

## TITLE PAGE

# Novel genetically-modified chimpanzee adenovirus and MVA-vectored respiratory syncytial virus vaccine safely boosts humoral and cellular immunity in healthy older adults.

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Christopher A Green<sup>\*1</sup>, Charles J Sande<sup>\*1</sup>, Elisa Scarselli<sup>2</sup>, Stefania Capone<sup>3</sup>, Alessandra Vitelli<sup>3</sup>, Alfredo Nicosia<sup>4,5,6</sup>, Laura Silva-Reyes<sup>1</sup>, Amber J Thompson<sup>1</sup>, Catherine M de Lara<sup>7</sup>, Kathryn S Taylor<sup>8</sup>, Kathryn Haworth<sup>1</sup>, Claire L Hutchings<sup>7</sup>, Tamsin Cargill<sup>7</sup>, Brian Angus<sup>1</sup>, Paul Klenerman<sup>7</sup>, Andrew J Pollard<sup>1</sup>.

<sup>1</sup> Oxford Vaccine Group, Department of Paediatrics and the NIHR Oxford Biomedical Research Centre, University of Oxford, Oxford, United Kingdom; <sup>2</sup> Nouscom Srl, via di Castel Romano 100, 00128 Roma, Italy; <sup>3</sup> ReiThera Srl, Via di Castel Romano 100, 00128 Roma, Italy; <sup>4</sup> Keires AG, Baumleingasse 18, CH 4051, Basel, Switzerland; <sup>5</sup> CEINGE, Via Gaetano Salvatore 486, 80145, Naples, Italy; <sup>6</sup> Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Via S. Pansini 5, 80131, Naples, Italy; <sup>7</sup> Experimental Medicine Division, Nuffield Department of Medicine, University of Oxford, Peter Medawar building, Oxford OX1 3SY, United Kingdom; <sup>8</sup> Nuffield Department of Primary Care Health Sciences, University of Oxford, Oxford OX2 6GG, United Kingdom.

\* CAG and CJS are shared first authors with equal contribution to this manuscript

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Correspondence: Dr Christopher Green  
Oxford Vaccine Group, Centre for Clinical Vaccinology & Tropical Medicine, Churchill Hospital, Oxford OX3 7LE, United Kingdom.  
email; christopher.green@paediatrics.ox.uk  
tel/fax; 0044 +1865 857 420

## ABSTRACT

**Objectives.** Respiratory syncytial virus (RSV) causes respiratory infection across the world, with infants and the elderly at particular risk of developing severe disease and death. The replication-defective chimpanzee adenovirus (PanAd3-RSV) and modified vaccinia virus Ankara (MVA-RSV) vaccines were shown to be safe and immunogenic in young healthy adults. Here we report an extension to this first-in-man vaccine trial to include healthy older adults aged 60-75 years.

**Methods.** We evaluated the safety and immunogenicity of a single dose of MVA-RSV given by intra-muscular (IM) injection (n=6), two doses of IM PanAd3-RSV given 4-weeks apart (n=6), IM PanAd3-RSV prime and IM MVA-RSV boost 8-weeks later (n=6), intra-nasal (IN) spray of PanAd3-RSV prime and IM MVA-RSV boost 8-weeks later (n=6), or no vaccine (n=6). Safety measures included all adverse events within one week of vaccination and blood monitoring. Immunogenicity measures included serum antibody responses (RSV- and PanAd3-neutralising antibody titres measured by plaque-reduction neutralisation and SEAP assays respectively), peripheral B-cell immune responses (frequencies of F-specific IgG and IgA antibody secreting cells and memory B-cells by *ex vivo* and cultured dual-colour ELISpot assays respectively), and peripheral RSV-specific T-cell immune responses (frequencies of IFN $\gamma$ -producing T-cells by *ex vivo* ELISpot and CD4+/CD8+/Tfh-like cell frequencies by ICS/FACS assay).

**Results.** The vaccines were safe and well tolerated. Compared with each individual baseline immunity the mean fold-changes in serum RSV-neutralising antibody, appearance and magnitude of F-specific IgG and IgA ASCs and expansion of CD4+/CD8+ IFN $\gamma$ -producing T-cells in peripheral circulation were comparable to the results seen from younger healthy adults who received the same vaccine combination and dose. There were little/no IgA memory B-cell responses in younger and older adults. Expansion of IFN $\gamma$ -producing T-cells was most marked in older adults following IM prime, with balanced CD4+ and CD8+ T cell responses. The RSV-specific immune responses to vaccination did not appear to be attenuated in the presence of PanAd3 (vector) neutralising antibody.

**Conclusions.** PanAd3-RSV and MVA-RSV was safe and immunogenic in older adults and the parallel induction of RSV-specific humoral and cellular immunity merits further assessment in providing protection from severe disease.

## INTRODUCTION

Human respiratory syncytial virus (RSV) is a globally distributed pathogen that causes respiratory infections throughout life. Infants, adults with severe immune-compromise and the elderly are especially at risk of severe lower-respiratory tract disease and death. Infection rates for the elderly living in the community and care homes range from 5 to 10% each year, which is broadly comparable to the infection rate observed in younger adults, and greatest in the oldest members of the elderly population(1-4). The elderly differ from younger adults in having a greater risk of disease progressing to the lower-respiratory tract causing respiratory failure (8-13%) and death (2-5%)(4). RSV infection is responsible for a significant proportion of elderly hospitalisations for pneumonia (10.6%), chronic obstructive pulmonary disease (11.4%), congestive cardiac failure (5.4%) and asthma (7.2%), with all-cause 30-day and 60-day mortality rates of 9% and 12% respectively(1, 5). Advanced age, senescence of the immune system and the accumulation of co-morbid conditions causes a hospital burden and mortality from RSV in the elderly comparable to seasonal influenza(5-11). (12). There is no effective treatment or licenced vaccine for RSV, and the magnitude of the disease burden has made the development of a safe and effective vaccine a major global health priority for many decades.

The novel genetic viral-vectored RSV vaccines, designated PanAd3-RSV and MVA-RSV, represent a new and promising approach to this problem. Each uses RSV proteins F (F0ΔTM), N and M2-1 as antigen delivered by replication-defective adenovirus (PanAd3) and modified vaccinia virus Ankara (MVA) vectors. Preclinical models that used homologous and heterologous combinations of these vaccines, including the intra-nasal route, found a single dose of intra-nasal (IN) or intra-muscular (IM) vaccine fully protected the lower respiratory tract from viral replication after challenge, with the IN route also capable of inducing sterilising immunity in the upper respiratory tract. Importantly the immunogenicity and protective efficacy of PanAd3-RSV and MVA-RSV was not associated with evidence of lung immunopathology(13-15). In 2015 we progressed to the first trials in humans and demonstrated that delivery of RSV antigen using these replication-defective viral-vectors was safe and capable of robustly boosting both humoral and cellular immune responses in healthy adults aged 18-50 years despite pre-existing natural immunity to RSV(16, 17). Here we report the safety and immunogenicity of these vaccines in healthy older adults, aged 60-75 years, with specific reference to the results from younger adult cohorts who received the same combinations of vaccine.

## MATERIALS & METHODS

### Study design.

RSV001 was an open-label, dose escalation, phase I clinical trial in 42 healthy adult volunteers aged 18-50 years that was later expanded to include an additional 30 healthy adult volunteers aged 60-75 years after a planned interim analysis of safety and immunogenicity data in younger adults. No formal sample size calculations were performed and the number of volunteers in each study group was considered standard to assess phase 1 (first-in-man) product safety and tolerability. The four prime/boost combinations tested in older adults were selected from combinations tested in the younger age cohort and from preclinical data. Study volunteers were self-selected individuals responding to open invitation to the trial and provided written informed consent 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 of the immune system, 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 (detailed in sTab. 1). Eligible volunteers were assigned to study groups by sequential allocation and were considered enrolled at the first vaccine visit. The primary and secondary objectives were the respective characterisation of safety and immunogenicity of each prime/boost combinations of vaccine (sTab. 2) and end points of the clinical trial were prospectively selected. The methods and results of the safety analysis are presented separately in the supplementary material (sFig. 1, sFig. 2, sTab. 3 and sTab. 4). Secondary end-point measures of immunogenicity were performed observer blinded by use of a randomly generated laboratory identifier. The collection and processing of samples and analysis of immune responses were pre-specified and performed as described previously, and only briefly described here(16, 17). The methods and analysis of intracellular staining with flow cytometry (ICS/FACS) analysis are described in supplementary material.

### Intervention.

The generation of the viral-vectored vaccines PanAd3-RSV and MVA-RSV and results of pre-clinical evaluation are described in detail elsewhere(13, 14). In brief, each vaccine was a replication-defective genetically modified organism engineered to deliver the fusion (F0 $\Delta$ TM, devoid of the trans-membrane region), nucleocapsid (N) and matrix (M2-1) RSV proteins by the insertion of the same single synthetic codon-optimised DNA fragment. A self-cleavage site derived from

foot and mouth disease virus 2A releases F0ΔTM into the supernatant while the N and M2-1 proteins remain intracellular. Deletion of the E1 and E4 loci of PanAd3 rendered the adenovirus vector replication-defective and MVA naturally cannot replicate in mammalian cells. The target-doses PanAd3-RSV and MVA-RSV used in older adults were  $5 \times 10^{10}$  viral particles (vp) and  $1 \times 10^8$  plaque forming units (pfu) respectively. PanAd3-RSV and MVA-RSV were given by intra-muscular injection of 0.5mls volume to the non-dominant deltoid muscle, and PanAd3-RSV 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).

#### Sample processing.

Blood samples were collected in heparinised tubes for assays that required peripheral blood mononuclear cells (PBMCs). PBMCs were isolated within 6hrs of sample collection. An aliquot of PBMCs was immediately used for fresh ELISpot assays and the remainder cryopreserved in Recovery™ Cell Freezing Medium. Serum samples were obtained by centrifugation of whole blood collected in clotted tubes, and then cryopreserved.

#### PanAd3-RSV vaccine virus shedding detection.

For volunteers primed with PanAd3-RSV by intra-nasal spray, an additional nasal sample was obtained three days later to detect vaccine virus shedding by PCR. Samples were collected in viral transport medium and analysed by WuXi AppTec, Inc, by inoculation onto cell lines to detect both replication competent and incompetent adenoviruses by the presence of cytopathic effects and immunofluorescence detection as confirmatory assay.

#### Serum PanAd3 vector neutralising antibody measurement.

Anti-PanAd3 (vector) neutralising antibody titres at baseline and before boost were assayed using a PanAd3 encoding for the reporter gene secreted alkaline phosphatase (SEAP) in a previously described neutralization assay(18). The neutralization titre was defined as the reciprocal of sera dilution required to inhibit SEAP expression by 50%.

#### Serum RSV-neutralising antibody measurement.

Plaque-forming units of RSV strain A2 were mixed with 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. The neutralising titre was defined as the sera dilution at which 50% of plaques survive and was calculated using the Spearman-Kärber method.

#### The quantification of F-specific IgG and IgA antibody secreting cells (ASCs) in peripheral blood.

Antigen-specific IgG and IgA ASCs were detected and quantified by dual-colour *ex-vivo* enzyme-linked immunosorbent (ELISpot) assay. In summary, plates were coated with F protein antigen and fresh PBMCs were added. Plates were developed using anti-human IgG and IgA secondary antibody. Responses measured as the antigen-specific spots per million PBMCs with human-serum albumin (HAS) background subtracted. A positive response was defined as any detection of spots above HAS background.

#### The quantification of F-specific IgG and IgA memory B-cells in peripheral blood.

Anti-F IgG and IgA memory B-cell responses were measured by dual-colour ELISpot and was a new assay not used on younger adult samples before. Frozen PBMCs were thawed and cultured for 6- days with CpG (BioScience UK), Pokeweed Mitogen (PWM, Sigma) and *Staphylococcus aureus* Cowans Strain (SAC, VWR International) in R10 media at a concentration of  $2 \times 10^6$  cells/mL. The cells were then harvested and a dual-colour ELISpot assay was performed as described for the ASC ELISpot assay above.

#### The quantification of RSV F, N and M<sub>2-1</sub> specific IFN $\gamma$ -producing T-cells in peripheral blood.

Antigen-specific IFN $\gamma$ -producing T-cells were detected and quantified by *ex-vivo* ELISpot assay, as previously described. Peptides pools consisted of mainly of 15-mer sequences with 11 amino acid overlaps and covering the sequence of proteins F, N and M2-1. 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). DMSO (the peptide diluent) and ConA were used as negative and positive controls, respectively. The mean+4StDev of the DMSO response from all samples identified a cut off whereby individual samples with background

DMSO values  $\geq 50$  spot forming cells per million PBMCs were excluded from analysis. Calculation of triplicate well variance was performed as described elsewhere and a threshold of 10 applied for exclusion(19). A response was considered positive when both (a) peptide pool responses were  $>50$  spots per million PMBCs and (b) greater than 3x DMSO background for the individual.

