti-Fab reagent, was included on each plate. Plates were developed by the addition of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Sigma) and optical density (OD) was read at 414 nm (Multiskan plate reader). For assay of antibody in faecal samples, faecal pellets were extracted in supplemented PBS as described previously [20]. In brief, 10 ml of cold PBS was prepared, supplemented with 1 tablet of complete mini protease inhibitor cocktail (Sigma) and 5 μl Tween 20 were added. To 0.1 g faecal pellets, 1 ml of supplemented PBS was added and left at room temperature for 5 min. Samples were vortexed for approximately 30 s, incubated on ice for a further 20 min. and then centrifuged (15,000g, 5 min.). Supernatants were retained and stored at −80 °C pending assay. The faecal extracts were assayed for specific IgG and IgA content, by ELISA, as for serum samples. Antibody concentrations in all samples were determined from the relevant standard curves using Ascent software with four-parameter logistic curve-fitting and reported in ng/ml or μg/ml serum or faecal extract, as appropriate.

2.5. Neutralisation of virus in vitro

To determine if the antibody induced by immunisation with to RBD-Fc was neutralising for MERS-CoV *in vitro*, plaque assays were performed. For this, two strains of MERS-CoV were used: London1-2012 (GenBank accession number KC164505.2) [22] and Erasmus Medical Center (EMC2012 Genbank accession number JX869059) (21). The London1-2012 strain was obtained from the National Collection of Pathogen Viruses, PHE Porton, Salisbury, UK and the EMC2012 strain was kindly provided by the Erasmus University Medical Center Rotterdam, the Netherlands. Both strains were prepared in serum-free media (Gibco) at a multiplicity of infection (MOI) of 0.01, equivalent to 10^3 plaque-forming units (pfu). The murine antiserum for testing was prepared at a dilution range from undiluted to 1:10 in PBS. Virus was incubated overnight (4 °C) with murine antiserum, negative control antibody (NIBSC, UK) or media, prior to infection of a confluent monolayer of Vero E6 cells (ECACC, Salisbury UK) with 200 μl of the mixture. The neutralising ability of the murine antiserum was tested in duplicate or triplicate at each dilution. After incubation (1 h, 37 °C), an overlay comprising a 1:1 dilution of carboxymethyl cellulose with serum-free media was added to the cells and incubation continued for a further 4 days (37 °C) prior to fixing (7.4% formaldehyde) and staining (0.2% crystal violet) with enumeration of the number of plaques per ml.

2.6. Murine infection with the MERS virus

Mice are not naturally susceptible to infection with MERS-CoV, since they lack the human DPP4 receptor. To induce transient susceptibility in Balb/c mice, we used an Ad5 construct (Oxford Genetics) to express the human DPP4 receptor (Ad5hDPP4), as previously described (23). Mice were administered the Ad5hDPP4 construct (2.5×10^8 pfu in 50 μl) by the intra-nasal (i.n.) route under light sedation with inhalational isofluorane and then monitored by serial blood sampling for serum levels of hDPP4/CD26 by ELISA (ThermoScientific). At peak levels of expression of hDPP4 (days 5–7), mice were lightly sedated as before and challenged by the i.n. route with MERS-CoV (EMC2012 strain) at 10^4 pfu in 50 μl per mouse. Mice were weighed prior to challenge on each subsequent day to monitor changes in body weight during infection.

2.7. Neutralisation of virus in vivo by passive transfer of antibody

To test the *in vivo* neutralising capacity of murine antiserum raised to the RBD-Fc construct, naïve mice (n = 10 per treatment group) were passively immunised by the i.p. route at 24 h. prior to i.n. challenge with the MERS Co-V (EMC2012 strain), as described above. The murine antiserum, pooled from 4 mice who had been primed with RBD-Fc PCMC and boosted orally (regimen 2, treatment group 2), was delivered at a dilution of 1:10 in PBS and delivered in a total volume of 100 μl per mouse. A further group of 10 mice received a purified polyclonal human IgG at a single dose level (150 μg/mouse in 100 μl i.p.), which had been raised to inactivated MERS-CoV. Control mice received a non-specific human IgG at a single dose-level (200 μg/mouse in 100 μl, i.p.). Both sets of human IgG (specific and non-specific) were raised in a bovine transchromosomal model and purified prior to use. A further group of 10 negative control mice were included, which received PBS in place of either the Ad5DPP4 construct or the MERS-CoV-specific antibody, and were also challenged i.n. with MERS-CoV (EMC2012 strain) at 10^4 pfu/mouse. To determine the protection afforded by the passive immunisation, pairs of mice from each treatment group were culled on days 1–8 after challenge and their lungs were removed and weighed and then rapidly frozen (−80 °C) prior to the determination of viral load.

