Published in final edited form as:
Sci Transl Med. 2015 August 12; 7(300): 300ra126. doi:10.1126/scitranslmed.aac5745.

Chimpanzee adenovirus and MVA-vectored respiratory syncytial virus vaccine is safe and expands humoral and cellular immunity in adults

CA Green[1], **E Scarselli**[2], **CJ Sande**[1], **AJ Thompson**[1], **CM de Lara**[3], **K Taylor**[1], **K Haworth**[1], **M Del Sorbo**[2], **B Angus**[1], **L Siani**[2], **S Di Marco**[2], **C Traboni**[2], **A Folgori**[2], **S Colloca**[2], **S Capone**[2], **A Vitelli**[2], **R Cortese**[4], **P Klenerman**[3], **A Nicosia**[2,5,6], and **AJ Pollard**[1]

[1]Oxford Vaccine Group, Department of Paediatrics and the NIHR Oxford Biomedical Research Centre, University of Oxford, Oxford, United Kingdom [2]ReiThera Srl, (former Okairos Srl), Viale Città d'Europa 679, 00144 Roma, Italy [3]Experimental Medicine Division, Nuffield Department of Medicine, University of Oxford, Peter Medawar building, Oxford OX1 3SY, UK [4]Keires AG, Bäumleingasse 18, 4051, Basel, Switzerland 5CEINGE, Via Gaetano Salvatore 486, 80145, Naples, Italy [6]Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Via S. Pansini 5, 80131, Naples, Italy

Abstract

Respiratory syncytial virus (RSV) causes respiratory infection in annual epidemics, with infants and the elderly at particular risk of developing severe disease and death. However, despite its importance, no vaccine exists. The chimpanzee adenovirus, PanAd3-RSV, and modified vaccinia virus Ankara, MVA-RSV, are replication defective viral vectors encoding the RSV proteins F, N and M2-1 for the induction of humoral and cellular responses. We performed an open-label, dose-escalation, phase 1 clinical trial in 42 healthy adults in which four different combinations of prime/boost vaccinations were investigated for safety and immunogenicity, including both intra-muscular and intra-nasal administration of the adenoviral vectored vaccine. The vaccines were safe and well tolerated, with the most common reported adverse events being mild injection site reactions. No vaccine-related serious adverse events occurred. RSV neutralising antibody titres rose in response to intramuscular (IM) prime with PanAd3-RSV, and after IM boost for individuals primed by the intra-nasal (IN) route. Circulating anti-F IgG and IgA antibody secreting cells (ASCs) were observed after IM prime and IM boost. RSV-specific T-cell responses were increased after IM PanAd3-RSV prime and were most efficiently boosted by IM MVA-RSV.

Correspondence: christopher.green@paediatrics.ox.uk, tel/fax; 0044 +1865 857 420.

Competing interests

AJP has previously conducted clinical trials of vaccines on behalf of Oxford University funded by GlaxoSmithKline Biologicals SA and ReiThera S.r.l, but does not receive any personal payments from them. AJP is chair of the UK Department of Health's (DH) Joint Committee on Vaccination and Immunisation (JCVI), but the views expressed in this manuscript do not necessarily represent the views of JCVI or DH. AV, RC, and AN are named inventors on patent applications covering RSV antigen expression system (WO 2012/089833). The remaining authors declare they have no competing interests.

Data and materials availability

RSV001 was registered with clinicaltrials.gov and EudraCT (ref NCT01805921 and 2011-003589-34 respectively).

Clinicaltrials.gov NCT01805921.

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IFNγ secretion after boost was from both CD4+ and CD8+ T-cells, without detectable Th2 cytokines that have been previously associated with immune pathogenesis following exposure to RSV after formalin inactivated RSV vaccine. In conclusion, PanAd3-RSV and MVA-RSV are safe and immunogenic in healthy adults. These vaccine candidates warrant further clinical evaluation of efficacy to assess their potential to reduce the burden of RSV disease.

Keywords

Respiratory syncytial virus; vaccine; phase I; adenovirus; MVA

INTRODUCTION

Respiratory syncytial virus (RSV) causes annual epidemics of respiratory infection throughout life with young infants and the elderly especially susceptible to developing severe disease. There is only supportive care for cases of infection. Despite decades of research effort there remains no licensed vaccine for the prevention of severe disease, and the use of palivizumab monoclonal antibody prophylaxis is limited to high-risk infants only. The peak incidence of disease requiring hospitalisation is in the first few months of life (1). Infants with bronchiolitis account for up to 18% of winter paediatric admissions (2) and infection by RSV is responsible for almost 80% of cases (3). Two thirds of infants are infected by RSV in the first year of life (1) and 2-3% of primary infections require admission to hospital (4, 5). Approximately 6% of these admissions will require management on dedicated paediatric intensive care units (6). Worldwide, RSV disease in children under the age of 5 years account for an estimated 33.8 million lower respiratory tract infections, 3.4 million hospitalisations and up to 200,000 deaths annually (8). RSV-associated deaths are almost exclusive to resource poor areas of the world where RSV is second only to malaria in all-cause infant mortality between 1 and 12 months of age (7, 8). Cumulative RSV exposure produces an immune response capable of protection against severe lower respiratory tract disease but not protection from infection. 50% of infants suffer at least one RSV re-infection by their second birthday (1) and there is increasing evidence for causality between RSV infection in infancy and subsequent wheezing and possibly asthma in later life (9, 10). Healthy adults can expect a 7-9% annual risk of infection with mild symptomatic disease (11, 12) and severe immune suppression can re-establish a risk of developing severe disease (13). Later in life, senescence of the immune system and comorbid conditions place the elderly at risk of developing severe RSV disease, and estimates of hospital burden and mortality from RSV in the elderly vary but may be comparable to seasonal influenza (14-16).

The high rate of emergency admissions, lack of universal and cost-effective preventative measures and the magnitude of seasonal disease incidence maintain RSV as a major priority for vaccine development (17). Concerns over safety and an incomplete understanding of the immune correlates of protection have hampered efforts to develop such a vaccine. The formalin-inactivated RSV vaccine candidate (FI-RSV), tested nearly 50 years ago, led to enhanced respiratory disease (ERD) upon RSV exposure. ERD, which had a propensity to manifest in the youngest infants, caused 80% to become hospitalised over the subsequent

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RSV transmission season (compared to 5% in controls) and two fatalities in FI-RSV recipients (18-21). Animal challenge experiments and post-mortem lung histology from the infant fatalities implicated ERD as a vaccine-primed Th2-associated immunopathology following exposure to natural RSV infection, and has cast a long shadow over subsequent vaccine design and development. Subunit vaccine formulations have therefore remained in development for seropositive target populations and as maternal vaccines. Intra-nasal live attenuated RSV vaccine candidates, whose development also started in the 1960's, continue to be evaluated in humans including RSV-naïve infants, but have been troubled by nasal congestion (young infants are obligate nasal breathers), genetic instability, vaccine virus transmission, and limited immunogenicity (22-26).

Genetically modified chimpanzee derived adenovirus and modified vaccinia virus Ankara (MVA) viral vectors are safe and highly successful biological platforms that induce robust immune responses when used as genetic vaccine carriers for several infectious diseases and cancer (27, 28). We have generated an artificial, consensus-based, RSV antigen including a soluble F protein for the induction of neutralizing antibodies and the conserved N and M2-1 internal proteins to elicit T-cell immunity. The replication-defective viral vectors PanAd3 and MVA encode this antigen as a vaccine. PanAd3 is a hexon group C chimpanzee derived adenovirus (ChAd) and one of the most immunologically potent adenoviral vectors tested in rodents and primates (29). PanAd3 has not been in tested in humans before, but its sequence is very similar to other group C adenoviral vectors, including human Ad5, human Ad6 and the chimpanzee adenovirus 3 (ChAd3), that have been used extensively in clinical trials and returned good safety and strong immunological potency in humans (30, 31). In developing this approach towards an RSV vaccine in humans, homologous and heterologous combinations of PanAd3-RSV, including intranasal vaccination route, and MVA-RSV were tested in preclinical models. The genetic vaccines elicited RSV-specific neutralizing antibodies and T-cell immunity in non-human primates and protective efficacy in challenge experiments in rodents with human RSV and in young sero-negative calves with bovine RSV (32)(see Taylor *et al* in this edition of STM). Of critical importance in both rodent and bovine challenge models was the absence of immunopathology associated with ERD after vaccination, with the calf model acting as a translational model for the development of a vaccine for the paediatric population. All regimens fully protected the lower respiratory tract from bovine RSV infection in the calf, and heterologous combinations resulted in sterilizing immunity in both upper and lower respiratory tracts (see Taylor *et al* in this edition of STM).

Here we report the translation of this preclinical research into a first-in-man clinical trial in healthy adult volunteers to test the safety and immunogenicity of these vaccine candidates administered in four different prime/boost combinations, including intranasal delivery.

RESULTS

40 healthy adult volunteers were selected for testing different prime/boost combinations of vaccine in an open-label, dose escalation study design

The vaccination schedules that defined each study group, and the baseline physical and demographic characteristics of volunteers within each group, are shown in Table 1 and in supplementary material (sFigure 2). In each experimental group, the first two enrolled

volunteers received a lower dose of PanAd3-RSV (5×10^9 vp, viral particles) and MVA-RSV (1×10^7 pfu, plaque forming units). The remaining volunteers received a target-dose of each vaccine, which was a ten fold higher dose of PanAd3-RSV (5×10^{10} vp) and MVA-RSV (1×10^8 pfu).

Study volunteers were self-selected members of the public responding to recruitment material that invited an expression of interest to take part in trial. 374 expressions were received and 101 were potentially eligible and invited for face-to-face physician screening. From these, 40 eligible volunteers were recruited to the study according to protocol defined inclusion and exclusion criteria (see supplementary material, sTable 1 and sFigure 1). Two volunteers withdrew after receiving the prime dose vaccination for reasons unrelated to the vaccine, and were replaced as per protocol resulting in a total number of 42 volunteers enrolled into the trial. A total of 82 doses of vaccine were administered according to the protocol-defined groups and 418/433 (96.5%) of scheduled visits were attended within the protocol-defined windows after vaccination.

PanAd3-RSV and MVA-RSV appear safe in healthy adult volunteers

There were two severe adverse events, each considered unrelated to the vaccine and are described in the supplementary material (sTable 2). Overall 18406/19027 (96.7%) of all expected safety data points were collected for analysis. Common adverse events were local site reactions typical to vaccines given by intramuscular injection. These events were self-limiting and generally mild to moderate in severity (see Figure 1). Only a few volunteers reported one or more solicited adverse event that lasted more than one week after vaccination, and all adverse events reached full resolution. IM MVA-RSV caused a greater frequency, severity and duration of pain and other local reactions relative to IM PanAd3-RSV. There were two recorded fevers. One was from a volunteer 3 days after IN PanAd3-RSV prime and was concurrent with an influenza-like illness that developed after vaccination and the detection of rhinovirus on nasal sample PCR. The second fever occurred after IM PanAd3-RSV boost. There were no cumulative adverse events with repeated doses of IM PanAd3-RSV for group 2 volunteers. Volunteers who received IN PanAd3-RSV reported very few adverse events within one week of vaccination.