#### Detection of respiratory viral infection by PCR from nasal swabs.

Nasal samples were collected from volunteers who reported an influenza-like illness at any stage of the trial for the detection of respiratory viral infection using a mid-turbinate swab. Viral diagnostics were performed by PCR for RSV, influenza A, parainfluenza 1/2/3, rhinovirus, coronaviruses, adenovirus, human metapneumovirus, enterovirus, parechovirus, bocavirus and *Mycoplasma pneumoniae*.

#### Statistics.

Analyses were based on the intention-to-treat (ITT) population that included all participants with any data and were planned as descriptive outcomes. We did not include hypothesis testing or the use of formal comparative statistics. Graphs and analyses were generated using GraphPad Prism for Mac version 6.0 for Mac (GraphPad Software) and SPSS version 21 for Mac (IBM Corporation).

## RESULTS

Each study group and a description of volunteer's characteristics are provided in Fig. 1. In total 1224 expressions of interest were received, and after initial screening 79 were invited and attended physician screening to identify and later enrol 30 eligible volunteers (see CONSORT, sFig. 3). All vaccine doses were administered between August and October 2014 which allowed for more than one month to elapse from when the last volunteer received a boost vaccine and the start of the 2014/15 RSV season, according to Public Health England (PHE) monitoring data, and minimised the risk of subclinical boosting of RSV-specific immune responses in the post-vaccination period (sFig. 4). A total of 376/379 (99.2%) of scheduled visits were attended and these data were used as the ITT population for analysis, and 373/376 (99.2%) of these visits were attended within the post-vaccination window specified in the trial protocol. One older adult volunteer was sadly diagnosed and died of oesophageal cancer before the final visit (study group 6, 43-weeks after single dose IM MVA-RSV) and was the only volunteer who failed to complete the trial. Overall each vaccine was safe and well tolerated in the older adult study population (see supplementary material, sFig. 1, sFig. 2, sTab. 3 and sTab. 4) with only one serious adverse event (SAE) which was considered unrelated to vaccination (sTab. 5). There was no vaccine virus shedding by PCR following IN PanAd3-RSV (study group 8) and volunteer-reported influenza-like illnesses identified only one PCR-confirmed case of natural RSV infection 12 weeks after IM MVA-RSV boost from a volunteer in group 8.

### Serum RSV-neutralising antibody induction in older adults was comparable to younger adults

The baseline antibody titres measured before prime were representative of the natural immunity generated from repeated exposure (sFig. 5). As an adjustment for baseline immunity we compared the fold change in antibody titres 28-days after each dose of vaccine which was when peak titres were recorded from younger adults. The mean fold-change in serum RSV-neutralising antibody titre in older adults was 2.21 (95% CI 1.1-3.3) 28-days following IM PanAd3-RSV prime and of a similar magnitude to younger adults (mean fold-change 2.06, 95% CI 1.4-2.7) (Fig. 2 and sFig. 6). Prime with IM MVA-RSV, a single dose vaccine strategy that was not tested in younger adults, induced a mean fold-change in serum RSV-neutralising antibody titre of 2.42 (95% CI 0.5-4.3), and was comparable to the IM PanAd3-RSV prime as well as the boost response observed in IN PanAd3-RSV/IM MVA-RSV vaccinated younger adults (study group 3). The IN route of prime failed to elicit any notable change in serum antibody titres (mean fold changes of 1.04 and 1.06 in younger and older adults respectively). As with our observations from younger adults, the greatest serum RSV-neutralising antibody responses were noted following the first dose of any (PanAd3 or MVA) IM vaccine. Subsequent doses of IM vaccine (IM



boost) failed to elicit any further incremental rise in serum antibody titres. For the 6 older adults primed with IN PanAd3-RSV (group 8) there was a demonstrable rise in serum RSV-neutralising antibody after IM MVA-RSV boost (mean fold-change in serum RSV-neutralising antibody titre of 1.71, 95% CI 0.8-2.6). For all study groups, in both younger and older adults, antibody titres appeared to inexorably wane towards pre-vaccination baseline titres following the peak response to the first dose of IM vaccine (sFig. 7).

F-specific IgG and IgA antibody secreting cells (ASCs) appear in peripheral circulation after vaccination at similar frequencies in both younger and older adults.

RSV F-protein specific IgG and IgA immunoglobulin producing cells were not detectable or appeared at low frequency in baseline blood samples (Fig. 3). When measured 7-days after vaccination we found all 12/12 and 10/12 older adults primed with IM PanAd3-RSV had developed IgG and IgA ASC responses. The responses were of comparable magnitude to those from younger adults who had received the same vaccine the previous year (median spots per million PBMCs of 92 and 31 for IgG and IgA respectively) (sFig. 8). For IM MVA-RSV prime we detected responses in 3/4 volunteers for both IgG and IgA (median spots per million PBMCs of 170 and 26 for IgG and IgA respectively). Approximately 50% of volunteers receiving IN PanAd3-RSV prime recorded a measurable IgG/IgA ASC response, indicating vaccine take in these volunteers, however the magnitude of these responses was approximately 10-fold less than the responses observed to the same vaccine given by IM injection at prime. As with the serum RSV-neutralising antibody measures, the ASC response to vaccination was consistently observed 7-days following the first dose of IM vaccine only (given as either prime or boost). Analysis of the F-specific IgG/IgA ASC response to boost recorded notable responses to IM MVA-RSV following IN PanAd3-RSV prime in all 6/6 older adult volunteers and were of comparable magnitude to the responses seen in younger adults (75 and 27 IgG and IgA spots per million PBMCs respectively). ICS/FACS analysis also recorded a transient expansion of plasmablasts 7-days after IM PanAd3-RSV prime (sFig. 20).

IgG, but not IgA, antibody producing F-specific memory B-cells were expanded in peripheral circulation by IM vaccination.

This assay tested younger and older cohort PBMCs simultaneously and allowed for direct comparison. The baseline results indicated a 2-log<sub>10</sub> range in F-specific IgG memory B-cell frequencies and responses were detectable from all volunteers, with a proportion of older adults who recorded >200 spots per million PBMCs which was the maximum response from younger adults. In contrast, the baseline IgA memory B-cell frequency was much lower and

from a large proportion of volunteer samples we could not detect any cells in circulation. The distribution of baseline memory B-cell frequencies was broadly comparable between younger and older adults (sFig. 9). Following IM prime there was a marked expansion of IgG memory B-cells 28-days later of broadly similar magnitude between younger and older study groups who received the same vaccine (Fig. 4, and sFig. 9). For IM PanAd3-RSV we observed mean geometric fold changes of 2.7 and 2.5 from younger and older adults respectively, and 1.9 from the smaller cohort of older adults who received IM MVA-RSV prime. In contrast, the IN route for PanAd3-RSV failed to expand the population of F-specific IgG memory B-cells in circulation. Measured again 28-days after boost this cell population was expanded in all study groups, which now included IN/IM combinations of vaccine, without clear distinction in magnitude between younger and older adults. By 180-days after boost the population of F-specific IgG memory B-cells appeared to have contracted to baseline levels. The observation for F-specific IgA memory B-cells was that of low baseline frequencies and poor expansion following vaccination irrespective of route, vector or volunteer age. ICS/FACS analysis also recorded a modest expansion of IgG (and not IgA) memory B-cells after IM PanAd3-RSV prime (sFig. 20).

IM prime induced expansion of RSV-specific IFN $\gamma$ -producing T-cells in peripheral blood in older adults than the younger cohort, with further re-expansion following MVA boost.

The population of RSV-specific IFN $\gamma$ -producing T-cells in peripheral blood was measured by *ex vivo* ELISpot using fresh PBMCs collected 14-days after prime and 7-days after boost. Baseline differences were observed from younger and older adults, with older adults recording fewer spots per million PBMCs compared with the younger cohorts measured one year earlier (sFig. 11). To allow for differences in baseline immunity we used the fold-change from baseline as an index of vaccine immunogenicity (Fig. 5 and sFig. 12). We found that IM prime in older adults reproduced and even exceeded the expansion of RSV-specific IFN $\gamma$ -producing T-cells in peripheral blood seen from the younger cohort. For IM PanAd3-RSV prime the measured geometric mean fold-changes were 3.5 and 6.1 for younger and older volunteers respectively. IM MVA-RSV prime given to 6 older adults recorded a geometric mean fold-change of 21.6. For IN prime there was no appreciable expansion in RSV-specific IFN $\gamma$ -producing T-cells in peripheral blood. The effect of the second dose of vaccine, measured 7-days after boost, was that of re-expansion of the circulating RSV-specific IFN $\gamma$ -producing T-cell population when the vector was MVA regardless of the route of prime. The MVA-RSV boost was responsible for the greatest rises in IFN $\gamma$ -producing T-cells, although the magnitude of this MVA vector-associated re-expansion was lower in the older adult study groups (geometric mean fold-changes of 6.6 and 3.0 for younger and older adults respectively

who received IM prime and 8.8 and 6.1 for younger and older adults respectively who received IN prime). PanAd3 IM homologous boost did not induce a further RSV-specific T-cell response expansion. The relative contribution of responses from each pool of peptides (Fa, Fb, M and N) resulting from natural exposure at baseline were preserved following vaccination (sFig. 13). ICS/FACS analysis, looking at PBMCs from baseline and 14-days after IM PanAd3-RSV prime, showed CD4<sup>+</sup> and CD8<sup>+</sup> responses for IFN $\gamma$ , IL2 and IL4 producing T-cells were broadly similar between younger and older adults (sFig. 17). T-follicular like helper cell frequencies and markers of activation (PD1 and ICOS) did not appear to be substantially altered by vaccination in older adults (sFig. 18 and sFig. 19).

Serum PanAd3 (vector)-specific antibody titres did not appear to abrogate the immune response vaccination using this vector.

As with our earlier observations in younger adults the IM route of prime resulted in similar significant increases in vector-neutralising antibody titres in older adults (geometric mean fold-changes of 6.7 and 11.9 in younger and older adults respectively) (Fig. 6 and sFig. 21)(16). The IN route did not impact on serum vector-specific immunity (geometric mean fold-changes of 0.8 and 0.1 in younger and older adults respectively). In an effort to see whether pre-prime anti-PanAd3 (vector) antibody had any impact on vaccine immunogenicity we correlated the baseline antibody titre against the fold-change in RSV-neutralising antibody, the F-specific IgG antibody secreting cell response and the fold-change expansion of IFN $\gamma$ -producing T-cells after IM PanAd3-prime. For both younger and older volunteers, there did not appear to be any relationship with these measures of vaccine immunogenicity.

## DISCUSSION

A safe and effective vaccine is needed to reduce the enormous burden of emergency hospital admissions and death from RSV infection each year in the elderly. PanAd3-RSV and MVA-RSV, the first novel recombinant viral-vectored vaccine candidates for RSV, were first tested in young adults and, in this study, were also found to be safe and induce humoral and cellular RSV-specific immune responses in a healthy older adult population.

Serum F-specific antibody alone has provides only partial protective efficacy from developing severe disease in infants, as shown by the 45-55% reduction in admissions for RSV-bronchiolitis with monoclonal antibody prophylaxis (palivizumab, MedImmune)(20-23). High RSV-specific neutralising antibody titres from natural exposure persist into later

life but relatively lower titre have, nevertheless, been associated with the development of severe disease in the elderly(24-27). Baseline antibody titres of volunteers from our trial were recorded in different years, limiting any direct comparison of natural humoral immunity, but several observational studies of natural infection have not found significant or consistent quantitative or qualitative deficits with antibody responses, mucosal immune responses or memory B-cell responses and increasing age(27, 28). However, a greater proportion of frail older adults recorded a  $\geq 4$ -fold rise in serum antibody after infection without a concurrent rise in viral neutralisation (measured by micro-neutralisation assay), suggesting an accumulation of non-protective antibody and immune dysregulation with age(28). In addition, natural boosting of humoral immunity seems imperfect when approximately 15% of PCR-confirmed RSV infections in elderly patients with chronic obstructive pulmonary disease, 73% of whom were symptomatic of infection, was not associated with changes in serum or nasal RSV-specific antibody(29). Antibody-mediated protection in the elderly could therefore be improved through vaccination. However, vaccine-induced antibody alone may not be sufficient to protect from severe lower-respiratory tract disease in many adults, and despite the worldwide prevalence of F-specific neutralising antibody the surface expressed F protein target has undergone little temporal evolution suggestive of being under relatively weak selective pressure. For younger adults undergoing human stem cell transplantation the depletion of lymphocytes (not antibody) has been associated with the risk of progression towards the lower-respiratory tract, severe disease and death and serum antibody did not confer protection from infection or appear to modulate disease severity of younger adults undergoing experimental RSV-challenge(30, 31). In addition to needing to generate biologically relevant neutralising antibody, the paralleled restoration of age-related losses of RSV-specific cellular immunity through vaccination may provide the added protection needed for limiting disease severity in the elderly.