2.8. Determination of viral load in lungs

Pairs of lungs from each of 2 mice per treatment group were individually thawed and homogenised in serum-free media (2 ml). RNA was extracted from 140 μl of each homogenate using the QiAamp Viral RNA kit (Qiagen), following the manufacturers' instructions. Real-time PCR was conducted on duplicate 5 μl

aliquots of each RNA extract, using the MERS-CoV-specific N3 assay and reaction conditions [24]. As in Lu et al, we used the forward primer GGGTGTACCTCTTAATGCCAATTC and reverse primer TCTGTCCTGTCTCCGCCAAT with probe ACCCCTGCGCAAAATGCT GGG. Each 25 μl reaction contained 6.25 μl TaqMan Fast Virus 1-Step mastermix (ThermoFisher Scientific); forward and reverse primers (0.5 μM each), probe (0.1 μM), 5 μl RNA template and 10.25 μl water. A standard curve was constructed by spiking naïve lung homogenate with MERS-CoV (EMC 2012) (final concentration 5×10^4 pfu/ml) and diluting in naïve lung homogenate to 0.5 pfu/ml. RNA was extracted from duplicate 140 μl aliquots of each concentration and PCR conducted using the above method. The amount of virus in tested samples was determined in duplicate using the standard curve and reported as pfu/g lung tissue.

2.9. Statistical analysis

All data were analysed using Graph Pad Prism software v.6 and expressed as mean ± s.e.m. Statistical comparisons were made using one-way ANOVA or unpaired *t*-test.

3. Results

3.1. Expression and purification of RBD-Fc

The RBD-Fc protein was expressed in both adherent 293FT and suspension human embryo kidney (HEK) cells, but with greater expression in adherent cells (Fig. 1). Purification of protein from adherent cells with Protein A was very effective, yielding protein which was >99% pure, with molecular weight of approximately 100 kDa (Fig. 1A). The use of 1 M urea for elution was optimum, as it was sufficient to solubilise the protein without denaturing it, yielding RBD-Fc in optimum yield (0.2 mg/ml) and predominantly in a dimeric form (Fig. 1B). This method of protein purification was therefore selected for forward use.

3.2. Optimisation of RBD-Fc immunogenicity and assay of neutralising activity

RBD-Fc, formulated for either sub-cutaneous (s.c.) or per oral (p.o.) immunisation, was tested for immunogenicity and the formulations optimised in an iterative approach. Initially, a s.c. dosing regimen was used in which RBD-Fc was formulated in either alhydrogel or MF59 to deliver 2.5 μg of protein on each of three occasions at 0, 10 and 31 days. Mice were monitored for 26 days after the final boost and IgG titre determined (Fig. 2A). At day 70, the total IgG titres achieved with RBD-Fc in alhydrogel or MF59 did not differ significantly. To determine if the presentation of RBD-Fc in either alhydrogel or MF59 influenced the ability to develop virus-neutralising antibody, antisera were selected from 2 mice in each immunisation group and tested in a plaque assay for neutralisation of both the EMC2012 and London1-2012 strains of MERS-CoV (Fig. 2B and C). All four sera gave some neutralisation of viral activity, although at a 1:20 dilution, sera 136 and 169 were most potent, against both viral strains. Sera 136 and 169 were derived from the treatment group immunised with MF59-adjuvanted RBD-Fc, whereas sera 132 and 150 were derived from alhydrogel-adjuvanted RBD-Fc (Fig. 2A). Based on this pilot data, we subsequently used MF59 as the conventional adjuvant for RBD-Fc, to compare with some novel formulations.

3.3. Induction of systemic, mucosal and functional antibody to RBD-Fc

Having demonstrated to proof-of-principle that the RBD-Fc, when delivered in MF59 can induce a high titre of antibody with neutralising activity, we next investigated how to tailor an