Unsolicited adverse event reporting identified that 5/21 recipients of IN PanAd3-RSV suffered short, mild and self-limiting sore throat reactions within one week of vaccination. No sore throat reactions were reported, or were required to be reported, after intra-muscular prime or boost. Nasal samples collected 3 days after IN PanAd3-RSV did not detect any shedding of vaccine virus. Adverse events detected from safety blood sampling and visit observations were generally mild and a drop in haemoglobin was detected in volunteers from all groups after vaccination, likely related to regular phlebotomy. Two transient drops within one week of boost vaccination (one after IM PanAd3-RSV, the other after IM MVA-RSV) were clinically significant and possibly related to the vaccine since there was a concurrent drop in other haematological indices. All measures returned to normal at the next sample collection one week later (see supplementary material, sTable 3). A vaccine-related, clinically non-significant and momentary rise in C-reactive protein above 10 mg/L was noted 3 days after IM vaccination in 3 volunteers after IM PanAd3-RSV prime (maximum

48.4), 9 volunteers after MVA-RSV boost (maximum 56.8) and one volunteer after IM PanAd3-RSV boost (maximum 11.2).

Influenza-like illnesses during the trial

Unscheduled visits were provided for volunteers reporting influenza-like illnesses throughout the study to identify RSV infections that could potentially impact the measured immune responses. Nasal swabs were collected and analysed by PCR for respiratory viral infections. A total of 30 unscheduled visits were performed. 11 of these unscheduled visits were within 5 weeks after either the prime or boost vaccine, and 9/11 tested PCR positive for rhinovirus with the other two cases failing to identify any pathogen. Overall 9/42 (21%) of the healthy adult volunteers suffered a symptomatic rhinovirus infection within one month of receiving one of the vaccines, with no increased risk attributed to a particular vaccine or route of immunization (3 following IN PanAd3-RSV, 2 following IM PanAd3-RSV, 3 following MVA-RSV and one following IM PanAd3-RSV boost). Three nasal swab samples were positive for RSV by PCR at different times after the vaccination and during the transmission season (2 volunteers from group 1, one volunteer from group 3). The first RSV detection was 10 weeks after boost and in all cases RSV detection was concomitant with the detection of other respiratory viruses. The only immunological assay performed after the detection of these RSV infections was the final assessment of antibody-mediated RSV neutralisation titres at week 34.

Serum RSV neutralising antibody titres increased in response to vaccination with PanAd3-RSV and MVA-RSV

A plaque reduction neutralisation assay (PRNA) was used to investigate the effect of vaccination on the functional antibody response to RSV (Figure 2). Baseline titres are representative of the background immune response to RSV from cumulative natural exposure in young healthy adults, with a geometric mean titre (GMT) for all 42 volunteers of 3191 (95% CI 2415 – 4217). Anti-RSV GMTs rose in response to IM PanAd3-RSV prime from 2771 (95% CI 2199 – 3495) at baseline and peaked at 4817 (95%CI 3731 – 6218), for groups 1 and 2 at both doses combined 4 weeks later. In contrast, serum anti-RSV GMTs remained indistinguishable from baseline levels following IN PanAd3-RSV prime, 2801 (95%CI 2094 – 3747) at baseline and 2547 (95%CI 1801 – 3602) 8 weeks later.

Serum GMTs from IM primed volunteers were observed lower at later recorded time points, indicating a waning of antibody titres towards baseline by week 30 and 34 despite the administration of a booster vaccine at week 4 (for group 2) or week 8 (for group 1). For volunteers primed with intra-nasal PanAd3-RSV, we observed a 4-week lag after IM boost before anti-RSV GMTs rose. Titres rose from 2540 (95%CI 1506 – 4281) to 3562 (95%CI 1718 – 7388) after IM MVA-RSV boost (group 3) and from 3015 (95%CI 1824 – 4984) to 4196 (95%CI 2936 – 5998) after IM PanAd3-RSV boost (group 4). Subsequent time points recorded a waning of neutralising antibody titres towards baseline levels at or before week 34. In all study groups there did not appear to be any relation between the dose of vaccine and the magnitude of the immune response (see supplementary material, sFigure 3). Volunteers with RSV-confirmed infections were not excluded from the final measure of neutralising antibody titres at week 34, which was performed 8 and 16 weeks after RSV

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detection for 2/3 cases and on the same day as detection for the final case. For these individuals there was no increase in titres from the last recorded titre.

Antibody secreting cells appear in blood in response to PanAd3-RSV and MVA-RSV

To investigate cellular immunogenicity of the vaccines in each group and the antibody response in more detail, we proceeded to measure the anti-F specific IgG and IgA plasma B-cell (antibody secreting cell, ASC) response 7-days after vaccination using a dual IgG and IgA *ex vivo* enzyme-linked immunospot (ELISpot) assay (Figure 3). Detectable responses were found in only 1/26 and 5/32 volunteers for anti-F IgG and IgA respectively at baseline. One week after IM PanAd3-RSV prime we could detect circulating anti-F IgG ASCs in 19/19 volunteers, and anti-F IgA ASCs in 18/20 volunteers with a median of 92 and 31 spots per million PBMCs respectively. Fewer volunteers made detectable responses to IN PanAd3-RSV prime (8/17 and 8/18 for anti-F IgG and IgA ASCs respectively) and these were of a lower magnitude (median 4 and 5 spots per million PBMCs for IgG and IgA respectively).

Interestingly, the hierarchy of responses to prime was reversed for the boost response. Volunteers primed with IN PanAd3-RSV after boost made comparable responses to IM PanAd3-RSV primed volunteers irrespective of whether the boost was IM MVA-RSV or IM PanAd3-RSV. At 7-days after boost, responses could be detected in 9/9 volunteers with a median of 217 and 27 spots per million PBMCs for anti-F IgG and IgA ASCs respectively in group 3 (IM MVA-RSV) and in 9/9 and 8/9 volunteers with a median of 109 and 27 spots per million PBMCs for anti-F IgG and IgA ASCs respectively in group 4 (IM PanAd3-RSV). In contrast, responses could be detected in 4/5 and 3/5 volunteers with a median of 38 and 5 spots per million PBMCs for anti-F IgG and IgA ASCs respectively in group 1 after IM MVA-RSV boost, and in 2/9 and 0/9 volunteers with a median of 0 spots per million PBMCs in group 2 after IM PanAd3-RSV boost (see supplementary material, sFigure 4).

PanAd3-RSV and MVA-RSV expand interferon-gamma (IFNγ) T-cell responses in healthy volunteers

To further characterise the cellular immune response to vaccination we examined the IFNγ T-cell response before and after vaccination using an *ex vivo* enzyme-linked immunospot (ELISpot) assay (Figure 4). This assay employed four peptide pools encompassing the whole amino-acid sequence of the vaccine antigen. At baseline, PBMCs from 19/33 (58%) of volunteers had detectable responses to at least one peptide pool. Analysis of fresh PBMCs collected two weeks after prime recorded the frequency of subjects showing RSV-specific IFNγ T-cell responses had increased to 16/18 (89%) and 16/19 (85%) after IM and IN PanAd3-RSV respectively (see supplementary material, sFigure 5). Consistent with the post-prime ASC response, the magnitude of the T-cell IFNγ response was greater 2 weeks after IM prime (geometric mean of 306 spots per million PBMCs, 95%CI 199 – 471) than after IN prime (geometric mean of 123 spots per million PBMCs, 95% CI 68 – 224).

IFNγ T-cell responses were comparable to pre-vaccination responses before boost at week 4 (group 2) and week 8 (groups 1, 3 and 4). The heterologous PanAd3-RSV prime/MVA-RSV boost generated the highest magnitude and breadth of RSV-specific T-cell responses with a

10-fold increase over the baseline recorded one week after boost, irrespective of the route of prime. The geometric mean responses reached 1643 (95%CI 1152 – 2344) and 1588 (95%CI 1077 – 2342) spots per million PBMCs in groups 1 and 3 respectively. The response to IM PanAd3-RSV boost was 598 (95%CI 437 – 820) and 400 (95%CI 211 – 758) spots per million PBMCs in groups 2 and 4 respectively. The kinetics of individual responses within each study group showed no effect of vaccine dose on the magnitude of responses after prime or boost vaccination, and IFNγ T-cell responses post-boost were distributed over the peptide pools covering F, N and M2-1 vaccine antigens. Most of the T-cell responses were directed to the F protein, possibly due to the larger size of this antigen (524aa) as compared to N (391aa) and M2-1 (256aa) (see supplementary material, sFigure 6).

PanAd3-RSV and MVA-RSV expand both CD4+ and CD8+ T-cell responses

Next we tested the functional phenotype of vaccine induced T-cells by intracellular cytokine staining (ICS) and FACS analysis on frozen PBMC stimulated with the same peptide pools used in the IFNγ ELISpot (see Figure 5 and sFigure 7). In keeping with the ELISpot data we observed low levels of CD8+ and CD4+ T cell responses at baseline. There was a small but consistent increase in IFNγ-producing CD4+ T cells, of a magnitude consistent with the ELISpot data, and responses to all peptide pools. This was seen most clearly in groups 1 and 3 who were boosted with IM MVA-RSV. Similar data (in terms of magnitude, breadth and group responses) were observed on analysis of CD8+ T cells, indicating the IFNγ responses were balanced between CD4+ and CD8+ T-cells. Similar data were obtained for CD4+ and CD8+ IFNγ secreting cell populations by analysis of total response pooled across the four proteins. Using a threshold for positivity of 0.02% to detect a T cell response (33, 34), we observed an increase in the number CD4+ and CD8+ IFNγ responses, again most obviously in groups 1 and 3 (see supplementary material, sFigure 8). In terms of Th2 responses, we did not observe any responses against these peptide pools at either time point by parallel analyses of IL-5 secretion, although reactivity from positive control stimuli was observed (see supplementary material).

To further explore the phenotype of vaccine-induced immune responses, we measured the production of cytokines by a cytometric bead array (CBA) using supernatants from the IFNγ ELISpot of three volunteers in each target-dose study group at baseline and one week post-boost. No detectable IL2, IL4, IL10 and TNFα responses were observed above background, and IL6 and IL17 production was detected mostly in response to N peptide pool stimulation with no consistency in changes across baseline or after vaccination (sFigure 12).