We first explored the cellular immune responses that support antibody production and maintenance. A clear signal of vaccine-induced immunogenicity across the spectrum of younger and older adult volunteers came from the F-specific IgG and IgA antibody secreting cells (ASCs) measured 7-days after vaccination. Mean F-specific IgG ASC counts of 149 and 173 spots per million PBMCs 7-days following IM PanAd3-RSV prime in younger and older adults respectively compares with 200 spots per million PBMCs observed 10-16 days following natural infection in younger and older adults(32). IM MVA-RSV prime in older adults mounted comparable F-specific ASC responses in 3/4 samples. Our vaccine data, observations from natural infection, human experimental RSV-challenge and other vaccine trials have consistently demonstrated the transient appearance of F-specific IgG and IgA ASCs in peripheral circulation in the days after stimulation which soon disappear as the cells presumably traffic towards the bone marrow and mucosa(16, 33). The next

logical step was to look at how vaccination affected long-lived cells that underpin pathogen-specific adaptive humoral immunity. F-specific IgG memory B-cells circulated in all volunteers but a significant proportion of volunteers had no detectable F-specific IgA memory B-cells in the baseline samples, and IgA responses that were detected were significantly lower cell frequencies compared with the resting IgG memory pool. This dichotomy has been observed in children and paediatric healthcare workers and under controlled experimental RSV-challenge conditions in healthy adults, when there was also a noticeable absence of an IgA memory B-cells in peripheral blood before and after challenge(33, 34). The ICS/FACS analysis appeared to independently corroborate the ELISpot findings of plasmablast and memory B-cell activity together with an absence of IgA memory B-cell responses. The role for IgA memory B-cells in protection from RSV is unclear, but an intriguing prospect concerns the effect of RSV-specific IgA can have at the respiratory mucosa. Under experimental RSV-challenge of healthy adults the quantity of nasal RSV-specific IgA can be associated with the risk of infection, and multivariate analysis from other observational studies have described low nasal IgA RSV-specific antibody titres as an independent risk factor for RSV infection(25, 33, 35). The biology of mucosal and systemic IgA requires further investigation to fully understand and exploit the host repertoire of RSV immunity.

PanAd3-RSV and MVA-RSV were able to robustly expanded RSV-specific (IFN $\gamma$ -producing) T-cell immunity in older adults, and in natural infection the loss or impairment of host T-cell function has revealed where these cells provide critical functions in the restriction of disease severity and duration(24, 27, 31, 36, 37). The MVA-RSV boost provided an expansion of IFN $\gamma$ -producing T-cells independent of the route of priming, indicating the added immunogenicity from a boost vaccine, and (in the case of group 6) confirmation that natural priming instead of IN prime was sufficient in supporting MVA-vectored responses which has been problematic in some antigen-naïve, single-dose MVA-vectored vaccine trials(38-40). MVA-vectored responses independent of vaccine priming have also been observed in naturally exposed influenza vaccine trials in the elderly and from phase I antigen-naïve Ebola vaccine trials. In these trials , responses to the MVA-vector at prime were observed which were then boosted with an adenovirus vector that resulted in T-cell responses that superseded the conventional adeno/MVA combination(41, 42). Ageing is associated with a contraction of the resting RSV-specific CD4+ and CD8+ memory T-cell populations in peripheral circulation and an expansion of suppressive regulatory T-cells, with relatively little change in central and effector T-cell function(27, 36, 37). Repeated seasonal exposure may be relatively ineffective at maintaining/boosting T-cell immunity and, confounded by immune senescence in the elderly, a particularly desirable target for vaccine protection.

Vaccine-induced antibody and cellular immune responses in blood were largely restricted to the first dose of IM vaccine and the value of the second IM dose was the added expansion of memory B-cells and, with the case of the MVA-vector, further expansion of IFN $\gamma$ -producing T-cells. Data from RSV infection in adults has showed no correlations between the magnitude of the RSV-specific ASC response, RSV neutralising antibody titres and serum anti-F IgG antibody titres(32). The preclinical animal challenge studies that involved IN PanAd3-RSV prime showed that sterilising immunity in both the upper and lower respiratory tracts was not associated with significant immune responses in blood(13, 14). The PanAd3 vector used as an IN influenza vaccine in mice induced greater IgG antibody responses in broncho-alveolar lavage samples and greater CD8+ IFN $\gamma$  T-cell responses in the lungs compared with the same vaccine given by intramuscular injection, which generated greater responses in the spleen(15). The intra-nasal, live-attenuated influenza vaccine (FluMist) has protective efficacy in infants and has indirectly reduced the incidence of influenza in the elderly, although equally fails to elicit a substantial immune response in blood(43, 44). The lungs are laden with resident antigen-specific T-cells that are not found in blood, especially for respiratory viral infections such as influenza and RSV(45). In adults and protection from severe RSV disease, the baseline population of resident CD8+ (CD69+ CD103+) memory T-cells in the lung (measured from serial bronchoscopy washings) appears to be the main determinant for RSV disease severity(30). The IN route, in younger and older adults, could have resulted in desirable immune responses at the mucosa that were not measured in this trial. The mechanisms behind mucosal vaccination that bring about protective immunity in the respiratory tract, seemingly independent of blood responses, remains a major focus for further investigation.

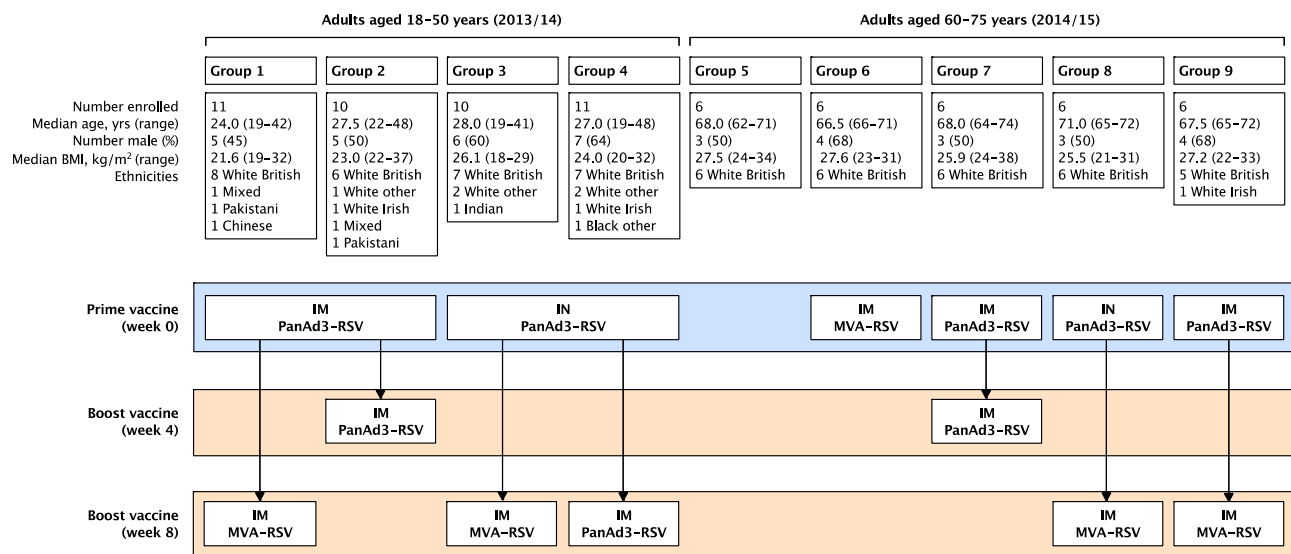
One obstacle to use of viral-vectored vaccines has been the prevalence of serum vector-neutralising antibody with the potential to abrogate vaccine responses. This was described in HIV vaccine trials where anti-adenovirus serotype 5 (Ad5) neutralising antibody titres >200 were associated with an impairment of vaccine immunogenicity to an Ad5-vectored HIV vaccine and increased rates of HIV-acquisition in circumcised men (although the association with HIV-acquisition waned over time)(46-48). In other studies, it was reported that adenovirus vector-neutralising antibody had little effect on T-cell responses(49). PanAd3 and MVA were selected by low human sero-prevalence rates in combination with potent *in vitro* immunogenicity, and although anti-PanAd3 antibody titres were greater than the 3% expected from US and European population estimates we could not detect an impact with vaccine responses in the older adult population(50-52). MVA vector-specific immune responses were not measured and, aside from the specified exclusion criteria, the potential remained for MVA-vector immune responses dating as far back as the 1960's and childhood smallpox vaccination to have persisted in our cohort of older adults. Vaccinia-specific serum antibody and T-cell responses

persist for life and cross-reactivity from earlier smallpox vaccination in adults was associated with a small but significant impairment in IFN $\gamma$  responses to malaria antigen delivered by heterologous prime/boost with recombinant attenuated fowlpox virus FP9 and MVA viral vectors(53). Several trials continue to use and report successes with repeated MVA-vector delivery, suggesting MVA-vector immunity may be a relatively ineffective obstacle to vaccination and older adults who were vaccinated with smallpox vaccine in childhood will become increasingly infrequent in the general population.

The major limitation from our trial expansion to include older adults was the small cohort of 24 vaccinated volunteers who were carefully screened for being healthy. In contrast, 90% of the elderly who are admitted to hospital and who die from severe RSV infection have a least one significant co-morbid condition and infection rates increase with age, and our small study population was not representative of the frail elderly at greatest risk of developing severe disease(5). Although direct and indirect benefit could come from the vaccination of healthy older adults, additional clinical trials will be needed to establish whether these vaccines remain safe and immunogenic in the adults over 75 years with significant co-morbidities. As immune correlates of protection remain elusive, further work is needed to determine whether the immune responses to these vaccines, an especially IN prime, can contribute towards protection from severe disease. Finally, the inclusion of the RSV F-protein in nearly all RSV vaccine candidate reflects the fact that this antigen is well conserved across RSV subtypes with little temporal variation and a target of both serum neutralising antibody and T-cell epitopes. Antibodies directed towards epitopes restricted to the pre-fusion (pre-F) trimeric structure of the RSV F protein are significantly more potent in conferring viral neutralisation, and it remains unknown whether the F-protein antigen used in PanAd3-RSV and MVA-RSV (F0 $\Delta$ TM) presented pre-F as well as the more stable post-F epitopes for the induction of serum neutralising antibody responses(54-57).

In conclusion, we report the first clinical study for replication-defective viral-vectored RSV vaccines for towards the elderly population in need of protection from severe RSV disease. PanAd3-RSV and MVA-RSV were safe and, compared with the data from younger adults who received the same vaccines, lost no potency in boosting desirable RSV-specific immune responses. This included the parallel induction of humoral and cellular immunity, and the inclusion of targeting broad T-cell immune responses may prove especially important for protection in the elderly.