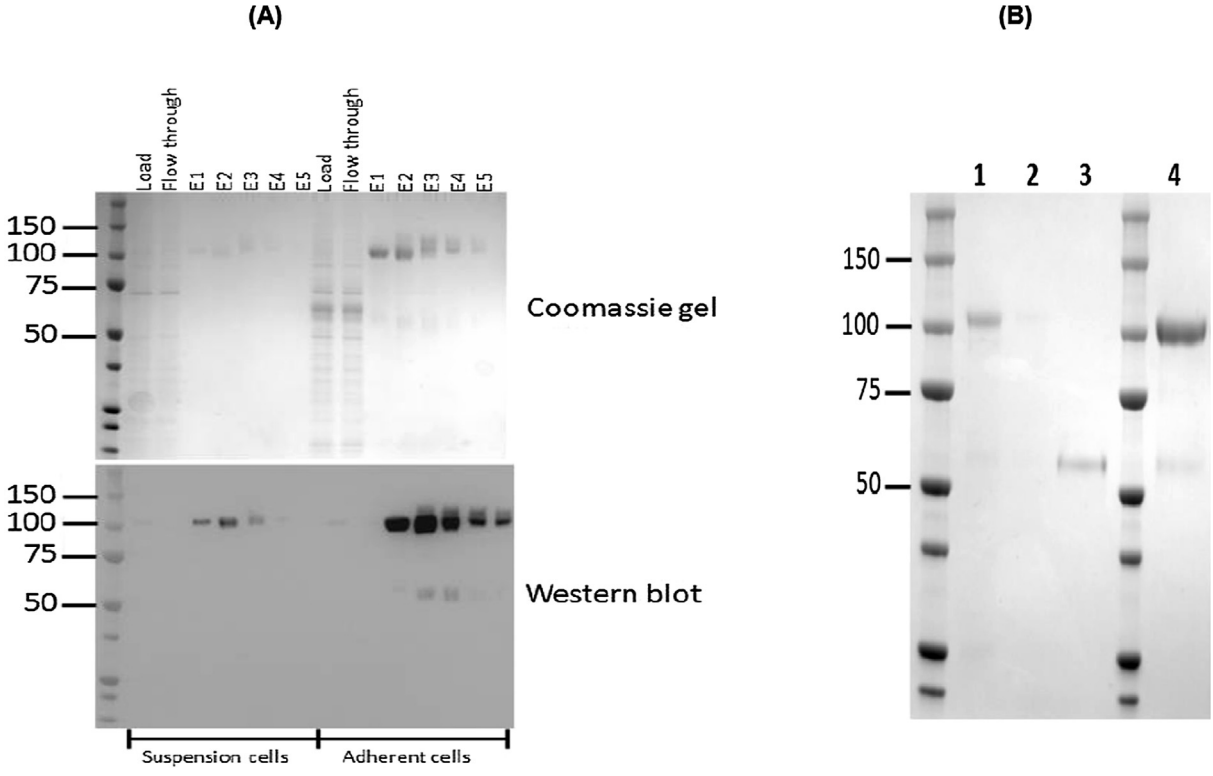

Fig. 1. Expression and purification of RBD-Fc from suspension and adherent Human Embryo Kidney (HEK) cells. (A) Expression of RBD-Fc in suspension and adherent cells as a 100 k Da protein by SDS-PAGE. Lanes 1 & 8: Load; Lanes 2 & 9: Flow through; Lanes 3–7 and 10–14: Eluates 1–5 (B) Coomassie-stained gel of protein purified from adherent cells only: Lane 1: soluble protein; Lanes 3 & 4: protein precipitated on Protein A beads and eluted in 6 M urea and 1 M urea respectively.