Anti-PanAd3 neutralising antibody titres were detectable at baseline and were efficiently boosted by IM PanAd3-RSV

At baseline 29/40 (73%) of volunteers had detectable neutralising antibody titres to the PanAd3 adenoviral vector (Figure 6) and a higher proportion of volunteers than expected, 15/40 (38%), recorded a titre >200 (29). There was no correlation between pre-existing vector antibody titres and volunteer age (sFigure 13). Baseline titres were higher in volunteers allocated to receive prime with the IN spray. After administration of the prime vaccine it was evident that IM PanAd3-RSV induced a significant rise in circulating anti-PanAd3 neutralising antibody where IN PanAd3-RSV did not. Importantly, there appeared

to be no correlation between the anti-PanAd3 titres before vaccination and any of the measured immunological responses after vaccination (see supplementary material, sFigure 14).

DISCUSSION

Development of an effective RSV vaccine remains a high public health priority for infants, the elderly and immune compromised adults. However, an incomplete understanding of the immune correlates of protection and concerns over vaccine immunopathology has slowed progress towards meeting this need. Here, we report the first human data on the safety and immunogenicity of viral-vectored vaccines expressing RSV proteins.

PanAd3-RSV and MVA-RSV appeared to be safe in this small population of healthy adults. Local adverse events at sites of vaccine injection were common, especially following MVA-RSV, consistent with the experience from other adenoviral- and MVA-vectored vaccine preparations (31). Intra-nasal PanAd3-RSV caused a proportion of volunteers to develop a mild, self-limiting sore throat shortly after vaccination. The reason for this is unknown, it was not reported or required to be reported following vaccination, and may be a non-specific effect of the mucosal immune response. None of the nasal samples from volunteers contained detectable vaccine virus at day three after IN PanAd3-RSV vaccination. The immunobiology of RSV is complex and can be both protective and harmful as observed after FI-RSV vaccination. In humans FI-RSV immunopathology featured non-neutralising antibody and a mononuclear cellular lung infiltrate in post-mortem lung histology (20, 21, 35). The human cytokine response following FI-RSV has not been characterised but is inferred from animal challenge data. Similar pulmonary immunopathology was observed to RSV G glycoprotein candidate vaccines in the mouse challenge model (36). Although the mechanisms of disease are different for FI-RSV and G glycoprotein vaccine ERD, the mouse model was characterised by the involvement of CD4+ cells (with a loss of CD8+ cell inhibition) and the production of IL-4, IL-5 and IL-13 cytokines (36-39). The absence of ERD-associated patterns of response to vaccination in the respective ICS and CBA assays in the current study, together with the pre-clinical results obtained with PanAd3-RSV and MVA-RSV vaccination followed by RSV challenge, provide important support for the further development of this approach. It is important to note that the assays presented here were performed after vaccination and not following natural RSV exposure, and our clinical study population had prior exposure to RSV, which was a significant factor that conferred protection to ERD in older infants following FI-RSV (40). The absence of detectable Th2 cytokine responses in a small number of volunteers after boost, while encouraging, does not fully discount the induction of undesirable vaccine immunogenicity in other populations after vaccination followed by natural infection. This will require continued evaluation in the elderly and throughout age de-escalating development of these vaccines in seropositive children and infants towards RSV-naïve infants.

RSV disease is propagated within the host by the release of virus (targets of neutralising antibody) from infected cells and by cell-to-cell transfer (targets of cellular immunity). Safe and optimal vaccine-induced protection from severe disease might therefore require the induction of desirable humoral and cellular RSV-specific immune responses. Serum

neutralising antibodies alone constitute a significant obstacle for RSV and mitigate of the risk of developing severe disease. Passive immune prophylaxis (palivizumab) for high-risk infants can reduce hospitalization by 45-55% (41, 42), and these circumstances demonstrate how RSV F protein-specific antibodies alone can sometimes be sufficient to confer protection to severe disease. Although adult sera contains high titres of naturally acquired F- and G-specific neutralising antibody, as measured at baseline, IM administered PanAd3-RSV and MVA-RSV were able to induce up to a two-fold rise in neutralising titre after vaccination. A similar fold-change in neutralising antibody titres was observed following a promising RSV nanoparticle vaccine, now entering late stage clinical evaluation as maternal vaccine candidate (43). High RSV-specific neutralising antibody titres from natural exposure can persist into later life (44), though, according to some reports, lower titres are associated with the development of severe disease in the elderly (45-47). Vaccine induced F-specific antibodies might therefore fulfil functional and biologically relevant roles in protection against RSV in infants and the elderly. The neutralising antibody response wanes from a few weeks after the first IM vaccination, but the relevance of this for a paediatric sero-negative population cannot be anticipated. Further preclinical studies in calves could model the longevity of vaccine-induced immune responses in naive populations. A clear signal of vaccine take after IM PanAd3-RSV, and later IM MVA-RSV, was observed in all subjects by the detection of circulating anti-F IgG and IgA ASCs which were undetectable at baseline. The mean anti-F IgG ASC response 7 days post-IM PanAd3-RSV prime was 149 spots per million PBMCs (±SD 136.1), which compares with post-infection clinical data in elderly adults who, approximately 7 days into symptomatic RSV infection, recorded a mean anti-F IgG ASC response of 200 spots per million PBMCs (±SD 256)(48). The absence of detectable ASC responses 7 days after boost in IM primed volunteers, and not IN primed volunteers, may be an effect of vector neutralising antibody or imply differences in the kinetics and magnitude of ASC responses after primary and secondary immunisations. In other vaccine trials the peak ASC response following a booster vaccine appeared around day 7 for rabies, capsular group C meningococcal, pneumococcal and oral cholera vaccines (49-52). Furthermore, where a second influenza dose is not indicated in healthy adults and the elderly it remains a requirement for naïve infants for an effective immune response, indicating that favourable and demonstrable immunogenicity after two intramuscular injections remains possible in sero-negative infants when it was not observed in an adult population

In vivo viral neutralisation by antibody is supported by cellular immune components to protect from severe RSV disease. T-cell deficiencies in infants and T-cell immune senescence in the elderly confer a substantial risk of developing severe disease (44, 45, 53, 54). It seems likely that an optimal vaccine for the elderly should also re-establish T-cell effectiveness and a heterologous prime/boost regimen may be preferred for optimal restoration of RSV-specific immunity. For other vaccine antigens, the heterologous prime-boost vector combination induces strong antigen-specific T-cell responses (30) and consistent with these observations we showed PanAd3-RSV prime/MVA-RSV boost was able to induce robust RSV specific T-cell responses independent of the route of priming. This indicates additional immunogenicity from a boost vaccine, within the context of a previous vaccine ‘prime’ in naturally primed healthy adults, and of potential value in

considering the development of prime/boost combinations for seropositive children and the elderly where impaired T-cell responses have been associated with severity of disease. We did not seek to identify de novo priming of naïve T cells after prime or after boost, but in several volunteers, who later developed clear responses after vaccination, we were not able to detect T-cell reactivity to RSV peptide pools at baseline. Phenotypic analysis of the RSV-specific T-cells showed baseline CD4+ responses were generally below 0.1%, which was within the range for healthy adults reported elsewhere (0.05 and 0.3%) (55), and baseline CD8+ T-cell responses were similar to those against HLA-B7 restricted epitopes to RSV nucleoprotein (56). Analysis of the cell population frequencies one week after PanAd3-RSV or MVA-RSV boost showed an expansion of CD4+ and CD8+ T-cells producing IFNγ. A similar RSV-specific Th1-biased CD4+ T-cell response was observed after immunization of PanAd3-RSV prime/MVA-RSV boost in relevant preclinical models such as non-human primates and sero-negative calves (32) (see Taylor et al in this edition of STM). The identification of RSV epitopes for CD4+ and CD8+ cells remain an active area of investigation (57), but in the context of influenza a positive correlation was reported between the frequency of memory IFNγ CD4+ cells and protection from clinical symptoms (58).

All our immunology endpoints indicate that intra-nasal prime did not induce detectable immune responses in peripheral blood for many volunteers. However, responses measured in blood are not representative of the overall immune response to vaccination or infection. The vaccination regimens selected for clinical evaluation were determined from challenge experiments in rodents and sero-negative calves, where they appeared safe and capable of inducing sterilising immunity without FI-RSV associated pulmonary pathology (32) (see Taylor et al in this edition of STM). The regimens based on intranasal vaccination showed improved protective efficacy in the upper respiratory tract, and were therefore included in the human trial. Preclinical experiments in mice demonstrated that the PanAd3 vector, used an influenza vaccine candidate delivered intra-nasally, induced greater IgG antibody responses in broncho-alveolar lavage samples and greater CD8+ IFNγ T-cell responses in the lungs compared to the same vaccine given by intramuscular injection, which generated greater responses in the spleen (59). When applied as a vector for RSV antigen, IN PanAd3-RSV induced lower levels of systemic RSV-specific T-cells than did IM PanAd3-RSV, but intranasal prime generated comparable levels of immunity in the lung (32). Selective accumulation of memory T-cells in the lung against respiratory pathogens like RSV has been described in humans (60), and pulmonary T-cells may have contributed substantially to protection afforded by IN PanAd3-RSV in animal challenge studies. The only licensed intra-nasal vaccine, the live attenuated influenza vaccine (LAIV, Flumist®), protects despite its limited ability to induce humoral and cellular effector responses in blood compared to the injectable trivalent inactivated vaccine (TIV) (61, 62), signifying a crucial role for local mucosal immunity in protection to respiratory pathogens. Recent data from healthy adults infected with RSV under experimental challenge conditions implicated an important role in RSV-specific IgA muscosal immune responses (as well as IgA B-memory immunity) and a putative correlate for protection to infection (63). Although one obstacle facing intra-nasal live attenuated RSV vaccine candidates has been their limited capacity to induce serum antibody in sero-negative infants, these infants were able to significantly restrict, by 100-

fold, the replication of a 'second challenge' subsequent vaccines dose (25). Therefore intra-nasal PanAd3-RSV prime may have generated desirable immune responses at mucosal sites. The mucosal immune response to IN PanAd3-RSV in humans remains potentially important for protection to infection of the upper respiratory tract and supports the use of an IM boost combination (PanAd3-RSV or MVA-RSV), even in naturally primed vaccinees, to induce RSV-specific immune responses in blood for the purpose of supporting protection to severe disease in the lower respiratory tract.