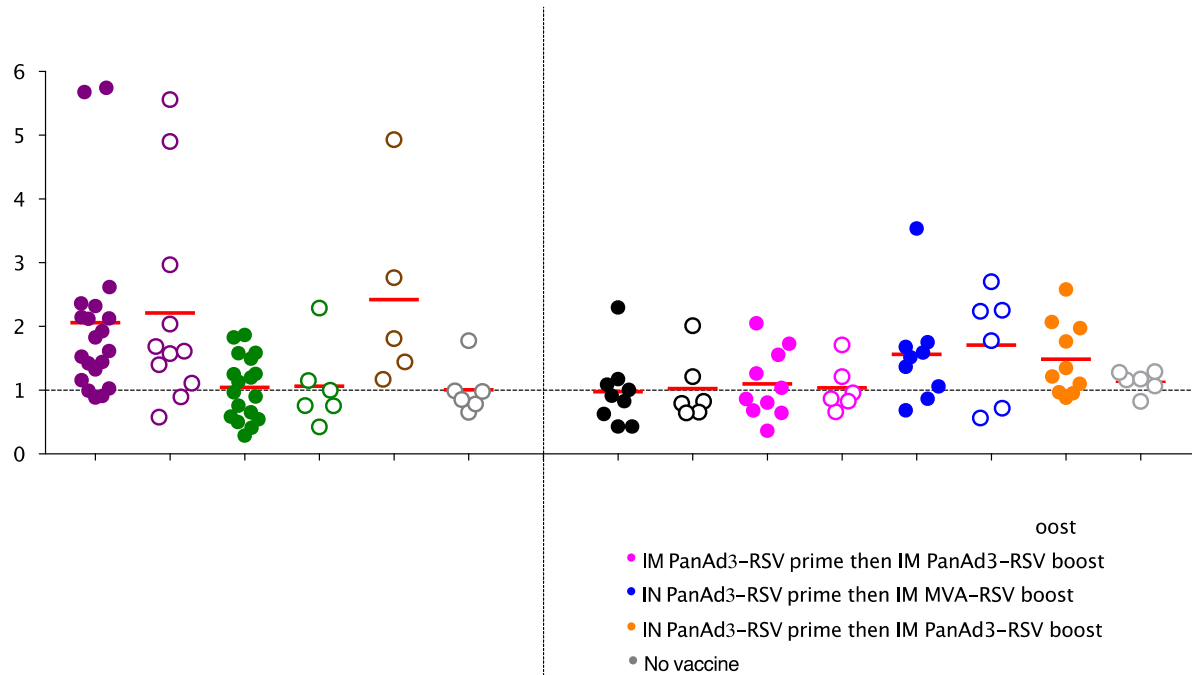
## FIGURES



**Fig. 1. Study groups defined by prime/boost combination and age, with the baseline physical characteristics of volunteers enrolled into each group.**

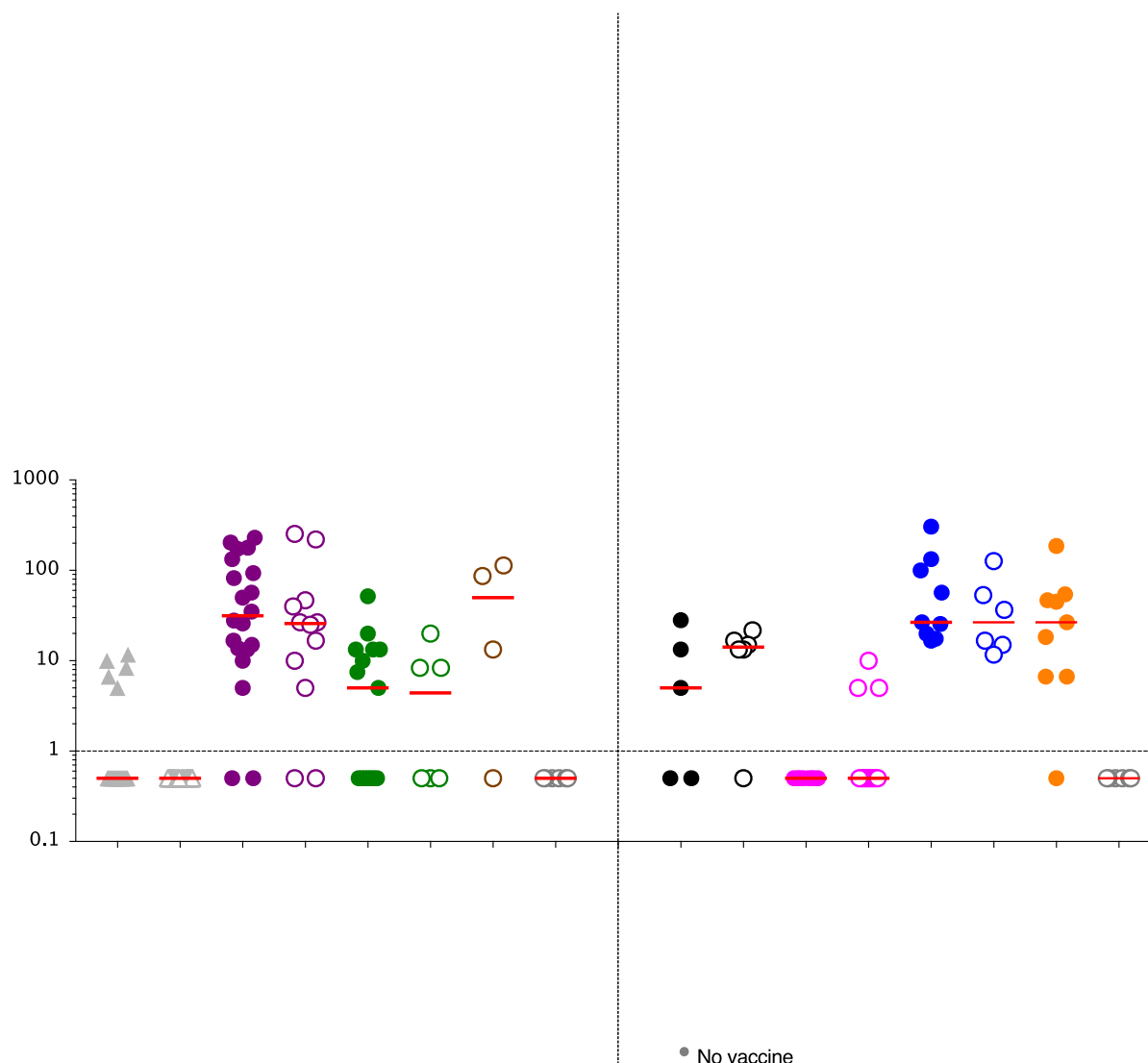
The terms 'prime' and 'boost' are conventional terms and used here to indicate the first and second dose of vaccine. These terms are inherited from previous adenoviral and MVA-vectored vaccine research in immunologically naïve subjects and our population was already primed from repeated natural exposure. Prime vaccines were delivered by intra-muscular injection (IM) or intra-nasal spray (IN), and all boost vaccines were delivered by IM injection. The cohort of younger adults, study groups 1-4, were enrolled, vaccinated and followed up between 2013 and 2014, the results of which are published elsewhere(16). Here we report the analysis of an extension to the trial that included 30 healthy adults aged 60-75 years (study groups 5-9). Prime/boost combinations in older adults were selected from earlier trial data and Group 5 was a non-vaccinated control arm.





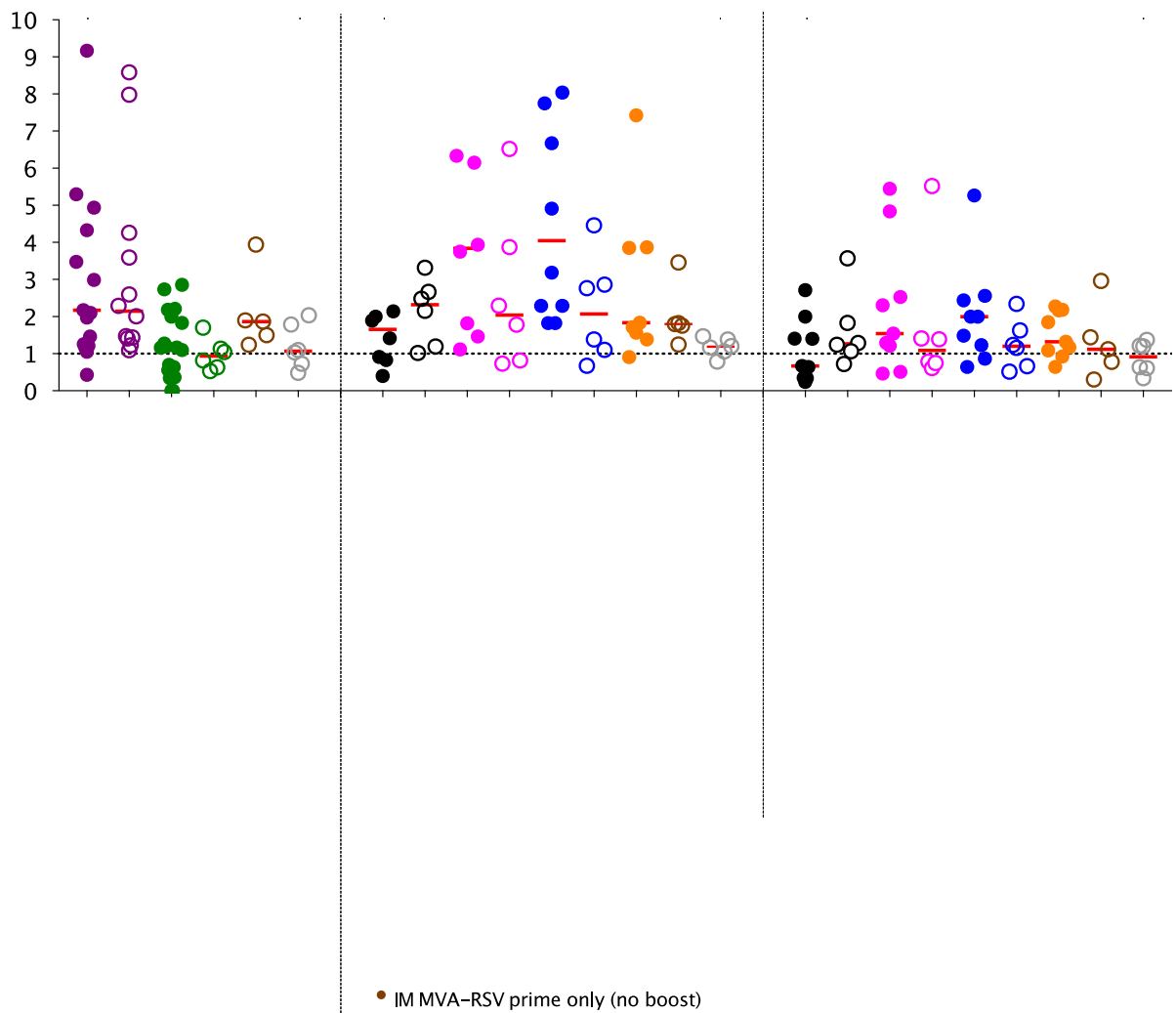
**Fig. 2. Serum RSV-neutralising antibody response to vaccination.**

The serum RSV-neutralising antibody titre was measured by plaque-reduction neutralisation assay, expressed as the dilution required for 50% plaque survival (ND<sub>50</sub>). Coloured circles represent adults aged 18-50 years (study groups 1-4) and empty circles represent adults aged 60-75 years (study groups 5-9). Grey circles are non-vaccinated older adults (group 5). Red bars to denote the mean. The left panel shows the fold change from baseline and the right panel the fold change from pre-boost.



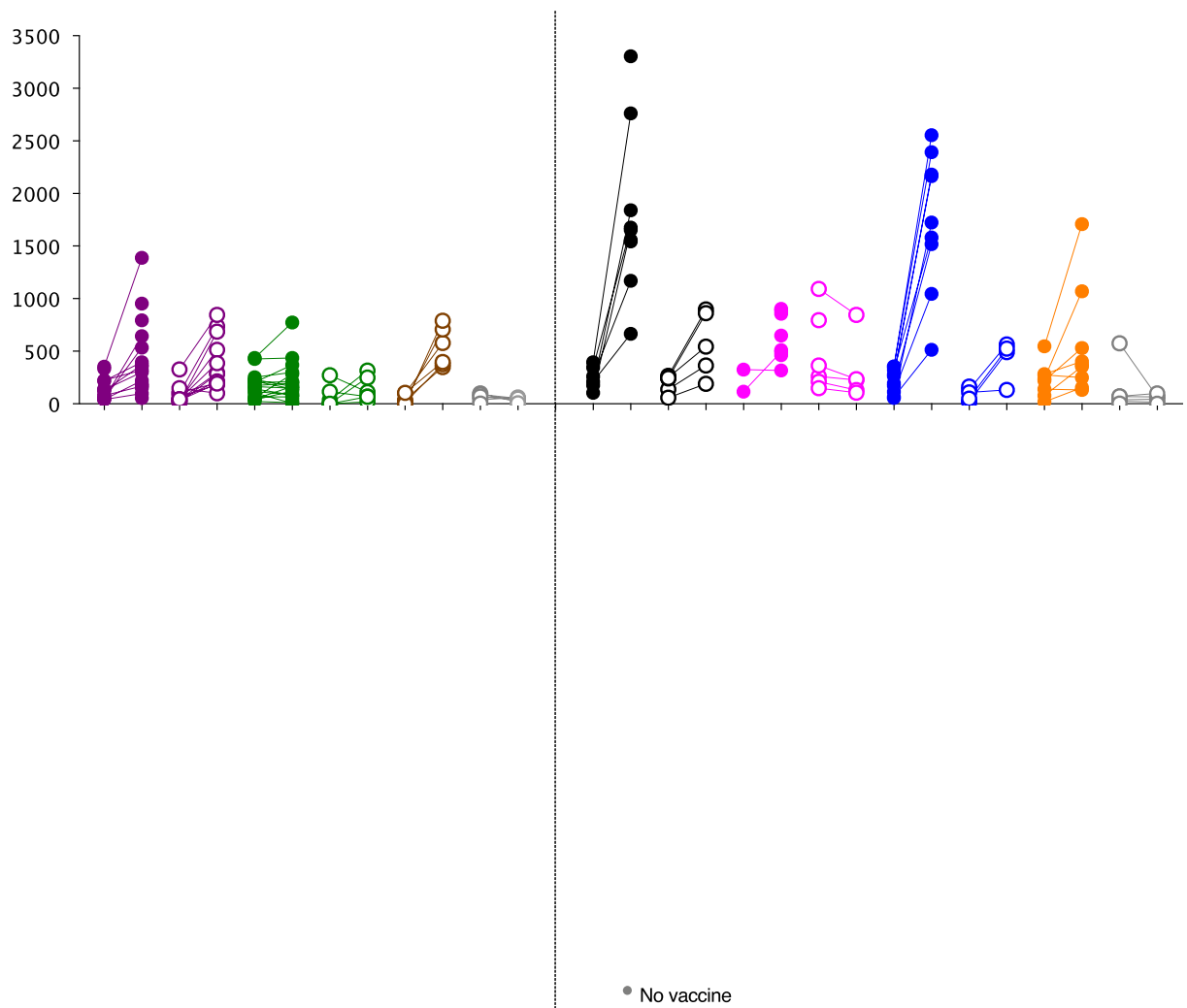
**Fig. 3. The F-specific IgG and IgA antibody secreting cell (ASC) response to vaccination.**