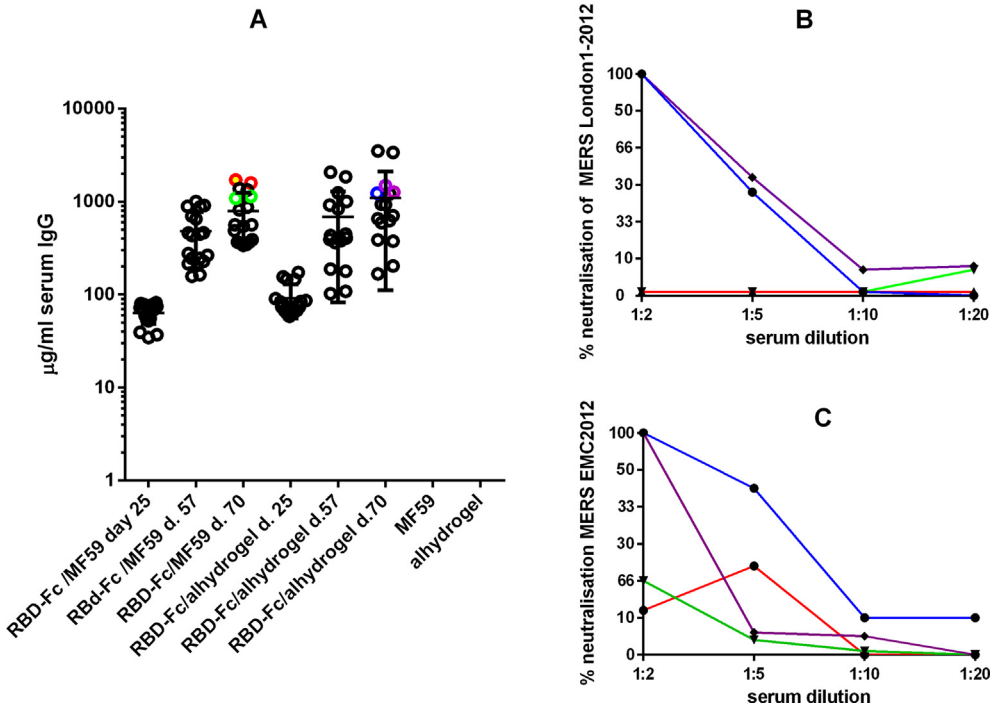

Fig. 2. A. Development of RBD-specific murine IgG titres with time in response to RBD-Fc in MF59 or alhydrogel immunisation by the s.c. route on days 0, 10 and 31. The coloured replicates indicate the serum samples from each group assayed (132-blue and 150-purple in the alhydrogel group) and 136-red and 169-green in the MF59 group) and demonstrated to have neutralising activity *in vitro* for clinical strains of MERS-CoV. (B) shows the *in vitro* neutralisation of the London1-2012 strain by individual murine antisera to RBD-Fc whilst (C) shows neutralisation of the EMC2012 strain.

RBD-Fc vaccine optimally to induce both systemic and mucosal immunity, with the aim also of reducing to a 2-dose immunisation regimen and increasing functional antibody. For this, we selected novel formulations in which RBD-Fc protein was presented as RBD-Fc-PCMC for s.c. priming and incorporated into mineral oil (MO) for p.o. boosting. We compared the serum IgG response achieved from this 2-dose dual route immunisation with that induced to RBD-Fc delivered in MF59 in a 2-dose s.c. regimen (Fig. 3). At 1 month after the booster dose, at day 49, there was no significant difference in the serum IgG titres achieved, so that the 2-dose dual-route immunisation with RBD-Fc-PCMC for s.c. priming and incorporated in MO with excipients for p.o. boosting, was just as immunogenic as the 2-dose s.c. immunisation with RBD-Fc in MF59 (Fig. 3A). At day 49, the serum response to

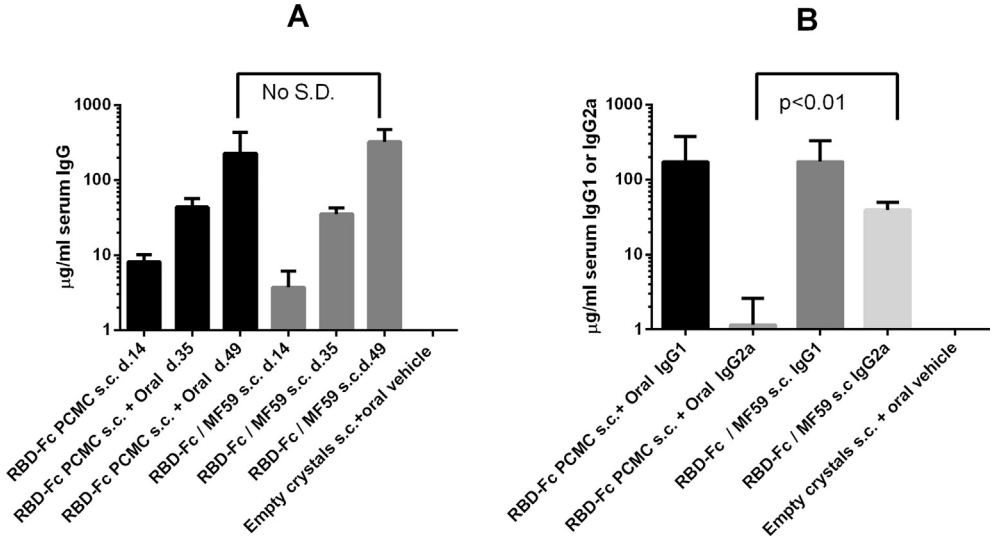

Fig. 3. (A) Serum IgG to RBD-Fc after dual- or single-route immunisation. Mice were immunised with RBD-Fc on PCMC or with RBD-Fc in MF59 s.c. and boosted p.o. with RBD-Fc in the oral formulation, or with RBD-Fc in MF59 s.c., each on day 21. The serum IgG response at days 14 and 35 of the schedule is shown in response to the priming and booster doses. (B) shows the distribution of IgG1 or IgG2a isotypes induced by day 49 of the immunisation schedule. Statistical significance was determined at the $p < 0.05$ level, by unpaired *t*-test.

RBD-Fc in the dual-route regimen was predominantly IgG1 biased, whereas s.c. dosing with RBD-Fc in the presence of MF59 induced both IgG1 and IgG2a (Fig. 3B).