An alternative consideration is that IFNγ T-cell responses to IM MVA-RSV, in the case of group 4, were independent of the vaccine-priming route in naturally exposed adult volunteers. Previous use of single dose MVA-vectored vaccines, or use of these vectors for priming, has not proved sufficiently immunogenic in formulations developed for malaria, tuberculosis or HCV because of the presumed need for effective priming by an alternative vaccine or natural exposure (31, 64, 65). In the context of RSV, this opens the possibility of IM MVA-RSV acting as a single dose vaccine candidate for populations with past exposure to RSV and this is currently being explored in an extension to the clinical trial in healthy adults aged 60-75 years (clinicaltrials.gov NCT01805921). These data from healthy younger adults supports the evaluation of prime/boost combinations in the older adults, and the trial extension also includes the combinations used in group 1 (IM PanAd3-RSV/MVA-RSV boost 8 weeks apart), group 2 (IM PanAd3-RSV/IM PanAd3-RSV 4 weeks apart) and group 3 (IN PanAd3-RSV/IM MVA-RSV boost).

The potential limiting effect of pre-existing and *de novo* induced anti-vector neutralising antibody on the magnitude of immune responses to viral vectored vaccines remains an area of active investigation. Anti-Ad5 neutralising antibody titres >200 were associated with lower immune responses to an Ad5-vectored HIV vaccine candidate (66). The PanAd3 vector was selected on the basis of low prevalence of neutralising antibodies in humans and potent immunogenicity in animal models. The same criteria were applied to select successful chimpanzee derived adenoviral vectors for HCV, malaria and Ebola antigen in humans (ChAd63 and ChAd3)(29, 30, 67). Our study volunteers were excluded at screening if they had had, at any time, received another adenoviral or MVA-vectored vaccine, and yet the proportion of volunteers with pre-existing neutralising anti-PanAd3 titres >200 exceeded the 3% expected from earlier estimates from US and European populations (29). The source of these antibodies remains unclear, but the PanAd3 hexon protein results in its classification within subgroup C of human adenovirus (29), and therefore the potential for cross-neutralising antibodies from other adenovirus exposure. The predictive value of in vitro neutralization assays on adenovirus-vectored vaccination has been a matter of debate (68). While our data do not identify a clear effect from anti-PanAd3 neutralising antibody on immune responses to vaccination, the group sizes are small and immune responses we report are from robustly pre-primed individuals. Cumulative natural exposure to RSV produces highly functional antibodies that may mask loss of vaccine immunogenicity caused by vector neutralisation. Data from RSV infected adults showed no correlation between the magnitude of the RSV-specific ASC response, RSV neutralising antibody titres and anti-F IgG antibody titres in serum (48). Furthermore, anti-AdHu5 neutralising antibody had no effect on T-cell responses to AdHu5 vectored vaccines (69) implying the mechanisms of

vector antibody interference with vaccine responses may not be readily measured using standard assays. The study was not designed to infer protective efficacy of these vaccines, and we detected three mild RSV infections (concomitant with other respiratory viruses) that occurred within the RSV transmission season 18, 10 and 10 weeks after the last vaccination. Infection rate estimates in healthy adults by volunteer reported (and subjective) influenza-like illnesses would not capture very mild or sub-clinical RSV exposure, and the potential this has to alter RSV-specific immunity after vaccination. There was no non-vaccinated control arm to this study and the sample size is small which makes it difficult to evaluate the finding of three confirmed RSV infections, though this frequency of infection is consistent with estimates of the annual risk of symptomatic RSV infection in young healthy adults (11, 12). In healthy adults, vaccine safety and protective efficacy could be further explored using controlled human challenge experiments. However, while sterilising immunity may be desirable, this is not achieved with wild-type RSV infection and is therefore a high bar for a vaccine. The key driver for immunisation is prevention of death, severe disease, and hospitalisation.

The principal limitations lie within the study design and study population of healthy adults. The open-label design of the study with volunteer reported symptoms after vaccination means we cannot exclude or quantify any bias in adverse event reporting, and the requirement for fist-in-man evaluation in a small group of healthy adults introduces inherent risks of confounding chance observations. There was no non-vaccinated control group and protection to infection or severe disease cannot be inferred. Furthermore the post-vaccination immunological measures used to infer sub-clinical safety and describe vaccine-induced immunity would have been heavily influenced by repeated RSV exposure. While these data from RSV-exposed adults may not provide a clear indication of the potential immunogenicity of an RSV vaccine in infants and the elderly, important information has been obtained that seems to indicate that RSV vaccine candidates PanAd3-RSV and MVA-RSV are safe, well tolerated and induce desirable immune responses in healthy adult volunteers which supports the further investigation of this approach. On the critical issue of progressing from these highly encouraging phase 1 data from healthy young adults, RSV viral-vectored vaccines are now under evaluation in healthy older adults aged 60-75 years and will enter a paediatric development programme later this year (EudraCT number 2014-005333-31). Careful surveillance of safety and immunogenicity data will continue as these vaccines progress through clinical development towards target populations.

In conclusion, we report the successful transition of genetic vaccine technology for RSV from preclinical investigation to phase 1 safety and immunogenicity in humans. Vaccine immunogenicity was generated despite the presence of vector neutralising antibodies and achieved immune responses above the background of immune responses to RSV derived from repeated seasonal exposure. The immune response to vaccination included a rise in serum RSV neutralising antibody titres, and was supported by anti-F IgG- and IgA-secreting B-cells and Th1 (IFNγ) responsive T-cells. The safe and potent immunogenicity of PanAd3-RSV and MVA-RSV observed in this study warrant their further clinical development towards target populations in need of an RSV vaccine, and represent a new and exciting development in over 50 years of RSV vaccine research.

MATERIALS & METHODS

Study design

RSV001 was an open-label, dose escalation, single-site, phase I clinical trial in 42 healthy adult volunteers aged 18-50 years. The primary objective was to characterise the safety and tolerability of four prime/boost vaccination regiments, as depicted in Table 1. The terms 'prime' and boost' are conventional and used here to indicate the first and second dose of vaccine. The terms are inherited from previous adenoviral and MVA-vectored vaccine research in immunologically naïve subjects and our population was already primed from repeated natural exposure. Volunteers were assigned to study groups by sequential allocation with the first two volunteers in each study group assigned to receive the low-dose of each vaccine. One month following the last low-dose prime an analysis of the safety data were submitted for approval from the data safety monitoring committee (DSMC) to proceed to low-dose boost vaccinations and to commence target-dose vaccination schedules of the remaining volunteers. Halting rules for dose escalation included whether any volunteer experienced a severe adverse event related to the vaccine, or if two or more volunteers experienced a severe adverse events that were clinically significant and had reasonable possibility of it being related to the vaccine. Each volunteer was invited to attend 12 visits over 34 weeks for study groups 1, 3 and 4, and 11 visits over 30 weeks for group 2. Vaccinations were performed between May and the first week of November 2013 to minimise the potential for natural sub-clinical boosting of RSV-specific immune responses in the immediate post-vaccination period. Each volunteer was followed up for 6 months after boost and the last volunteer completed the study in May 2014. The trial was conducted in accordance with the clinical trial protocol and the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization (ICH) Good Clinical Practices standards.

Clinical Trial Authorisation was granted by the United Kingdom Medicines and Healthcare Products Regulatory Agency (ref 35082/0003/001-0001). Ethical approval and amendments were granted by NRES Berkshire (ref 13/SC/0023). The trial was performed by the University of Oxford at the Centre for Clinical Vaccinology and Tropical Medicine, Oxford, and monitored by the Clinical Trial Research Governance department, University of Oxford.

Intervention

Each vaccine was a replication-defective genetically modified organism engineered to deliver the Fusion (F), Nucleocapsid (N) and Matrix (M2-1) RSV proteins. The generation of PanAd3-RSV and MVA-RSV and results of pre-clinical evaluation are described elsewhere (see Taylor *et al* in this edition of STM). In brief, the genetic insert for both PanAd3 and MVA vectors constituted a single synthetic DNA fragment encoding all three proteins. Upon transfection into a mammalian cell, cleavage of a Foot and Mouth Disease Virus 2A region between the F and N and M2-1 released a soluble truncated F protein (devoid of the transmembrane region) into the supernatant while the N and M2-1 proteins remain intra-cellular. Deletion of the E1 and E4 loci of PanAd3 rendered the vaccine vector replication-defective and MVA naturally cannot replicate in mammalian cells. Clinical grade PanAd3-RSV and MVA-RSV vaccine products were manufactured under Good

Manufacturing Practice conditions by Advent and Impfstoffwerk Dessau-Tornau. Safety and characterization tests were performed on well-defined stages of the production processes of the two vaccine products and comply with the European Pharmacopoeia (Ph. Eur). Testing included sterility, endotoxins, residual host cell DNA and proteins, genome sequencing and extensive screening for extraneous virus contamination. In PanAd3-RSV, absence of replication-competent adenovirus (RCA) was verified. The biological activity (potency) of PanAd3-RSV vaccine product was quantified using anti-hexon immune staining that quantitatively measures infectious virus titre, quantitation of total vector particle concentration by q-PCR, a calculated determination of total versus infectious particle ratio and determination of expression of RSV transgene by Western blot. Testing of the MVA-RSV vaccine product included virus titre, identity by PCR, purity by PCR (free of non-recombinant MVA) and expression of RSV transgene by Western blot. The stability of PanAd3-RSV and MVA-RSV vaccine products, at the recommended storage condition of ≤ −60°C, has been followed since product manufacturing and for the entire duration of the clinical trial. Both PanAd3-RSV and MVA-RSV vaccine products met product stability specifications throughout this period of time. The methods for stability testing included sterility with some characterisation tests as part of the stability-monitoring plan. Potency related tests (Western blot, vector particle concentration, infectious virus titre) were performed at each time point since these parameters are considered to be main indicators of the stability of the vaccine products.

Vaccines were stored cryopreserved at the trial site in monitored −80°C freezers until use. Each vaccine was granted use under GMO (Contained Use) Regulations 2000 by the Oxford University Hospitals NHS Trust Genetic Modification Safety Committee (ref GM462.11.64). Doses of vaccine were prepared by diluting the concentrated product with 0.9% sterile saline solution to the required concentration and volume. Low-dose and target-dose PanAd3-RSV was 5×10^9 and 5×10^{10} viral particles (vp) respectively. Low-dose and target-dose MVA-RSV was 1×10^7 and 1×10^8 plaque forming units (pfu) respectively. PanAd3-RSV was given either by intra-muscular injection of 0.5mls vaccine product to the non-dominant deltoid muscle, or by intra-nasal spray of 0.15mls volume to each nostril in the sitting position using a syringe attached to an LMA MAD Nasal™ needle-free drug delivery system (LMA). MVA-RSV was administered by intra-muscular injection of 0.5mls vaccine product to the non-dominant deltoid muscle only. All intra-muscular injections used the ‘Z’ technique to avoid tracking of the vaccine through the needle track.