The F-specific IgG (**top figure**) and IgA (**bottom figure**) antibody secreting cell (ASC) response to vaccination was measured by dual-colour ex vivo ELISpot at baseline and 7-days post-prime and boost vaccine. Coloured circles represent adults aged 18-50 years (study groups 1-4) and empty circles represent adults aged 60-75 years (study groups 5-9). Grey triangles denote the pre-prime baseline and grey circles are non-vaccinated older adults (group 5). The red bar denotes the median and the dotted line the lower limit of detection.



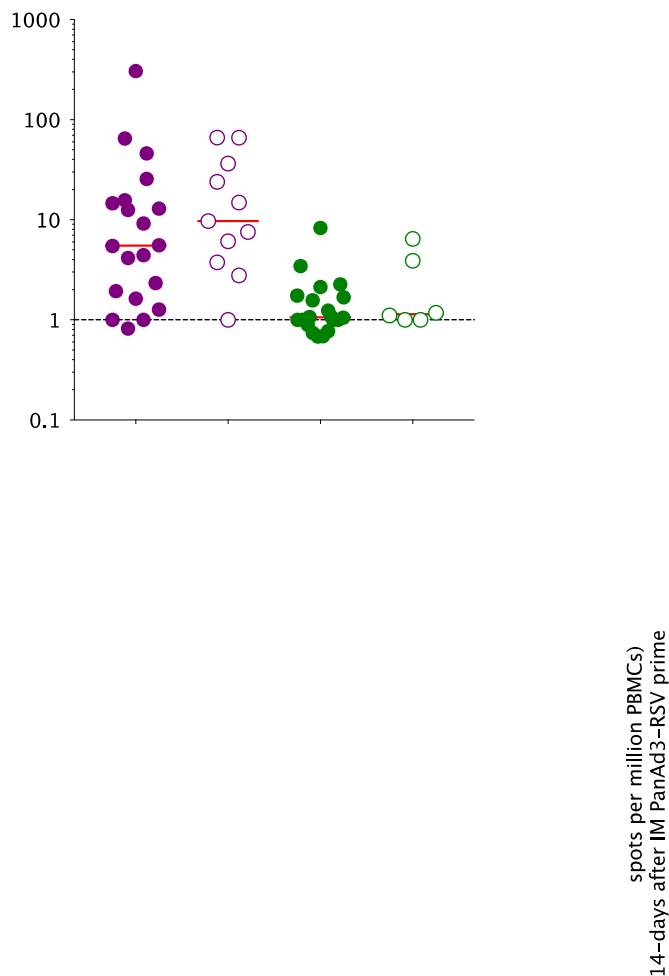
**Fig. 4. The F-specific IgG and IgA memory B-cell response to vaccination.**

The F-specific IgG (**top figure**) and IgA (**bottom figure**) memory B-cell response to vaccination expressed a fold change in spots per million PBMCs from baseline. F-specific IgG and IgA memory B-cell frequencies were measured by dual-colour ex vivo ELISpot at baseline and 28-days post-prime, 28-days after boost and 180-days after boost vaccine. Coloured circles represent adults aged 18-50 years (study groups 1-4) and empty circles represent adults aged 60-75 years (study groups 5-9). The red bar denotes the median. The left panel shows the fold change from baseline and the middle and right panels the fold change from pre-boost at 28-days and 180-days respectively.



**Fig. 5. The total T-cell IFN $\gamma$  response to vaccination.**

The total T-cell IFN $\gamma$  response to vaccination was by ex vivo ELISpot (sum of Fa, Fb, M and N peptide pools with 4x DMSO background subtraction). Coloured circles represent adults aged 18-50 years (study groups 1-4) and empty circles represent adults aged 60-75 years (study groups 5-9). Grey circles are non-vaccinated older adults (group 5). (**Top panel**) IFN $\gamma$  responses before vaccination (pre-prime or pre-boost) paired to the post-vaccination response from each individual 14-days after prime and 7-days after boost. (**Bottom panel**) The same data expressed as a fold-change in IFN $\gamma$  spots per million PBMCs with red bars to denote the median. The left panel shows the fold change from baseline and the right panel the fold change from pre-boost. There was a loss of samples from volunteers in study group 2 (pink dots) at the time of pre-boost due to laboratory error (n=2).



**Fig. 6. Serum anti-PanAd3 (vector) neutralising antibody response to IM/IN PanAd3-RSV prime and the effect on immune responses to prime.**

Serum PanAd3-vector neutralising antibody titres were measured by SEAP assay before prime and before boost. The vertical dotted line denotes the lower limit of detection for the assay. Coloured circles represent adults aged 18-50 years (study groups 1-4) and empty circles represent adults aged 60-75 years (study groups 5-9). **(Top left)** IM, and not IN, route of PanAd3-RSV prime induced a significant rise in PanAd3-specific antibody 4- or 8-weeks after vaccination. The red bars denote the median. The baseline titre of PanAd3 vector neutralising antibody did not appear to have any impact on the vaccine response as illustrated by correlation with the fold-change in serum RSV neutralising antibody 28-days after prime **(top right)**, F-specific antibody secreting cell frequency 7-days after prime **(bottom left)** and with the fold-change expansion of IFN $\gamma$ -producing T-cells 14-days after prime **(bottom right)**.

## SUPPLEMENTARY MATERIAL

### Study design

Inclusion criteria – all must be satisfied
1. Volunteer must be willing and able to consent to take part in the clinical trial
2. At the time of enrolment; aged 18-50 years inclusive for study groups 1-4, or aged 60-75 years inclusive for study group 5-9
3. In good health as determined by medical history, physical examination and in the judgement of the study investigators
4. Willing to use effective contraception (if sexually active); Females: Oral contraceptive pill, contraceptive implant or barrier methods from one month prior and for the duration of the study Males: Barrier contraceptive from V1 until 3 months after the last dose of vaccine
5. Willing to allow his/her General Practitioner and/or Consultant, if appropriate, to be notified of participation in the study
6. Confirmation from the General Practitioner that they are aware of the inclusion and exclusion criteria and are satisfied from their knowledge of the volunteer that they are suitable to enrol
7. Willing to provide their National Insurance/Passport number for the purpose of TOPS registration
Exclusion criteria – none can be satisfied
1. History of any significant organ/system disease that interfere with trial conduct or completion. These include any history of significant disease in the following; <ul style="list-style-type: none"> <li>Cardiovascular disease including congenital heart disease, previous myocardial infarction, valvular heart surgery (or history of rheumatic fever), previous bacterial endocarditis, history of cardiac surgery (including pacemaker insertion), personal or family history of cardiomyopathy or sudden death</li> <li>Respiratory disease such as asthma (excluding childhood asthma) and chronic obstructive pulmonary disease</li> <li>Endocrine disorders such as diabetes mellitus and Addison's disease</li> <li>Significant renal or bladder disease, including a history of renal calculi</li> <li>Biliary tract disease</li> <li>Gastro-intestinal disease such as inflammatory bowel disease, abdominal surgery within the last two years, coeliac disease and liver disease</li> <li>Neurological disease such as seizures and myasthenia gravis</li> <li>Metabolic disease such as glucose-6-phosphate dehydrogenase deficiency</li> <li>Psychiatric illness requiring hospitalisation or depression whose severity is deemed clinically significant</li> <li>Non-benign cancer, including squamous cell carcinoma, basal cell carcinoma of the skin and cervical carcinoma in situ</li> <li>Clinically significant contact dermatitis</li> </ul>
2. Have any known or suspected impairment or alteration of immune function, resulting from, for example: congenital or acquired immune deficiency, human immunodeficiency virus or symptoms/signs of an HIV-associated condition, autoimmune disease, receipt of immunosuppressive therapy such as chemotherapy or radiation therapy in the preceding 12 months or long-term corticosteroid therapy, receipt of immunoglobulin or any blood product transfusion within 3 months of the study start
3. A vaccination history indicative of; planning to receive another vaccine (other than the study vaccine) within 4 weeks of vaccination, a history of anaphylaxis reaction to a vaccine, history of allergic disease or reactions likely to be exacerbated by any component of the vaccine (e.g. Kathon), previously having received a recombinant simian or human adenoviral vaccine, previously having received a recombinant MVA vaccine
4. Detection of any of the following at screening; IgA deficiency, anti-HIV antibody, hepatitis B surface antigen, anti-HCV antibody, any other significant abnormalities at the discretion of the study investigator
5. Known or suspected drug and/or alcohol misuse (alcohol misuse defined as an intake > 42 units/week).
6. Nasal septal pathology including; congenital deformities such as an abnormal septum or polyps, previous cauterisation, rhinoplasty or surgery of any kind, recurrent epistaxis
7. Scheduled procedures requiring general anaesthesia during the study
8. Participation in another study involving an investigational product in the past 12 weeks, or are planning to do so during the study
9. Inability, in the opinion of the investigator, to comply with study requirements
10. Female participants who are pregnant, lactating or planning pregnancy during the course of the study
11. Has donated blood within 4 months before starting the trial, or is intending to donate blood during the trial and up to 12 weeks after completing the trial
12. Any other significant disease or disorder that, in the opinion of the investigator, may put the participant at risk because of participation in the study, influence the result of the study or impair the participant's ability to take part on the trial

sTab. 1. Summary of the eligibility criteria for study volunteers.

		Endpoint measures
Primary objective: vaccine safety and tolerability	Solicited and unsolicited symptoms recorded daily for one week after each vaccine dose	Oral temperature Solicited symptoms; headache, nausea and/or vomiting, malaise, myalgia and arthralgia Local injection site adverse events; pain and/or tenderness, erythema, induration and swelling Local nasal site adverse events; pain and/or tenderness, irritation and discharge Any other event not listed above (unsolicited symptoms)
	Nursing observations obtained at all visit attendances	Resting heart rate, resting respiratory rate, systolic and diastolic blood pressure and oral temperature
	Blood obtained at scheduled visit attendances for the routine safety monitoring of haematology and biochemistry	Haematology: haemoglobin, total white cell count, platelet count, haematocrit, red cell count, mean cell volume, mean haemoglobin, mean haemoglobin concentration, neutrophil count, lymphocyte count, monocyte count, eosinophil count and basophil count Biochemistry: sodium, potassium, urea, amylase, C-reactive protein, creatinine, bilirubin, alanine transaminase, alkaline phosphatase and albumin
Secondary objective: vaccine immunogenicity	Humoral immune response to vaccination	Serum anti-F IgG and IgA antibody titre by ELISA Serum antibody-mediated RSV neutralisation by PRNA assay Serum anti-PanAd3 neutralising antibody titre by SEAP assay
	Cellular immune response to vaccination	Quantification of circulating anti-F IgG and IgA antibody secreting cells by dual-colour ELISpot assay Quantification of circulating interferon-gamma (IFN $\gamma$ ) producing T-cells by ELISpot assay CD4+ and CD8+ subset analysis of the immune response to vaccination by ICS/FACS assay
	Exploratory immunology of the immune response to vaccination	Nasal and salivary anti-F IgG and IgA antibody titre by ELISA Cytokine response to vaccination by CBA assay Gene expression after vaccination analysis by DNA microarray

**sTab. 2. The primary and secondary endpoints of the RSV001 clinical trial.**

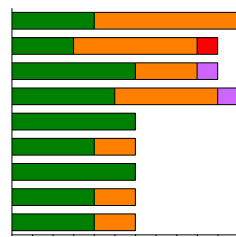
Enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunospot assay (ELISpot). Plaque-reduction neutralisation assay (PRNA). Secreted alkaline phosphatase assay (SEAP). Intra-cellular staining (ICS). Fluorescence-activated cell sorting (FACS). Cytometric bead array (CBA). Deoxyribonucleic acid (DNA).