Since dual route immunisation effectively induced serum IgG to RBD-Fc, it was of interest to determine whether it could also effectively induce mucosal immunity. In this study, the RBD-Fc-specific IgA response was determined in serum and in faecal pellet extracts from individual animals on day 49. In this case, the RBD-specific IgA responses of mice immunised in the 2-dose dual route regimen were compared with that of mice immunised by the oral route twice, and with mice immunised by the s.c. route in MF59 twice, on exactly the same days (0,21) (Fig. 4). This comparison showed that s.c. immunisation in MF59 did not induce serum IgA. However s.c. priming with RBD-Fc-PCMC with p.o. boosting effectively induced RBD-Fc-specific IgA and was not inferior to oral priming and boosting in this effect in either serum (Fig. 4A) or faecal extracts (Fig. 4B). However mice primed and boosted orally did not develop RBD-specific systemic IgG (data not shown).

Additionally, day 49 sera from mice in all treatment groups were tested for their ability to neutralise either strain of MERS-CoV *in vitro* (Table 2). From this it can be seen that sera from 4 out of 5 mice in the dual route regimen were fully neutralising

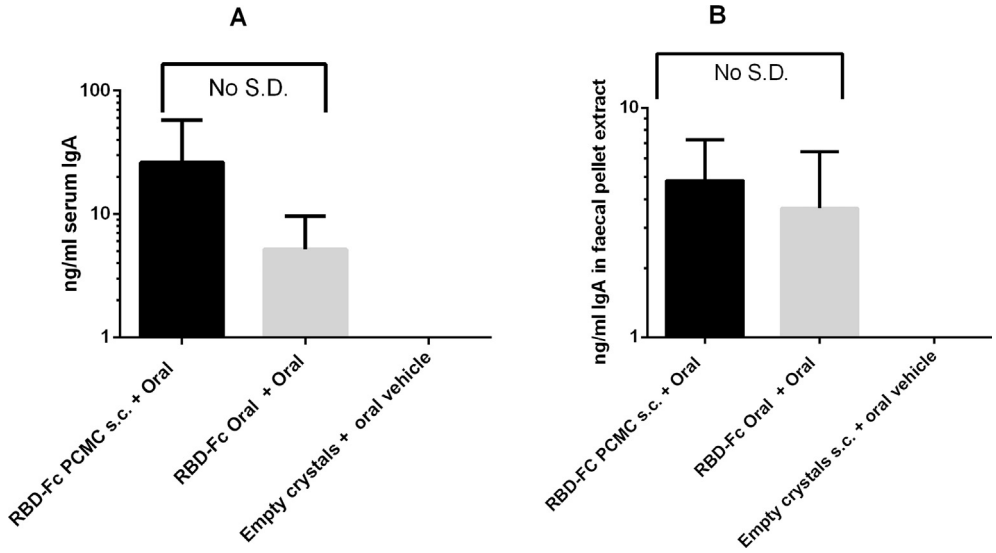

Fig. 4. Sub-cut priming with oral boosting is not inferior to oral priming and boosting in the induction of IgA, measured at day 49 in serum (A) and faecal extracts (B). The fig. shows IgA titres from mice immunised with RBD-Fc on PCMC s.c. and boosted p.o. with the oral formulation; or immunised and boosted p.o. with the oral formulation of RBD-Fc. Negative control mice were primed and boosted with RBD-Fc in MF59 s.c., or with empty PCMC s.c. plus oral vehicle p.o. Statistical significance was determined at the $p < 0.05$ level, by unpaired *t*-test.

Table 2
Neutralisation of MERS-CoV *in vitro*.