Study participants and eligibility criteria

Male and female participants were self-selected healthy volunteers aged 18-50 years responding to open invitation to the trial. Volunteers provided informed consent in writing prior to any study procedures. Potential volunteers were excluded if they had any history of significant organ or system disease, any known or suspected alteration in immune function (including IgA deficiency and autoimmune disease), previous receipt of a simian adenoviral or MVA-vectored vaccine of any kind, or any other significant disease or disorder that presented potential for risk, could influence the results or impair the participants ability to participate in the study. Further details of eligibility criteria are set out in the supplementary

material (sTable 1). Eligible volunteers were considered enrolled at the point of receiving the first dose of vaccine.

Objectives and endpoint measures

The primary objective was to investigate the safety and tolerability, and secondary objective was the characterisation of immunogenicity, of the replication-defective Chimpanzee Adenovirus and MVA vectors expressing RSV F, N and M2-1 in healthy adult volunteers. Primary endpoint measures were the frequency and severity of solicited and unsolicited local and systemic adverse events within one-week after vaccination, safety bloods (full blood count and differential, serum renal and liver biochemistry, C-reactive protein and amylase) and visit observations (pulse, respiratory rate, blood pressure) obtained at all visits. Adverse events were graded using modified Food and Drug Administration (FDA) and Division of AIDS (DAIDs) criteria. For volunteers primed with PanAd3-RSV by intra-nasal spray an additional nasal sample was obtained 3 days later to detect vaccine virus shedding. An independent Data Safety Monitoring Committee (DSMC) acting in accordance to a pre-specified charter provided safety oversight for the duration of the trial and formal approval before dose-escalation and boosting. Immunogenicity assays are detailed below and sample processing and analyses of all immunology data was performed observer blinded by use of a randomly generated laboratory identifier. The endpoints of the clinical trial were prospectively selected.

Sample processing

Blood samples were collected in heparinised tubes (400μL heparin per 50mLs whole blood) for assays that required Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were isolated within 6hrs of sample collection from a 1:1 mix of heparinised blood with R0 (RPMI with Penicillin/Streptomycin and L-Glutamine, stored 4°C) by density centrifugation through Lymphoprep™ (Alere). An aliquot of PBMCs was immediately used for fresh ELISpot assays and the remainder cryopreserved in Recovery™ Cell Freezing Medium (10% DMSO and calf serum, Invitrogen). Serum samples were obtained by centrifugation of whole blood collected in clotted tubes, and then cryopreserved.

PanAd3 vaccine virus shedding assay

Nasal swabs samples were collected in viral transport medium (see Materials and Methods) 3 days after intranasal vaccination and analysed by WuXi AppTec, Inc. Specimens were inoculated onto monolayers of HEK 293 and A549 cell lines, to detect both replication competent and incompetent adenovirus by the presence of virus-induced cytopathic effects (CPE) (14-day In Vitro assay), and immunofluorescence detection (IFA) as confirmatory assay. Briefly, each test article was thawed at 37±2°C, vortexed and filtered through a 0.8 μm and 0.2 μm filter. Filtered test articles were inoculated in 24-well plates and 35mm dishes of HEK-293 and A549 cells and incubated for 50-70 minutes in a humidified atmosphere of 37°C with 5% CO_2. Inoculum was removed, the monolayers washed with PBS and fresh culture medium added. Dish cultures were visually monitored for cytopathic effect (CPE) for 14 days. On day 3 or 4, 24-well plates were processed for immunofluorescence analysis (IFA) using a monoclonal antibody against hexon adenovirus. The nasal swab samples were considered positive if positive immunofluorescence and/or

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cytopathic effect consistent with viral infection were observed in the test article-inoculated HEK-293 or A549 cultures.

Anti-PanAd3 neutralisation assay

PanAd3 neutralising antibody titres at baseline and before boost were assayed as previously described using a secreted alkaline phosphatase (SEAP) assay (70). Briefly, 3.5×10^4 HEK293 cells per well were seeded in a 96-well-plate for 2 days. SEAP-expressing PanAd3 was pre-incubated for 1 hour at 37 °C alone or with serial dilutions (1:18, 1:72. 1:288, 1:1152 and 1:4608) of heat-inactivated serum from trial volunteers, added to the 95-100% confluent HEK293 cells for 1 hour at 37°C, and the supernatant was then removed and replaced with 10% FCS in DMEM. SEAP activity in the supernatant was measured after 22-26 hours using the chemiluminescent substrate (CSPD) from Phospha-Light kit (Tropix) following manufacturer's instructions. Light signal output expressed as relative light units (RLU) was measured 45 minutes after the addition of the CSPD substrate using a luminometer (Envision 2102 Multilabel reader, Perkin Elmer). The neutralization titre was defined as the reciprocal of sera dilution required to inhibit SEAP expression by 50%, compared to the SEAP expression of virus infection alone.

Plaque-Reduction Neutralisation assay for the detection of neutralising antibody

50 plaque-forming units of RSV strain A2 were mixed with doubling dilutions of heat-inactivated sera over a range of 1:20 to 1:10240. This mixture was incubated for one hour to facilitate the neutralisation reaction before adding to a confluent layer of HEp-2 cells that had been seeded onto 96 well plates at a frequency of 3×10^4 cells per well. The plates were then incubated for 60 hours at 37°C, 5% CO_2, 95% humidity. Cells were then fixed using cold acetone/methanol (80%/20% v/v) and RSV plaques detected by immuno-staining using amino-ethyl-carbazole. The neutralising titre was defined as the sera dilution at which 50% of plaques survive, and was calculated using the Spearman-Karber method.

Dual-colour ex-vivo ELISpot assay for the detection of anti-F IgG and anti-F IgA ASCs

$Multiscreen_{HTS}$ HA plates (Millipore, MSHAN4510) were coated with 5μg/mL F protein antigen (Sino Biological Inc), 10μg/mL Human Serum Albumin (HSA, Sigma), 5μg/mL tetanus toxoid protein (Statens Seruminstitute) and 10μg/mL polyvalent goat anti-human immunoglobulins (Caltag). After washing, plates were blocked with 1% skimmed milk for 45 minutes at 37°C before 100μL/well PBMCs in R10 (RPMI, 10% Foetal Bovine Serum, 2mM L-Glutamine, 50μg/ml Streptomycin, 50U Penicillin) were added at a starting dilution of 2×10^6/mL and incubated overnight at 37°C, 5% CO_2, 95% humidity. After washing plates were developed anti-human IgG-FITC (Sigma) and anti-human IgA-Biotin (AbD Serotec). After washing anti-FITC AP (Sigma) and Streptavidin-HRP (AbD Serotec) were added for 30 minutes at room temperature. Following final washes 3-Amino-9-ethylcarbazole (AEC) substrate kit (Sigma) was added for 30 minutes at room temperature, washed with dH_2O and 100μL/well Vector Blue substrate (Vector Laboratories) added for 10 minutes at room temperature before a final wash in dH_2O. After drying overnight plates were read using Autoimmun Diagnostika (AID version 5.0) and responses measured as the antigen-specific spots per million PBMCs with HSA background subtracted. A positive response was defined as any detection of spots above HSA background.

Ex-vivo T-cell ELISpot assay for the detection of IFN-gamma

Plates were coated overnight with mouse anti-human IFNγ, clone 1-D1K (MAbTec) in Dulbecco's PBS (dPBS). Plates were blocked before addition of 50μL/well RSV peptide pools consisting mainly of 15-mer sequences with 11 amino acid overlaps and covering the sequence of proteins F, N and M2-1 (JPT Peptide Technologies). The 269 peptides were dissolved in 100% DMSO and arranged in four pools designated as Fa (N terminus half of the F protein, 64 peptides), Fb (C terminal half of the F protein, 64 peptides), N (95 peptides) and M (46 peptides). Concentration of the four pools was adjusted at 0.3mg/mL single peptide in the mixture and used in the ELISpot assay at a final concentration of 3μg/mL of each peptide. DMSO (Sigma) was used as a negative control and CMV cell lysate, FEC (mixed HLA class–I restricted peptides from Flu, EBV and CMV) and ConA (Sigma) acted as positive controls. 50μl/well PBMCs were added to peptide wells in triplicate at a concentration of 4×10^6/ml and incubated overnight at 37°C, 5%CO_2, 95% humidity. Detection was with anti-human IFNγ, clone 7-B6-1, Biotin conjugate (MAbTec) and an anti-Biotin AP conjugate (Vector Laboratories) with 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride substrate (BCIP/NBT 1-step solution, Pierce). IFNγ producing cells were counted using AID software version 5.0. The mean+4StDev of the DMSO response from all samples identified a cut off whereby individual samples with background DMSO values ≥50spot forming cells per million PBMCs were excluded from analysis. Samples were also excluded from analysis if no spots were detected in any positive control well. Calculation of triplicate well variance was applied as described elsewhere (71) and a threshold of 10 applied for exclusion from analysis. A response was considered positive when both (a) peptide pool responses were >50 spots per million PMBCs and (b) greater than 3× DMSO background for the individual. A subject was considered a positive responder if reactivity against at least one of the four RSV peptides pools was observed using these criteria.

Peripheral blood mononuclear cell intracellular cytokine staining

Frozen PBMCs were thawed in 9ml Thawing medium (RPMI with 10% FCS, 1% PenStrep, glutamine), re-suspended in serum-free 1ml CTL-Wash™ (Cellular Technology Ltd) wash buffer with 100μL DNAase and rested in an incubator overnight (5% CO_2, 37°C) in 4mL R10 (RPMI, 10% FBS, 2mM L-Glutamine, 50μg/mL Streptomycin, 50U Penicillin) before plating at a concentration of 1×10^6 cells/well in a 96-well tissue culture plate. DMSO, Fa, Fb, M, N, FEC, phorbol 12-myrstate 13-acetate (PMA, Sigma)/Ionomycin (Sigma) and phytohemagglutinin peptides (PHA, Sigma) were added with 1 microL to each well brefeldin A (1 in 100 dilution of 0.5μg/mL stock) and the plate was incubated overnight (5% CO_2, 37°C). After 1xPBS wash flourochrome-conjugated monoclonal surface staining antibodies L/D-APC-Cy-7 (Life Technologies Limited) in PBS solution were added for 20 minutes before washing and adding 1% FACS Fix (1ml Formaldehyde in 36ml PBS) for 20 minutes. Plates were washed with permeabilization buffer (eBioscience Inc) and re-suspended in permeabilization buffer for 20 minutes. Flourochrome-conjugated monoclonal antibodies added in permeabilization buffer solution included CD3-eflour450 (Affymetrix, eBioscience Inc), CD4-APC (BioLegend), CD8-Viogreen (Miltenyi Biotec Ltd), IFN-gamma-FITC, IL2-PerCP-Cy5.5, TNF-alpha-PeCy7 and IL-5-PE. These antibodies and

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were allowed 25mins incubation with cells before wash, and samples were spun at 1500rpm for 5 minutes and re-suspended in 200μl PBS. FACS was performed using a MACSQuant® (Miltenyi Biotec) and analysed using FlowJo software (version X0.7 for Mac). Responses were background DMSO subtracted and a threshold of 0.02% was applied to define a positive T cell response (33, 34).