### Analysis of vaccine safety

An independent data safety monitoring committee (DSMC) acted in accordance to a pre-specified charter provided safety oversight for the duration of the trial. Primary endpoint measures of safety included the frequency and severity of solicited and unsolicited local and systemic adverse events within 7-days of 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). Adverse events were graded using modified Food and Drug Administration (FDA) and Division of AIDS (DAIDs) criteria.

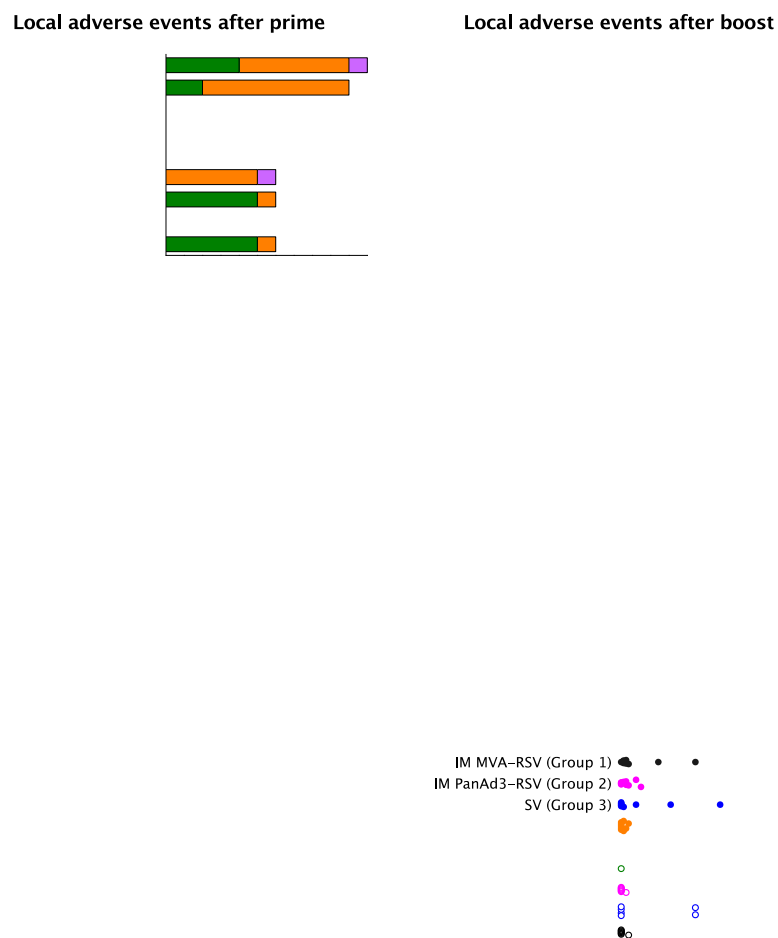
Overall each vaccine schedule was safe and well tolerated in the older adult study population (sFig. 1, sFig. 2, sTab. 3 and sTab. 4). There was only one serious adverse event (SAE) from the older adult population which was considered unrelated to vaccination by the study team and independent DSMC, in addition to the two non-vaccine related SAEs reported in younger adults (sTab. 5). The most common reported adverse event was local pain/tenderness at the site of IM vaccination, with a greater frequency, severity and duration reported to IM MVA-RSV that was comparable with the rates observed from younger adults. These reactions were self-limited and of mild/moderate severity. In contrast to the data from younger adults there were no recorded episodes of fever or grade 3 (severe) solicited adverse events in older adults. Analysis of blood parameters and visit observations did not identify any clinically significant adverse events in relation to vaccination. Intra-nasal PanAd3-RSV prime was well-tolerated and nasal samples taken 3-days after IN PanAd3-RSV prime failed to detect GMO vaccine virus shedding by PCR (group 8). Unsolicited adverse event reporting from younger adults had identified a 25% incidence of mild, self-limiting, sore throat reactions following IN PanAd3-RSV prime. Furthermore, volunteer-reported influenza-like illnesses, which triggered additional visits for nasal sampling and pan-respiratory viral PCR testing, had recorded an incidence of 21% for symptomatic rhinovirus infection in the weeks following vaccination (not specific to any vaccine or route) in younger adults. Neither of these observations was reproduced from our population of older adults following vaccination with the same vaccines given over the following summer.





**sFig. 1. The maximum severity systemic solicited adverse event reported within one week of prime or boost vaccination.**

Each volunteer maintained a daily record of severity grades from none (grade 0, green bars), mild (grade 1, yellow bars), moderate (grade 2, purple bars) and severe (grade 3, red bars). The number of volunteers is across the x-axis. Further safety data results are presented in sTab. 3.



**sFig. 2. The maximum severity local solicited adverse event reported within one week of prime or boost vaccination.**

Each volunteer maintained a daily record of severity grades from none (grade 0, green bars), mild (grade 1, yellow bars), moderate (grade 2, purple bars) and severe (grade 3, red bars). The number of volunteers is across the x-axis. The dotted red line for temperature, induration and redness are the grade 1 threshold. Further safety data results are presented in sTab. 4.

	Adults aged 18-50 years				Adults aged 60-75 years				
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Prime vaccine	IM PanAd3-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IN PanAd3-RSV	No vaccine	IM MVA-RSV	IM PanAd3-RSV	IN PanAd3-RSV	IM PanAd3-RSV
N volunteers	11	10	10	11	6	6	6	6	6
Any systemic adverse event after vaccination, n/N (%):									
<b>Headache</b>	<b>7/11 (64)</b>	<b>7/10 (70)</b>	<b>4/10 (40)</b>	<b>6/11 (55)</b>	0/6 (0)	<b>2/6 (33)</b>	0/6 (0)	<b>2/6 (33)</b>	<b>2/6 (33)</b>
Low dose	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	6/9 (66.7)	6/8 (75.0)	3/8 (37.5)	5/9 (55.6)	0/6 (0)	2/6 (33.3)	0/6 (0)	2/6 (33.3)	2/6 (33.3)
<b>Nausea</b>	<b>1/11 (9.1)</b>	<b>2/10 (20)</b>	<b>1/10 (10)</b>	<b>2/11 (18)</b>	0/6 (0)	0/6 (0)	0/6 (0)	<b>1/6 (17)</b>	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	1/9 (11.1)	2/8 (25.0)	1/8 (12.5)	2/9 (22.2)	0/6 (0)	0/6 (0)	0/6 (0)	1/6 (16.7)	0/6 (0)
<b>Malaise</b>	<b>4/11 (36)</b>	<b>4/10 (40)</b>	<b>2/10 (20)</b>	<b>3/11 (27)</b>	0/6 (0)	<b>3/6 (50)</b>	0/6 (0)	<b>1/6 (17)</b>	<b>1/6 (17)</b>
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	4/9 (44.4)	4/8 (50.0)	2/8 (25.0)	3/9 (33.3)	0/6 (0)	3/6 (50.0)	0/6 (0)	1/6 (16.7)	1/6 (16.7)
<b>Myalgia</b>	<b>6/11 (55)</b>	<b>5/10 (50)</b>	<b>1/10 (10)</b>	1/11 (9.1)	<b>1/6 (17)</b>	<b>2/6 (33)</b>	0/6 (0)	<b>2/6 (33)</b>	<b>1/6 (17)</b>
Low dose	1/2 (50.0)	1/2 (50.0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	5/9 (55.6)	4/8 (50.0)	1/8 (12.5)	1/9 (11.1)	1/6 (16.7)	2/6 (33.3)	0/6 (0)	2/6 (33.3)	1/6 (16.7)
<b>Arthralgia</b>	<b>3/11 (27)</b>	<b>2/10 (20)</b>	<b>1/10 (10)</b>	0/11 (0)	0/6 (0)	<b>2/6 (33)</b>	0/6 (0)	<b>2/6 (33)</b>	0/6 (0)
Low dose	1/2 (50.0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	2/9 (22.2)	2/8 (25.0)	1/8 (12.5)	0/9 (0)	0/6 (0)	2/6 (33.3)	0/6 (0)	2/6 (33.3)	0/6 (0)
<b>Fever</b>	<b>0/11 (0)</b>	0/10 (0)	0/10 (0)	1/11 (9.1)	0/6 (0)	<b>2/6 (33)</b>	0/6 (0)	0/6 (0)	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	0/9 (0)	0/9 (0)	0/9 (0)	1/9 (11.1)	0/6 (0)	2/6 (33.3)	0/6 (0)	0/6 (0)	0/6 (0)
Any local adverse event after intra-muscular (IM) vaccination, n/N (%):									
<b>Pain</b>	<b>6/11 (55)</b>	<b>7/10 (70)</b>	n/a	n/a	n/a	<b>6/6 (100)</b>	0/6 (0)	n/a	<b>1/6 (17)</b>
Low dose	1/2 (50.0)	2/2 (100)	-	-	-	-	-	-	-
High dose	5/9 (55.6)	5/8 (62.5)	-	-	-	6/6 (100.0)	0/6 (0)	-	1/6 (16.7)
<b>Induration</b>	0/11 (0)	<b>4/10 (40)</b>	n/a	n/a	n/a	<b>2/6 (33)</b>	0/6 (0)	n/a	<b>1/6 (17)</b>
Low dose	0/2 (0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	0/9 (0)	3/8 (37.5)	-	-	-	2/3 (33.3)	0/6 (0)	-	1/6 (16.7)
<b>Redness</b>	<b>3/11 (27)</b>	<b>5/10 (50)</b>	n/a	n/a	n/a	<b>1/6 (17)</b>	0/6 (0)	n/a	<b>1/6 (17)</b>
Low dose	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	2/9 (22.2)	4/8 (50.0)	-	-	-	1/6 (16.7)	0/6 (0)	-	1/6 (16.7)
<b>Swelling</b>	<b>7/11 (64)</b>	<b>8/10 (80)</b>	n/a	n/a	n/a	<b>2/6 (33)</b>	0/6 (0)	n/a	<b>1/6 (17)</b>
Low dose	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-	-	-
High dose	5/9 (55.6)	7/8 (87.5)	-	-	-	2/6 (33.3)	0/6 (0)	-	1/6 (16.7)
Any local adverse event after intra-nasal (IN) vaccination, n/N (%):									
<b>Pain</b>	n/a	n/a	0/10 (0.0)	1/11 (9.1)	n/a	n/a	n/a	0/6 (0)	n/a
Low dose	-	-	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	1/9 (11.1)	-	-	-	0/6 (0)	-
<b>Discharge</b>	n/a	n/a	<b>1/10 (10)</b>	1/11 (9.1)	n/a	n/a	n/a	<b>3/6 (50)</b>	n/a
Low dose	-	-	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	1/9 (11.1)	-	-	-	3/6 (50.0)	-
<b>Irritation</b>	n/a	n/a	<b>1/10 (10)</b>	<b>2/11 (18)</b>	n/a	n/a	n/a	<b>2/6 (33)</b>	n/a
Low dose	-	-	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	-	-	0/8 (0)	2/9 (22.2)	-	-	-	2/6 (33.3)	-

**sTab. 3. Counts and proportions of any systemic adverse event reported after prime vaccination by dose and study group within 7-days of vaccination.**

Counts and proportions for the occurrence of any solicited symptom (grade 1-3) in each study group within one week of vaccination. The data are presented as the number of volunteers who reported an adverse event (n) and the number of volunteers in the study group (N). The frequencies of mild (grade 1), moderate (grade 2) and severe (grade 3) adverse events for each study group and each day after vaccination are presented in supplementary results. n/a (not applicable). Adverse events that occurred in  $\geq 10\%$  of volunteers are shown in bold.