In vitro neutralisation of MERS EMC2012							*In vitro* neutralisation of MERS London 1–2012						
Pfu virus at dilutions of mouse serum							Pfu virus at dilutions of mouse serum						
Treatment	Mouse ID	1:10	1:20	1:40	1:60	% neutralisation	Treatment	Mouse ID	1:10	1:20	1:40	1:60	% neutralisation
RBD-Fc PCMC s.c/s.c.	1	0	0	0	0	100	RBD-FC PCMC s.c/s.c.	1	0	0	0	0	100
	2	0	0	0	0	100		2	0	0	0	0	100
	3	0	0	0	0	100		3	0	0	0	0	100
	4	0	0	0	0	100		4	0	0	0	9,3	100
	5	0	0	0	0	100		5	0	0	0	0	100
RBD-Fc PCMC s.c/oral	**1**	**0**	**0**	**0**	**0**	**100**	**RBD-FC PCMC s.c/oral**	**1**	**0**	**0**	**0**	**0**	**100**
	2	**0**	**0**	**4,6**	**4,8**	**100,100,85,85**		**2**	**0**	**7;6**	**6;5**	**25;26**	**100, 89,89,0**
	3	**0**	**0**	**1,1**	**0**	**100**		**3**	**0**	**2;0**	**5;6**	**14;4**	**100, 97,91, 86**
	4	**0**	**1.0**	**2,1**	**0**	**100**		**4**	**0**	**1;0**	**4;1**	**8;7**	**100,100,96, 88**
	5	0	0	0	0	100		5	0	1;0	2;2	6;2	100,100,97,91
RBD-Fc oral/oral	1	24,15	30,22	22,17	21,22	0	RBD-FC oral/oral	1	25, TNTC	44, TNTC	TNTC	TNTC	0
	2	19,12	11,21	26,16	17,32	0		2	TNTC	TNTC	TNTC	TNTC	0
	3	16	5	21	n.d.	62		3	18	28	TNTC	N.D.	0
RBD-Fc/MF59	1	0	0	0	0	100	RBD-Fc/MF59	1	0	0	0	0	100
	2	0	0	0	0	100		2	0	0	0	0	100
Empty microcrystals	1	13,12	17,13	20,15	25,18	0	Empty microcrystals	1	23;19	22;28	38;29	33;41	0
	2	9,8	14,9	16,14	12,16	0		2	35;27	34;24	43;32	39;37	0
	3	15,23	13,20	21,21	20,9	0		3	33;30	34;44	44;29	21;46	0
Virus only	1	23	26	33	22	0	Virus only	1	61	60	65	45	0
	2	28	32	42	29	0		2	58	46	52	52	0
	3	28	31	22	29	0		3	70	56	56	71	0
	4	31	19	24	28	0		4	47	52	44	55	0

in vitro for both strains (EMC2012 and London1-2012), when tested at 1:60 dilution (Fig. 5A and B). In order to test whether the *in vitro* neutralising activity translated into viral neutralisation *in vivo*, sera from these 4 mice (highlighted in Table 2) were pooled in equal aliquots at 1:10 dilution to enable a subsequent passive transfer study.

3.4. Use of Ad5hDPP4 to induce CD26 expression in naïve mice

In order to design the passive transfer study, it was necessary to define the duration of expression of CD26 in murine lungs *in vivo*, following induction with the Ad5hDPP4 construct. Mice dosed with Ad5hDPP4 i.n. at T_0 were culled in pairs and lung homogenates prepared and assayed for CD26 expression. CD26 in lung tissue was expressed in a time-dependent manner, with levels peaking at day 3 and declining to day 17 (Fig. 6A), setting a sufficient window to use the model for the determination of the protection against viral challenge afforded by the passive transfer of MERS-specific antibody.

3.5. Neutralisation of virus in vivo by passive transfer of antibody

To determine the protection afforded by the passive transfer of murine antiserum raised in the dual route immunisation regimen, against infection, susceptibility to MERS-CoV was induced at T_0 with i.n. administration of Ad5hDPP4 to groups of 10 mice. Passive transfer by the i.p. route of the pooled serum sample derived from the 4 mice highlighted in Table 2, which had previously been shown to be neutralising *in vitro* (Table 2) was conducted 5 days later and mice were challenged after a further 24 h with MERS-CoV EMC2012. Additional groups of mice, which had been transduced with Ad5hDPP4, were passively immunised with a MERS-CoV specific human IgG and a non-specific human IgG. At 1–8 days after challenge, pairs of mice were culled for the determination of viral load in lungs, which was determined to peak at 3 days p.i. (data not shown). At 3 days p.i., the pooled murine antiserum significantly reduced viral titres in lungs, to the same extent as the specific human IgG, and contrasting with the negative control human IgG, demonstrating significant *in vivo* neutralising activity (Fig. 6B).

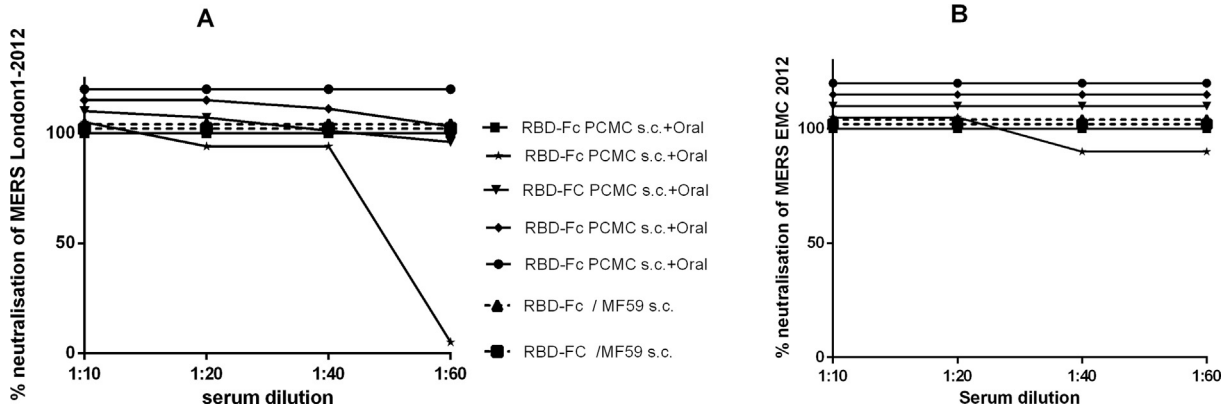

Fig. 5. *In vitro* neutralisation of (A) MERS-CoV (London1-2012 strain) and (B) MERS-CoV (EMC2012 strain) by individual murine antisera induced to RBD-Fc in either the dual-route or single-route immunisation regimen.