Cytokine quantification by Cytometric Bead Array

Cytokine quantification was performed using a BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 cytokine kit using supernatants from the ex-vivo IFNγ ELISpot. 35μL of the pooled peptide triplicate supernatant from the DMSO, Fa, Fb, M and N wells were mixed with 5μL aliquot of each cytokine capture bead (human IL2, IL4, IL6, IL10, TNF, IFNγ and IL17A) and 35μL detection reagent (phycoerythrin(PE)-conjugated antibody) for 3 hours at room temperature and protected from light. 800μL of wash buffer was then added and each sample centrifuged at 200g for 5 minutes. The supernatant was discarded and the bead pellet was re-suspended in 200μL wash buffer. Cytokine detection was performed using an LSRII FACS machine (BD), BD FACSDiva software (version 6.0 for Windows) and FlowJo software (version X0.7 for Mac).

Detection of respiratory viral infection by PCR from nasal swabs

Nasal samples were collected using a mid-turbinate swab and Copan Universal Transport Medium kit (UTM-RT mini, Copan Diagnostics Inc) according to the manufacturers instructions. Viral diagnostics were performed by PCR for respiratory syncytial virus, influenza A, parainfluenza 1/2/3, rhinovirus, coronaviruses, adenovirus, metapenumovirus, enterovirus, parechovirus, bocavirus and mycoplasma pneumoniae.

Statistics

The purpose of the study was to characterise the safety and immunogenicity of different prime/boost combinations of vaccine and therefore analyses were descriptive in nature. There was no pre-specified hypothesis on which to power the study and pre-planned analyses did not included hypothesis testing. Statistical analyses of the data have thus been kept to a minimum and results instead presented as descriptive statistics using graphical presentations. Analyses were based on the intention-to-treat population that included all participants with any data. Comparative statistics and the generation of p-values are pot-hoc analyses.

Graphs and analyses were generated using GraphPad Prism for Mac version 6.0 for Mac (GraphPad Software), STATA version 13.1 (StataCorp LP), SPSS version 21 for Mac (IBM Corporation) and SAS version 9.3 (SAS Institute).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to acknowledge the support and active input of the Data Safety Monitoring Committee, which was chaired by Prof Stephen Gordon together with Prof Saul Faust, Dr Stephane Paulus and Dr Christina Yap (Statistician). We thank Virgina Ammendola for setting up the shedding assay. Merryn Voysey provided statistical input to the protocol, data analysis and wrote the statistical analysis plan. We also wish to acknowledge the team of research staff at the Oxford Vaccine Group and thank the many volunteers who were willing to contribute to this research.

Funding

The study was funded and sponsored by ReiThera S.r.l. (formerly Okairos s.r.l), which was acquired by GlaxoSmithKline Biologicals SA during the trial, the NIHR Oxford Biomedical Research and salary support for Charles Sande and Paul Klenerman from the Wellcome Trust.

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Author contributions

CAG was the lead physician; KH was the lead research nurse; KT was the trial statistician; CAG, ES, RC, PK, AN, AJP, CT, AF, SCo, SCa, AV designed the study/protocols; CJS, AJT, CMdL, MDS, LS, SDM optimized and performed the assays; CAG, KT, ES, CJS, SCa, AV and PK performed data analysis; CAG, BA and AJP provided clinical safety oversight throughout the trial; CAG, ES, AV, SCa, AN, PK, AJP wrote the manuscript; AJP was the chief investigator. All authors had input into the manuscript and have approved the manuscript for publication.

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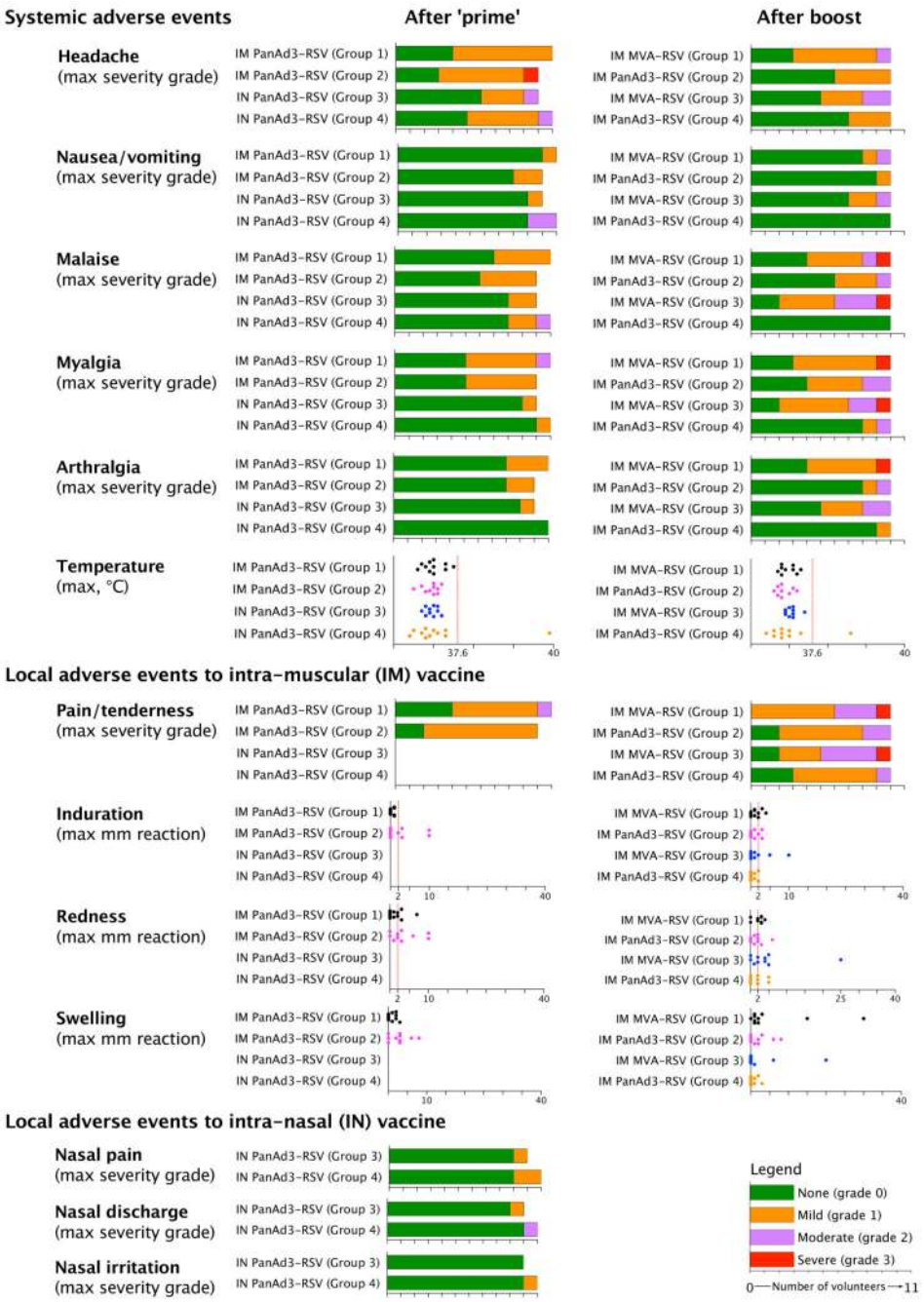

Figure 1. Frequency of the maximum severity solicited adverse event, oral temperature and size of local injection site reactions within one week of vaccination

The number of volunteers is represented across the x-axis without distinction between low-dose and target-dose recipients; n=10 or 11 for events after prime and n=10 for events after boost due to withdrawals. Volunteers reported subjective symptoms as none, mild (does not interfere with routine activities), moderate (interferes with routine activities) and severe (unable to perform routine activities). Redness, swelling and induration at the site of injection used the maximal recorded diameter of any reaction for objective severity grading. Redness and induration were graded as none (0-2 mm), mild (3-50 mm), moderate (51-100

mm) and severe (≥101mm). Swelling graded as none (no visible reaction), mild (1-20 mm), moderate (21-50 mm) and severe (≥51mm). Fever was graded as none (≤37.6°C), mild (37.6.0-38.0°C), moderate (38.1-39.0°C) and severe (≥39.1°C). Overall 5587/5593 (99.9%) of expected data points for solicited adverse events within one week after vaccination were collected for analysis. The only missing data was for temperature recordings. Sore throat reactions were not a solicited symptom although occurred as an unsolicited event in 5/21 IN primed volunteers.

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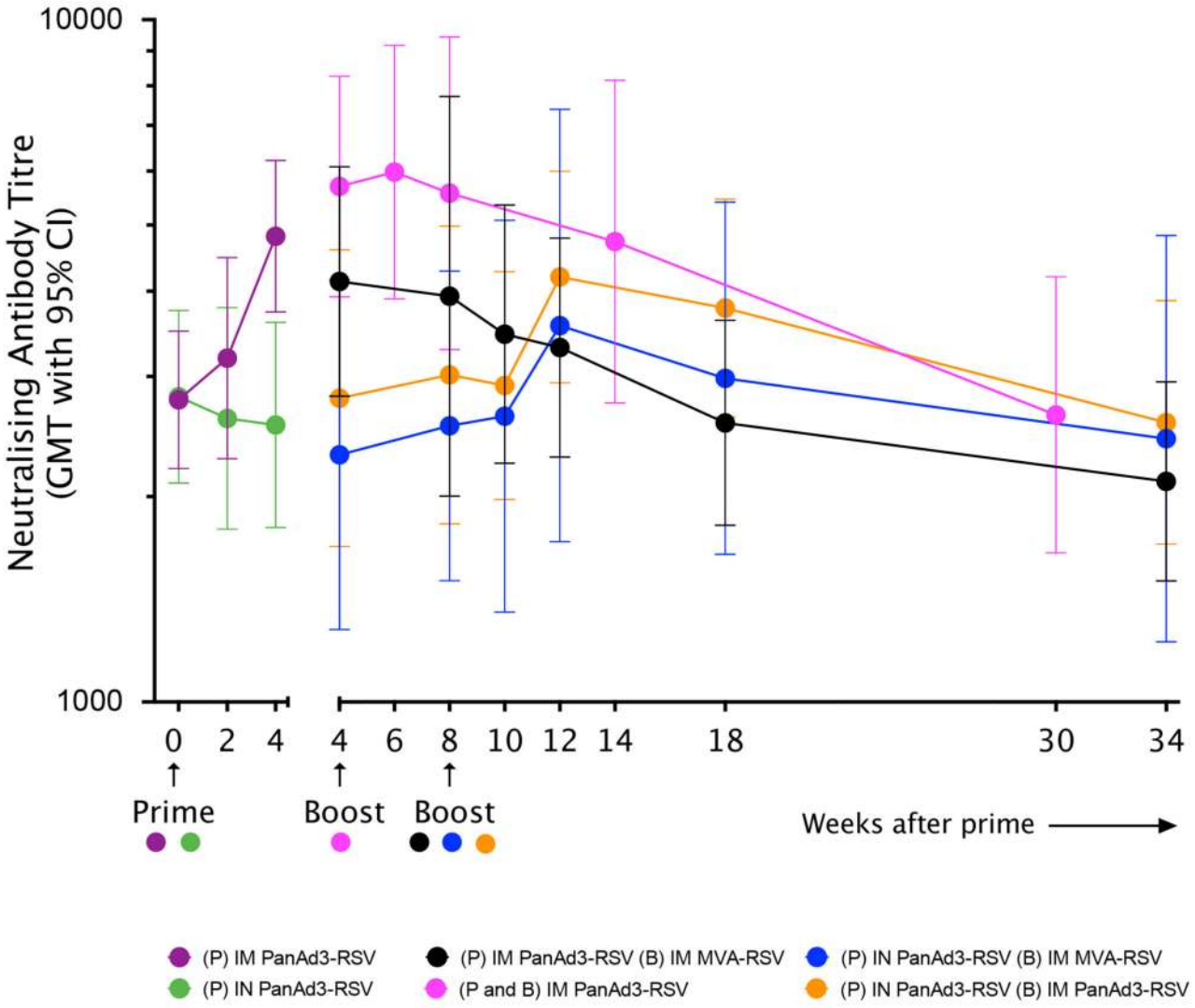