	Adults aged 18-50 years				Adults aged 60-75 years				
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Boost vaccine	IM MVA-RSV	IM PanAd3-RSV	IM MVA-RSV	IM PanAd3-RSV	No vaccine	No vaccine	IM PanAd3-RSV	IM MVA-RSV	IM MVA-RSV
N volunteers	10	10	10	10	6	-	6	6	6
Any systemic adverse event after vaccination, n/N (%):									
<b>Headache</b>	<b>7/10 (70)</b>	<b>4/10 (40)</b>	<b>5/10 (50)</b>	<b>3/10 (30)</b>	<b>1/6 (17)</b>	n/a	0/6 (0)	<b>5/6 (83)</b>	<b>3/6 (50)</b>
Low dose	1/2 (50.0)	0/2 (0)	0/2 (0)	1/2 (50.0)	-	-	-	-	-
High dose	6/8 (75.0)	4/8 (50.0)	5/8 (62.5)	2/8 (25.0)	1/6 (16.7)	-	0/6 (0)	5/6 (83.3)	3/6 (50.0)
<b>Nausea</b>	<b>2/10 (20)</b>	<b>1/10 (10)</b>	<b>3/10 (30)</b>	0/10 (0)	0/6 (0)	n/a	0/6 (0)	0/6 (0)	<b>1/6 (17)</b>
Low dose	1/2 (50.0)	1/2 (50.0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	1/8 (12.5)	0/8 (0)	3/8 (37.5)	0/8 (0)	0/6 (0)	-	0/6 (0)	0/6 (0)	1/6 (16.7)
<b>Malaise</b>	<b>6/10 (60)</b>	<b>4/10 (40)</b>	<b>8/10 (80)</b>	0/10 (0)	0/6 (0)	n/a	<b>2/6 (33)</b>	<b>5/6 (83)</b>	<b>2/6 (33)</b>
Low dose	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	6/8 (75.0)	4/8 (50)	7/8 (87.5)	0/8 (0)	0/6 (0)	-	2/6 (33.3)	5/6 (83.3)	2/6 (33.3)
<b>Myalgia</b>	<b>7/10 (70)</b>	<b>6/10 (60)</b>	<b>8/10 (80)</b>	<b>2/10 (20)</b>	<b>1/6 (17)</b>	n/a	0/6 (0)	<b>2/6 (33)</b>	<b>2/6 (33)</b>
Low dose	1/2 (50.0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	6/8 (75.0)	6/8 (75.0)	7/8 (87.5)	2/8 (25.0)	1/6 (16.7)	-	0/6 (0)	2/6 (33.3)	2/6 (33.3)
<b>Arthralgia</b>	<b>6/10 (60)</b>	<b>2/10 (20)</b>	<b>5/10 (50)</b>	<b>1/10 (10)</b>	0/6 (0)	n/a	<b>1/6 (17)</b>	<b>1/6 (17)</b>	<b>2/6 (33)</b>
Low dose	1/2 (50.0)	0/2 (0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	5/8 (62.5)	2/8 (25.0)	4/8 (50.0)	0/8 (0)	0/6 (0)	-	1/6 (16.7)	1/6 (16.7)	2/6 (33.3)
<b>Fever</b>	0/10 (0)	0/10 (0)	0/10 (0)	<b>1/10 (10)</b>	0/6 (0)	n/a	0/6 (0)	0/6 (0)	0/6 (0)
Low dose	0/2 (0)	0/2 (0)	0/2 (0)	0/2 (0)	-	-	-	-	-
High dose	0/8 (0)	0/8 (0)	0/8 (0)	1/8 (12.5)	0/6 (0)	-	0/6 (0)	0/6 (0)	0/6 (0)
Any local adverse event after intra-muscular (IM) vaccination, n/N (%):									
<b>Pain</b>	<b>10/10 (100)</b>	<b>8/10 (80)</b>	<b>8/10 (80)</b>	<b>7/10 (70)</b>	n/a	n/a	0/6 (0)	<b>6/6 (100)</b>	<b>4/6 (67)</b>
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	8/8 (100)	7/8 (87.5)	7/8 (87.5)	6/8 (75.0)	-	-	0/6 (0)	6/6 (100)	4/6 (66.7)
<b>Induration</b>	<b>2/10 (20)</b>	<b>2/10 (20)</b>	<b>3/10 (30)</b>	0/10 (0)	n/a	n/a	0/6 (0)	<b>2/6 (33)</b>	<b>2/6 (33)</b>
Low dose	0/2 (0)	0/2 (0)	1/2 (50.0)	0/2 (0)	-	-	-	-	-
High dose	2/8 (25.0)	2/8 (25.0)	2/8 (25.0)	0/8 (0)	-	-	0/6 (0)	2/6 (33.3)	2/6 (33.3)
<b>Redness</b>	<b>6/10 (60)</b>	<b>2/10 (20)</b>	<b>5/10 (50)</b>	<b>2/10 (20)</b>	n/a	n/a	0/6 (0)	<b>4/6 (67)</b>	<b>1/6 (17)</b>
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	4/8 (50.0)	1/8 (12.5)	4/8 (50.0)	1/8 (12.5)	-	-	0/6 (0)	4/6 (66.7)	1/6 (16.7)
<b>Swelling</b>	<b>10/10 (100)</b>	<b>6/10 (60)</b>	<b>4/10 (40)</b>	<b>3/10 (30)</b>	n/a	n/a	<b>1/6 (17)</b>	<b>2/6 (33)</b>	<b>1/6 (17)</b>
Low dose	2/2 (100)	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	-	-	-	-	-
High dose	8/8 (100)	5/8 (62.5)	3/8 (37.5)	2/8 (25.0)	-	-	1/6 (16.7)	2/6 (33.3)	1/6 (16.7)

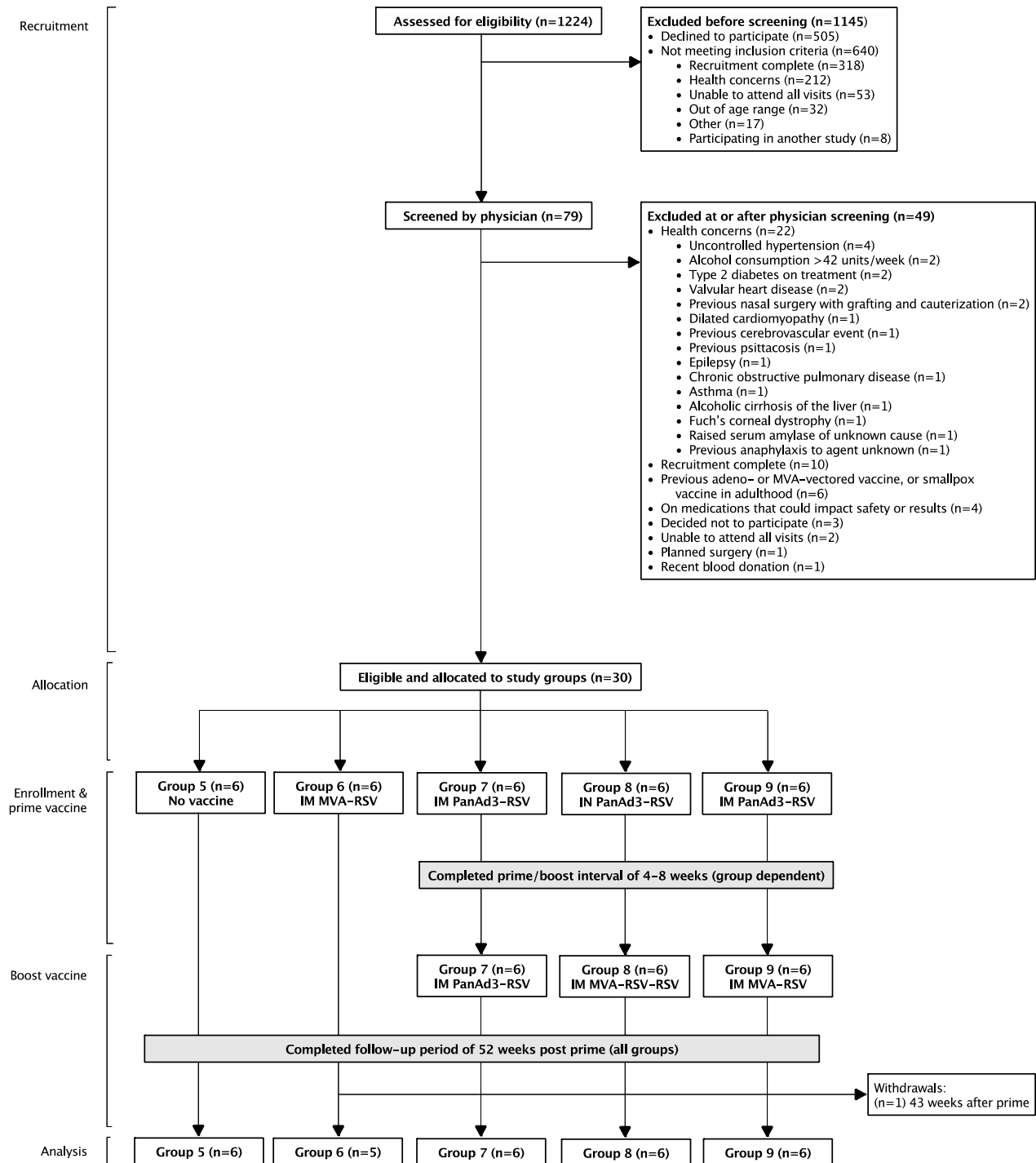
sTab. 4. Counts and proportions of any local adverse event reported after boost vaccination by dose and study group within 7-days of vaccination.

Counts and proportions for the occurrence of any solicited symptom (grade 1-3) in each study group within one week of vaccination. The data are presented as the number of volunteers who reported an adverse event (n) and the number of volunteers in the study group (N). The frequencies of mild (grade 1), moderate (grade 2) and severe (grade 3) adverse events for each study group and each day after vaccination are presented in supplementary results. n/a (not applicable). Adverse events that occurred in  $\geq 10\%$  of volunteers are shown in bold.

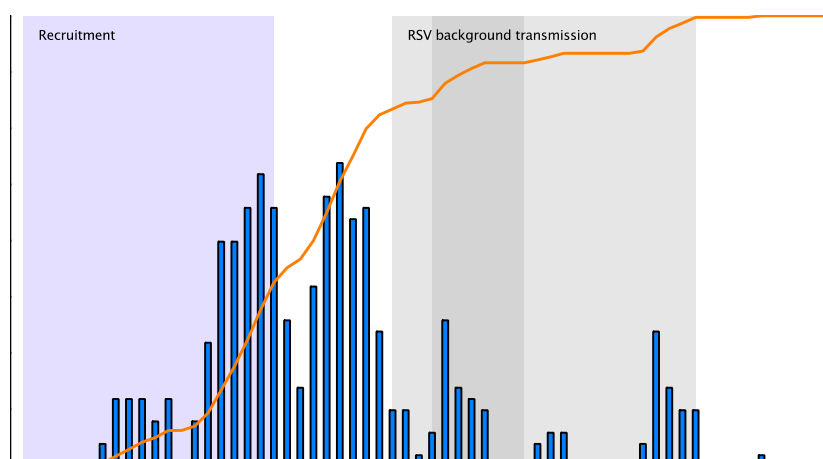
SAE event number	Temporal relationship to vaccination	Description of the event	Outcome
1	16-weeks after high-dose IN PanAd3-RSV prime and 8-weeks after high dose IM MVA-RSV boost. [Group 3 volunteer]	Admitted to hospital for serologically confirmed scrub typhus infection on return from travelling through Myanmar.	Resolved.
2	22-weeks after low-dose IN PanAd3-RSV prime, 16-weeks after a Yellow Fever travel vaccination, and 7-weeks after low-dose IM PanAd3-RSV boost. [Group 4 volunteer]	A recurrence of transient hypnopompic visual hallucinations with flaccid paralysis that occurred at a frequency of 3-4 times per week. After consultation with the DSMC, the frequency and severity of her symptoms warranted designation as an SAE. She recalled milder and shorter episodes (once per week for 2-3 weeks) of these symptoms had occurred 6-months before enrolment, and these were not declared at physician screening or known to her GP. This discounted causality from the vaccine.	She was referred to a sleep specialist by her GP, and her symptoms improved and resolved spontaneously after 7 weeks intermittent duration. The specialist referral was cancelled.
3	43-weeks after high-dose IM MVA-RSV (single dose). [Group 6 volunteer]	Developed a short history of dysphagia in early 2015 and he was diagnosed with terminal oesophageal carcinoma complicated by multiple pulmonary emboli.	Died.