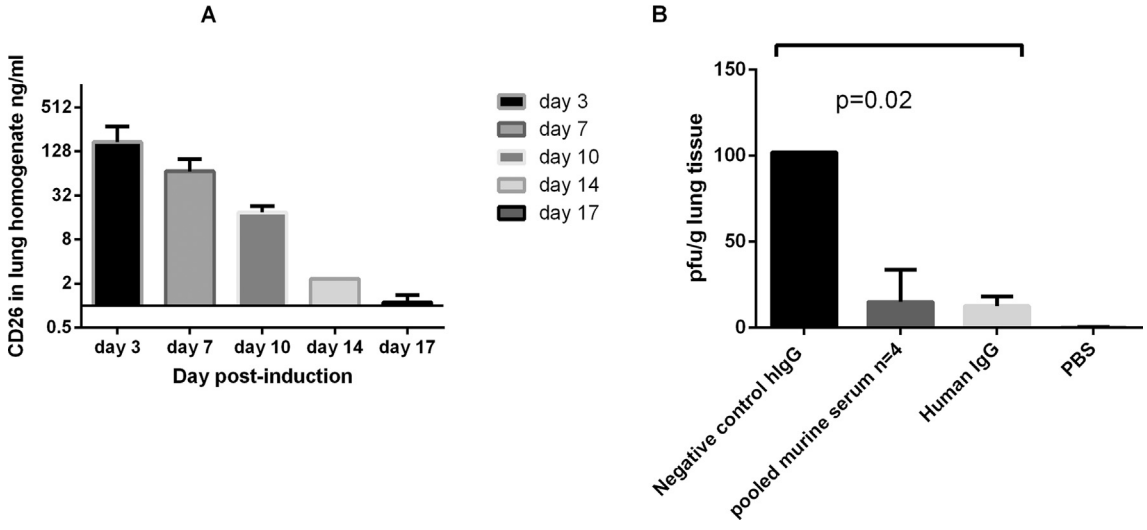

Fig. 6. A. Expression of CD26 was induced in lung tissue by the administration of Ad5hDPP4 (2.5×10^8 pfu) to mice by the i.n. route at T_0. Subsequently, mice were culled in pairs on the days shown and their lungs assayed for the expression of CD26. The plot shows the time-course of CD26 expression from 3 to 17 days post-induction. All data points were normalised for background values from control mice. 6B: Content of MERS-CoV (EMC2012 strain) in murine lungs (pfu/g tissue) determined by RT-PCR at day 3 post-infection, (equivalent to day 4 after passive transfer with murine antisera to RBD-Fc which had previously been shown to neutralise the EMC2012 strain *in vitro*). Mice received either a MERS-CoV-specific human IgG (150 μg) or non-specific human IgG (200 μg) in 100 μl /mouse i.p.; or murine antisera to RBD-FC, which had been pooled from 4 murine donors and which was delivered at 1:10 dilution (100 μl/mouse i.p.). Negative control mice received PBS in place of Ad5hDPP4 or antiserum All mice were challenged with MERS-CoV EMC2012 i.n. at 10^4 pfu/mouse. Statistical significance was determined at the $p < 0.05$ level by one way Anova and unpaired *t*-test.

No significant differences in body weight were detected between treatment groups challenged with MERS-CoV, which was attributed to the short time period of the study.

4. Discussion

MERS is a serious endemic respiratory infectious disease for which there is no licensed vaccine, although there are several vaccines in clinical trial currently including adenovirus-vectored delivery of the Spike protein and sub-units [27], DNA vaccines and nanoparticle-delivered sub-unit approaches [28]. We were interested in determining the advantage of r a vaccine which could induce mucosal as well as specific systemic immunity to the key target, the RBD protein, in order to achieve optimum protective efficacy. Vaccination to induce effective immunity at mucosal surfaces, should prime the immune system to respond rapidly to invading pathogens such as MERS-CoV. Previous studies have used adenovirus delivery of the MERS Spike protein with intra-muscular (i.m.) or intra-gastric (i.g.) delivery to induce neutralising systemic IgG, but not IgA; further, whilst i.m. delivery also induced specific T-cell immunity, i.g. delivery did not [29]. Others have shown that intra-nasal delivery of a live attenuated adenovirus-vectored sub-unit vaccines does induce specific mucosal as well as systemic immunity, although translation of this approach to the clinic may raise safety issues [30]. Here, we have relied on novel formulations of a sub-unit protein to enable dual route vaccination (parenteral and oral) to induce mucosal as well as systemic immunity.