Figure 2. The RSV neutralising antibody in response to vaccination

Data for all volunteers at both doses of vaccine, summarised as the geometric mean titre with 95% confidence intervals for each study group (•Group 1, •Group 2, •Group 3, •Group 4). Responses after prime (P) are grouped by the route of PanAd3-RSV administration (•IM or •IN). At week 4 the IM PanAd3-RSV boost (B) was administered to group 2•. At week 8, IM MVA-RSV and IM PanAd3-RSV boost (B) vaccines were given to the remaining volunteers. The results of individual volunteers are presented in the supplementary material (sFigure 3). The antibody titre 4 weeks following IM PanAd3-RSV prime was significantly elevated from baseline ($p < 0.001$, paired t-test) but not following the IN route ($p = 0.816$, paired t-test). For volunteers who received IM prime, the titres 4 weeks after boost were not statistically significant from pre-boost titres (group 1 between week 8 and week 12, $p = 0.152$; group 2 between week 4 and 8, $p = 0.872$; paired t-tests). Final measures of serum neutralising antibody titres were statistically indistinguishable from

baseline in all groups (group 1 p=0.316, group 2 p=0.416, group 3 p=0.587, group 4 p=0.152; paired t-tests).

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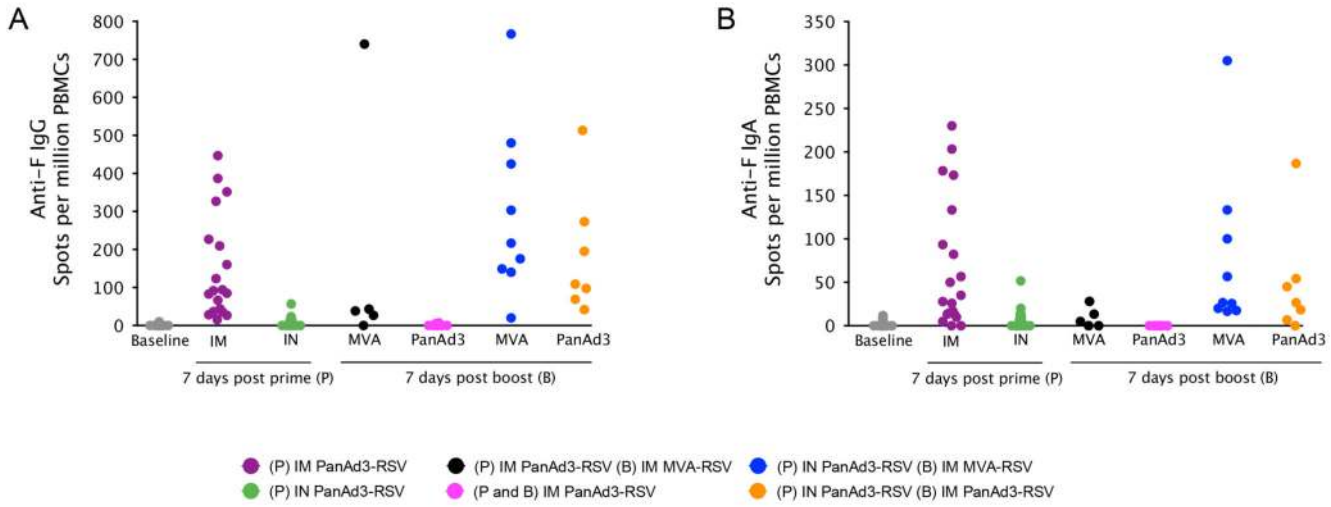

Figure 3. *Ex-vivo* B-cell (antibody secreting cell, ASC) response to vaccination

Fresh PBMCs were collected for analysis at baseline and one-week after prime and one-week after boost vaccinations and subjected to a dual-colour ELISpot. The responses are represented by scatter plot of ASC spots per million PBMCs after HSA background subtraction. (A) The anti-F specific IgG ASC response (B) the anti-F specific IgA ASC response to vaccination. The greatest ASC responses were detected after administration of the first IM vaccine. Overall 13/50 (26%) of plates for anti-F IgG and 10/50 (20%) of plates for anti-F IgA were rejected due to contamination or laboratory error. A total of 90/214 (73%) and 95/124 (77%) of data points were available for the analysis of anti-F IgG and anti-F IgA ASC responses respectively. Study groups; •Group 1, •Group 2, •Group 3, •Group 4. Combined groups; by route of PanAd3-RSV prime administration •IM and •IN.

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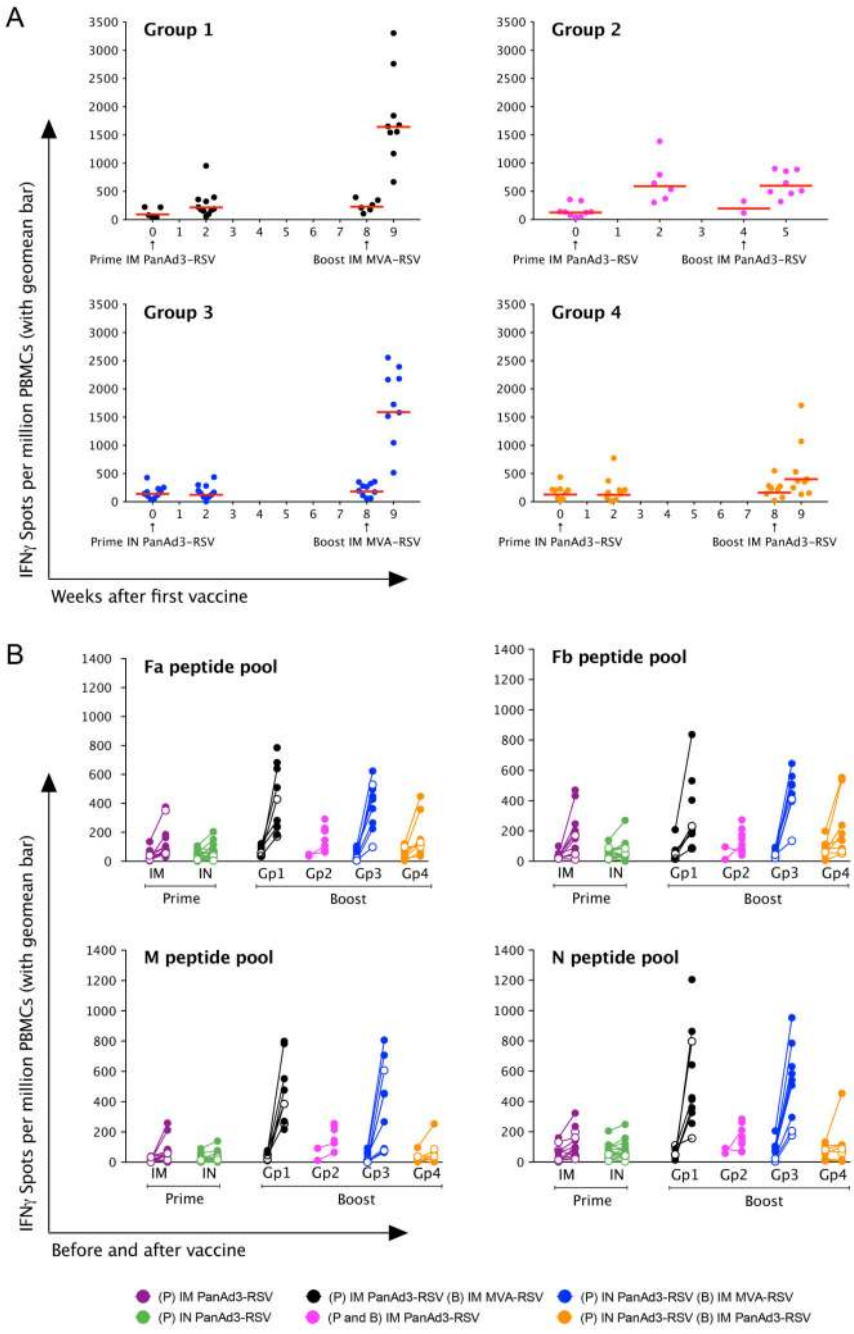

Figure 4. The >*ex-vivo* T-cell IFNγ response to vaccination

Fresh PBMCs were collected for ex-vivo IFNγ ELISpot analysis at baseline, two weeks after prime, before boost and one week after boost. Cells were stimulated overnight by peptide pools Fa, Fb, M and N being representative of the vaccine antigens. (Panel A) The results for each group presented by scatter plot of the summed response for each volunteer [(Fa+Fb +M+N) − (4xDMSO)]. The red line denotes the geometric mean. (Panel B) Individual responses to the separate peptide pools linked between before vaccination and after prime (P) and boost (B). Empty circles denote volunteers who received the lower dose (n=2 per

group). Overall 13/68 (19%) of plates failed due to contamination or laboratory error resulting in the loss of 30/163 (18%) of samples. There was a disproportionate loss of group 2 pre-boost samples. A further 5 peptide responses from 3 volunteers were rejected with a triplicate variance greater than 10. Study groups; •Group 1, •Group 2, •Group 3, •Group 4. Combined groups; by route of PanAd3-RSV prime administration •IM and •IN.

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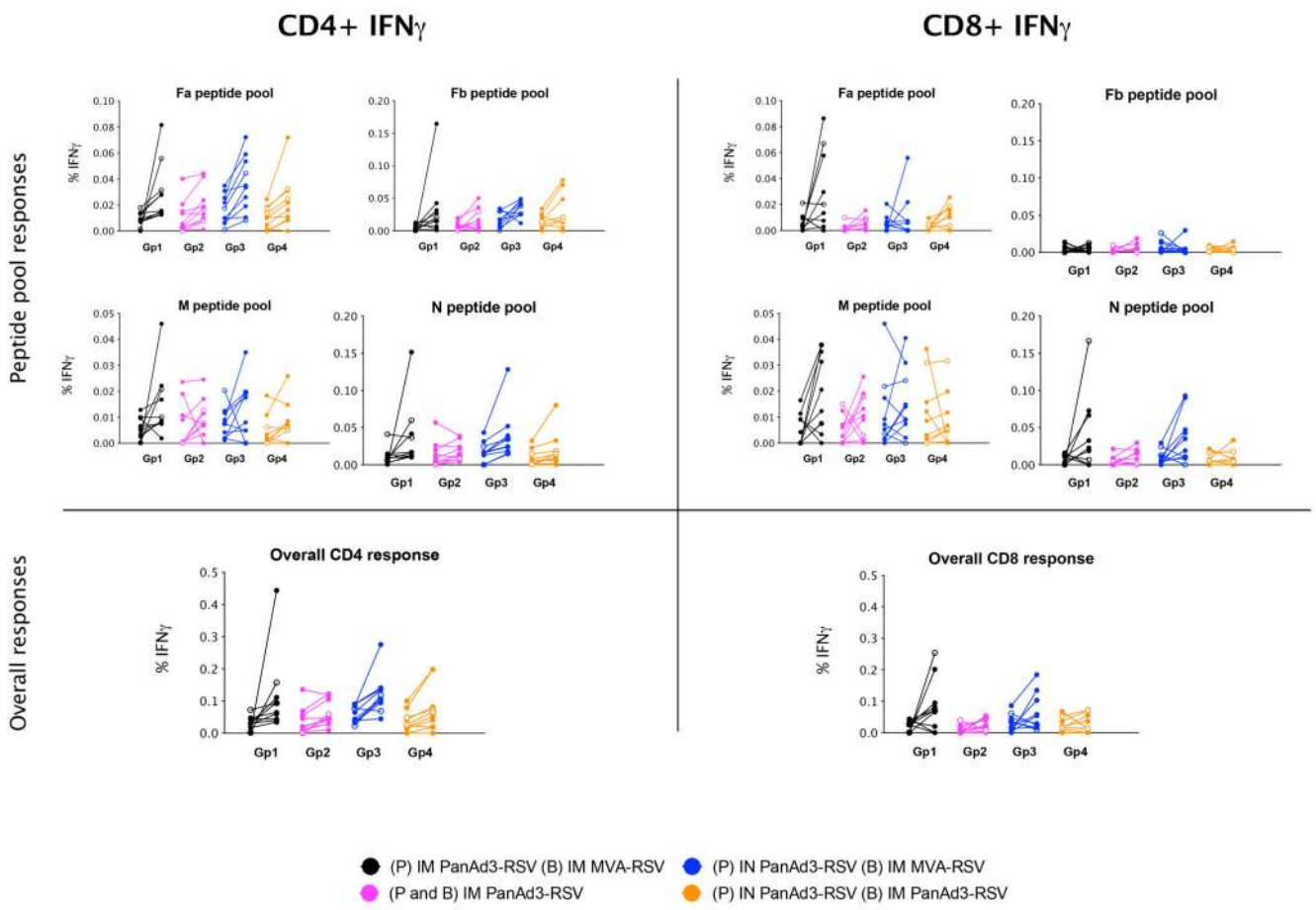

Figure 5. CD4+ and CD8+ IFNγ responses at baseline and one-week post boost by ICS

Empty circles are low-dose vaccine recipients (n=2 per group). Within each group the baseline response (left) is matched with the response one week after boost (right, at week 5 for group 2 and week 9 for the other groups). Overall the responses to Fa and Fb peptide pools were greater, with similar responses to N and fewer responses to M. The overall CD4+ and CD8+ responses were greatest following MVA-RSV boost compared to baseline. Study groups; •Group 1, •Group 2, •Group 3, •Group 4. The frequency of responses is presented in supplementary material (sFigure 8).

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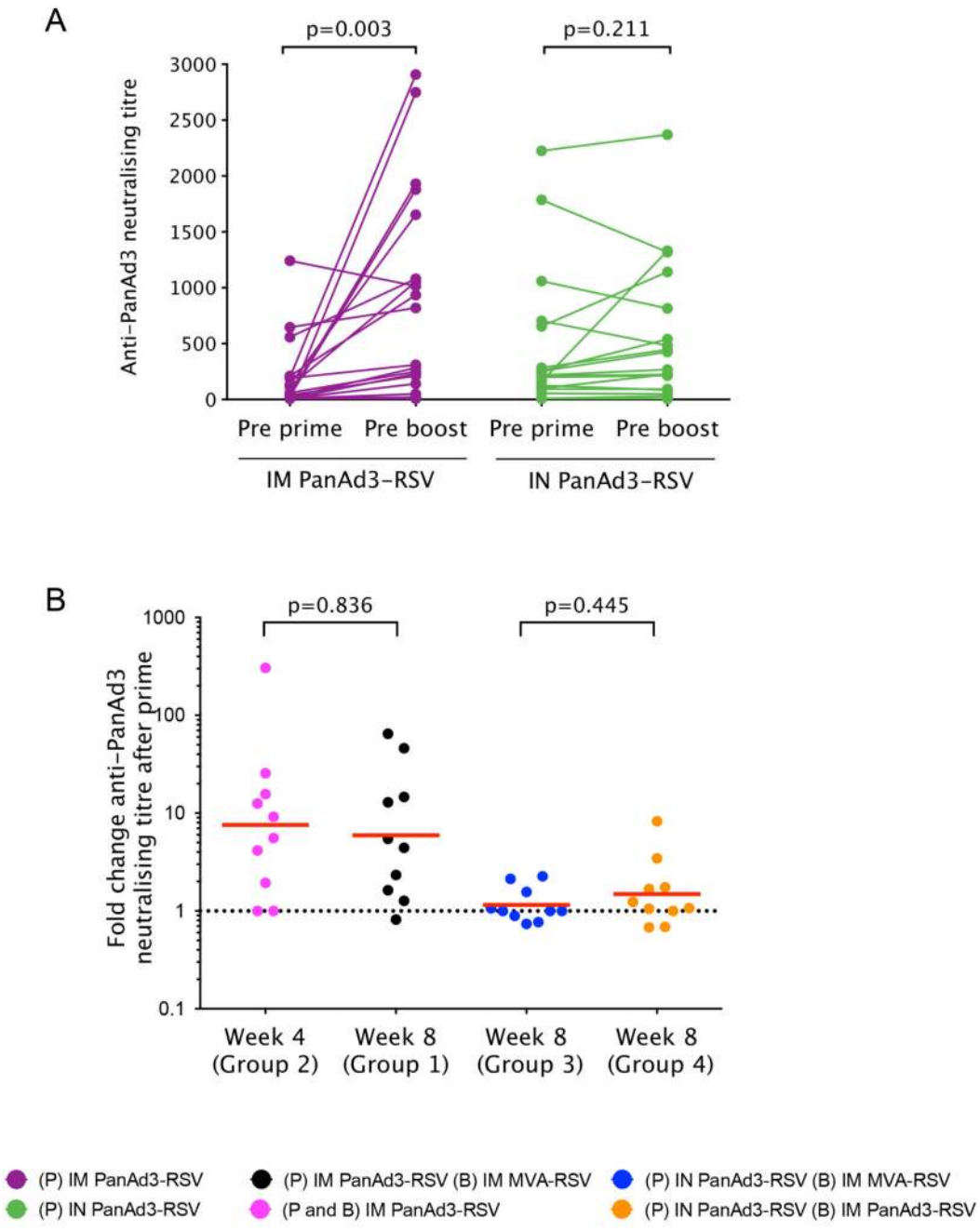

Figure 6. Vector neutralising antibody (anti-PanAd3) titres before prime and before boost vaccination

Anti-PanAd3 titres were measured for the 40 volunteers who completed the trial. No pre-screening of anti-PanAd3 titres was performed before enrolment and study group allocation. (A) Scatter plot of the anti-PanAd3 titre from before prime (baseline) and before boost vaccine. The lower limit of detection for the assay was a titre of 18, and titres ≤18 were arbitrarily assigned a titre of 9. (B) Fold change in anti-PanAd3 neutralising antibody after IM and IN PanAd3-RSV prime. The red bar denotes the geometric mean. Study groups; •

Group 1, Group 2, Group 3, Group 4. Combined groups; by route of PanAd3-RSV prime administration IM and IN.

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Table 1

Definition of each study group by prime/boost vaccine combination, and the baseline physical characteristics of volunteers enrolled into each group

Prime vaccines were delivered by intra-muscular injection (IM) or intra-nasal spray (IN), and all boost vaccines were delivered by IM injection. Recorded details include the age at enrolment in years and the body mass index (BMI). A CONSORT flow diagram from recruitment to completion of the trial, and further information on the study population is available in the supplementary material, sFigure 1.

Study groups:	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**All**
Figures symbol	●	●	●	●	
Vaccine schedules					
Prime vaccine	**PanAd3-RSV**	**PanAd3-RSV**	**PanAd3-RSV**	**PanAd3-RSV**	
Route of prime	IM injection	IM injection	IN spray	IN spray	
Boost vaccine	**MVA-RSV**	**PanAd3-RSV**	**MVA-RSV**	**PanAd3-RSV**	
Route of boost	IM injection	IM injection	IM injection	IM injection	
Prime/boost interval	8 weeks	4 weeks	8 weeks	8 weeks	
Study population at enrolment					
Number of volunteers	11	10	10	11	42
Number male (%)	5 (45)	5 (50)	6 (60)	7 (64)	23 (55)
Median age, yrs (range)	24 (19-42)	27.5 (22-48)	28 (19-41)	27 (19-48)	25.5 (19-48)
Mean BMI, kg/m^2 (range)	21.6 (19.1-31.8)	23.0 (21.6-37.2)	26.1 (18.3-28.7)	24.0 (19.7-32.2)	23.9 (18.3-37.2)