In this study, we have achieved the expression and purification of a recombinant RBD-Fc protein in milligram quantities. We have also demonstrated that when formulated as a sub-unit vaccine, the construct induced murine antibody which effectively neutralised two different clinical strains of MERS-CoV *in vitro*. Additionally, we have shown that these murine antisera, when passively transferred into naïve mice transduced to express the hDPP4 /CD26 receptor, conferred protection against viral challenge in the recipients, with significantly reduced viral loads in the lung tissue of the recipient mice.

Whilst the use of conventional adjuvants such as MF59 or alhydrogel to formulate the RBD-Fc protein resulted in high titres of specific IgG in serum, the MF59 formulation did not induce specific IgA in serum. In order to promote both systemic and mucosal immunity to RBD-Fc, we have formulated this protein for injected priming and p.o. boosting, entailing the optimisation of the CaP PCMC and of the reverse micelles in oil emulsion, respectively. This has enabled the achievement of a vaccination regimen comprising only two doses and rapidly inducing RBD-Fc-specific systemic and mucosal immunity.

Whilst the PCMC formulation of RBD-Fc was as effective as RBD in MF59 in inducing a primary IgG response, we have shown that an oral formulation of RBD-Fc in mineral oil with selected immunostimulants was as effective as MF59 when used as a booster immunisation. Additionally, we have shown that non-invasive oral priming and boosting is as effective at inducing a specific mucosal response, measured as specific IgA in serum and faeces, as is injected priming with RBD-Fc in the PCMC formulation together with oral boosting, leading to the exciting concept of a potential orally-dosed sub-unit vaccine for MERS.

Whilst both alhydrogel and the combination of PCMC and oral formulations are Th2-polarising, as evidenced by the predominantly IgG1 titres raised to RBD-Fc, the influence of MF59 on the response to RBD-Fc was a mixed Th-2/Th1 effect, with a significant induction of specific IgG1 and IgG2a. To counter a viral infection, it would be expected that a Th1 response would be most appropriate. However, the fact that neutralising antibody to RBD-Fc was raised under either Th1 or Th2-polarising influences, suggests that either isotype can be protective and primes the immune system sufficiently and would allow for cross-presentation to occur on subsequent exposure to the virus [31]. In this study, we have not examined the induction of a cell-mediated memory response to the RBD-Fc protein, although this will play a significant role in protection against the virus.

Currently, we are presenting the RBD protein in our formulations with an Fc tag, derived from human IgG1 and useful in purifying the protein. The Fc tag may contribute additional adjuvantising activity by engaging antigen-presenting cells in the vaccinee [32] and it may aid mucosal immunity since the Fc receptor, an MHC1 transmembrane protein, is also expressed at mucosal surfaces e.g. in the respiratory tract [33]. Vaccination of the zoonotic host, the dromedary camel, may also effectively curb outbreaks of MERS in endemic regions and limit the risk of viral recombination [34] and significant progress with an orthopox-vectored vaccine for MERS has recently been reported [35]. The potential use of a sub-unit vaccine for MERS in camel vaccination could be aided by varying the sequence of the RBD protein [36] and substituting the human Fc tag with an alternative tag recognised by the camel, to design approaches tailored for animal vaccination, bearing in mind that a single dose vaccine would be ideal in this context. However, future work in our laboratory will also address the value of retaining or removing the Fc tag from the RBD protein for clinical or veterinary iterations of the vaccine.

In this study we have determined vaccine efficacy by demonstrating *in vitro* and *in vivo* neutralising ability of murine antisera raised in the dual route two-dose regimen against two virulent clinical strains of MERS-CoV which have greater than 99% genome sequence homologyy. In future work, it will be worthwhile to test the efficacy of this approach against other clinical isolates of MERS-CoV. This is the first report of a dual route dosing regimen applied to a sub-unit vaccine for MERS-CoV. Future development of this approach would require the direct testing of efficacy in the immunised transduced mouse model. As well as giving a direct readout of vaccine efficacy, this will enable the identification of the immune correlates of protection, ready for transitioning this candidate vaccine into more extensive pre-clinical testing and clinical development.

Data availability statement

The authors declare that all the data supporting the findings of this study are available within the paper.

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Declaration of Competing Interest

The authors declare no conflict of interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.074.

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