**sTab. 5. Summary description of the 3 severe adverse events (SAEs) during the RSV001 clinical trial.**

*None were considered related to vaccination.*



**sFig. 3. CONSORT flow diagram for the recruitment and completion of the RSV001 clinical trial in healthy adults aged 60-75 years in 2014/15.**



Healthy adults aged 60–75 years

Cumulative % completion of scheduled visits

Study week →

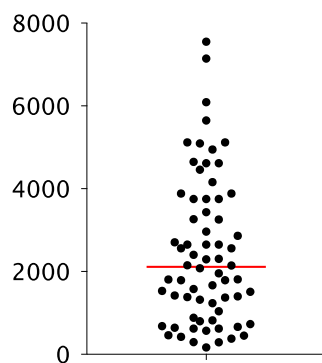
—

ompletion of all scheduled clinical visits

**sFig. 4. Data collection in relation to the RSV transmission periods.**

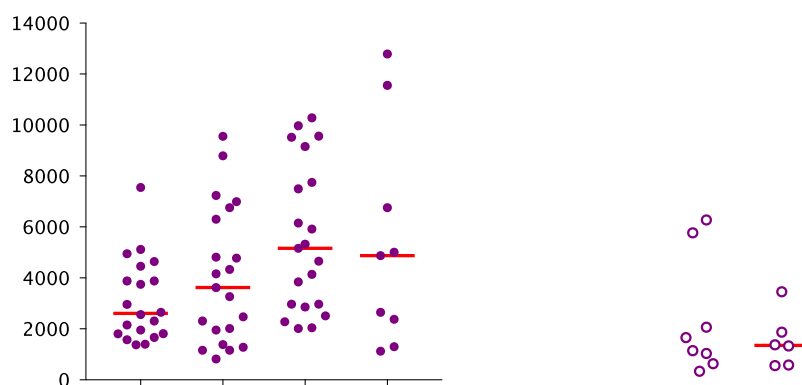
Each column represents one week of the clinical trial. The blue columns denote the number of scheduled visits performed in each study week, with the orange line showing the cumulative completion of clinical visits. The purple area shows the recruitment period. The light grey boxes illustrate the likely RSV transmission period based on Public Health England (PHE) data, with the dark grey box denoting the peak of period of RSV cases reported to PHE. (**Top figure**) Data from 42 healthy adults aged 18-50 years in 2013. Dose and group size escalation rules meant the clinical and laboratory workload was scheduled to expand rapidly after initial safety assessments. (**Bottom figure**) Data from 2014 and 30 older adults aged 60-75 years, without the need for dose or group size escalation rules.

## RSV-specific antibody



**sFig. 5. Serum RSV-neutralising antibody response to natural exposure in younger and older adults.**

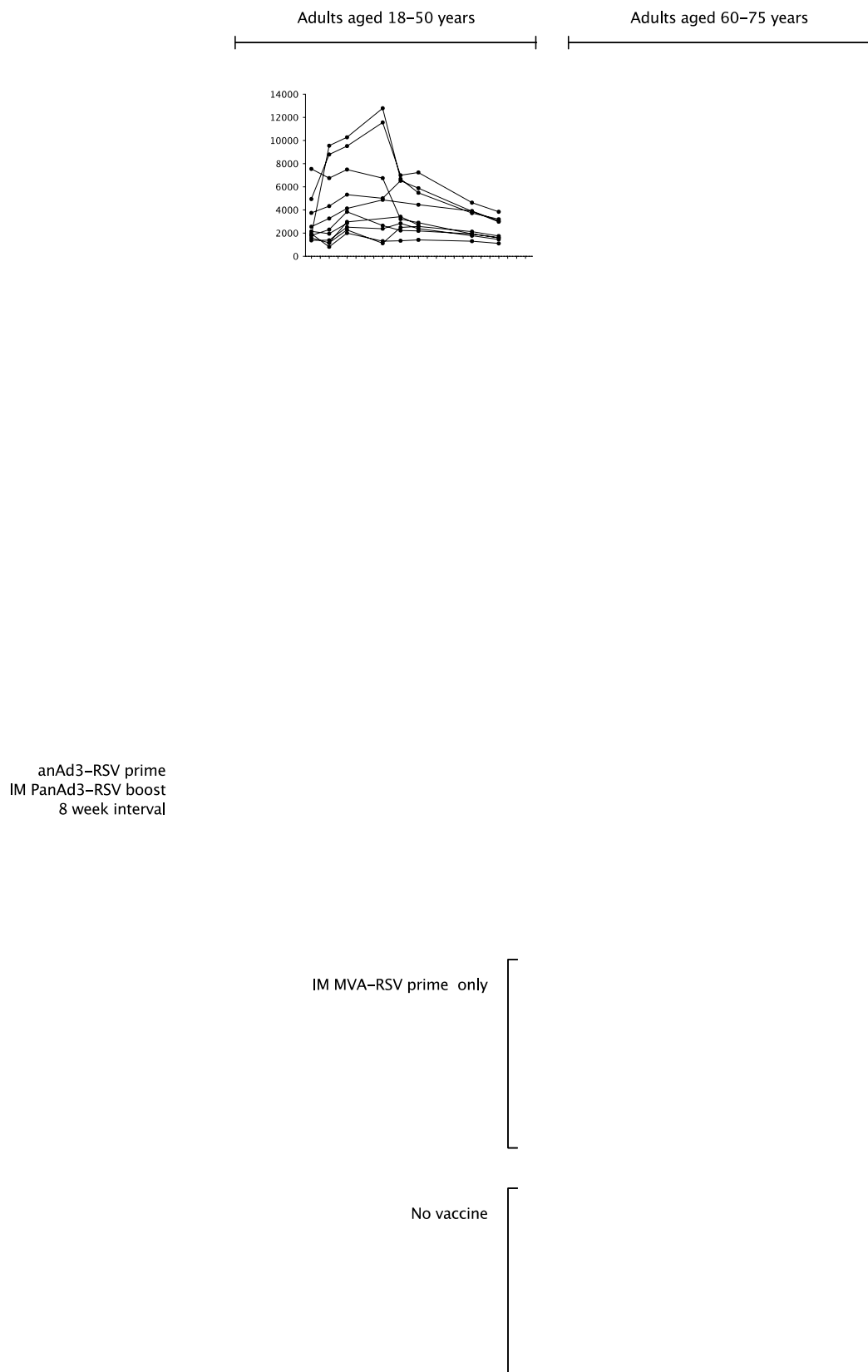
Serum RSV-neutralising antibody titre measured by plaque-reduction neutralisation assay. Each dot represents a single volunteer and the red line the median.



**sFig. 6. Serum RSV-neutralising antibody response to IM PanAd3-RSV prime vaccination.**

Serum RSV-neutralising antibody titre measured by plaque-reduction neutralisation assay. Each dot represents a single volunteer and volunteers pooled by the route of prime (groups 1 and 2 combined for younger adults and groups 7 and 9 combined for older adults). Time points are annotated as days in relation to last vaccine. For example, P14 denotes 14-days post-prime. Red lines denote the median.

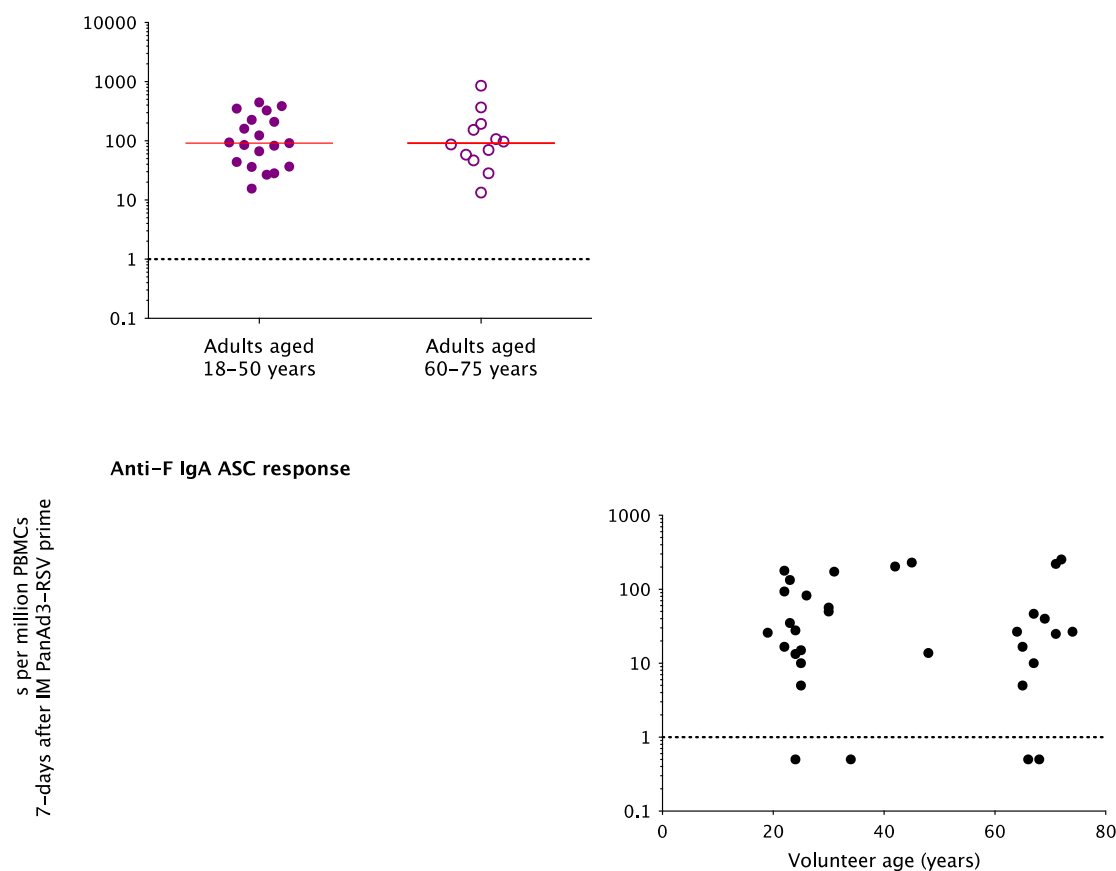




**sFig. 7. Serum RSV-neutralising antibody response to vaccination across all time-points.**

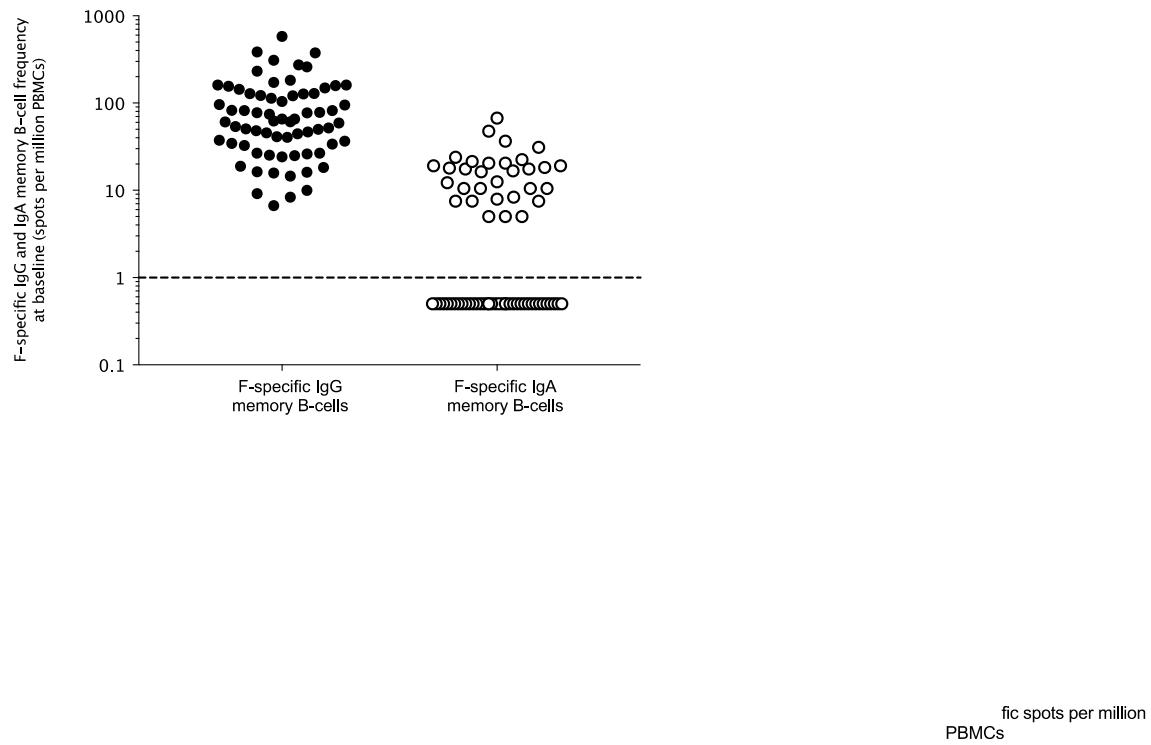
Serum RSV-neutralising antibody titre measured by plaque-reduction neutralisation assay. Each line represents a single volunteer. Time points are annotated as days in relation to last vaccine. For example, P14 denotes 14-days post-prime.

# B-cell immune responses (ASC and memory B-cells by ELISpot)



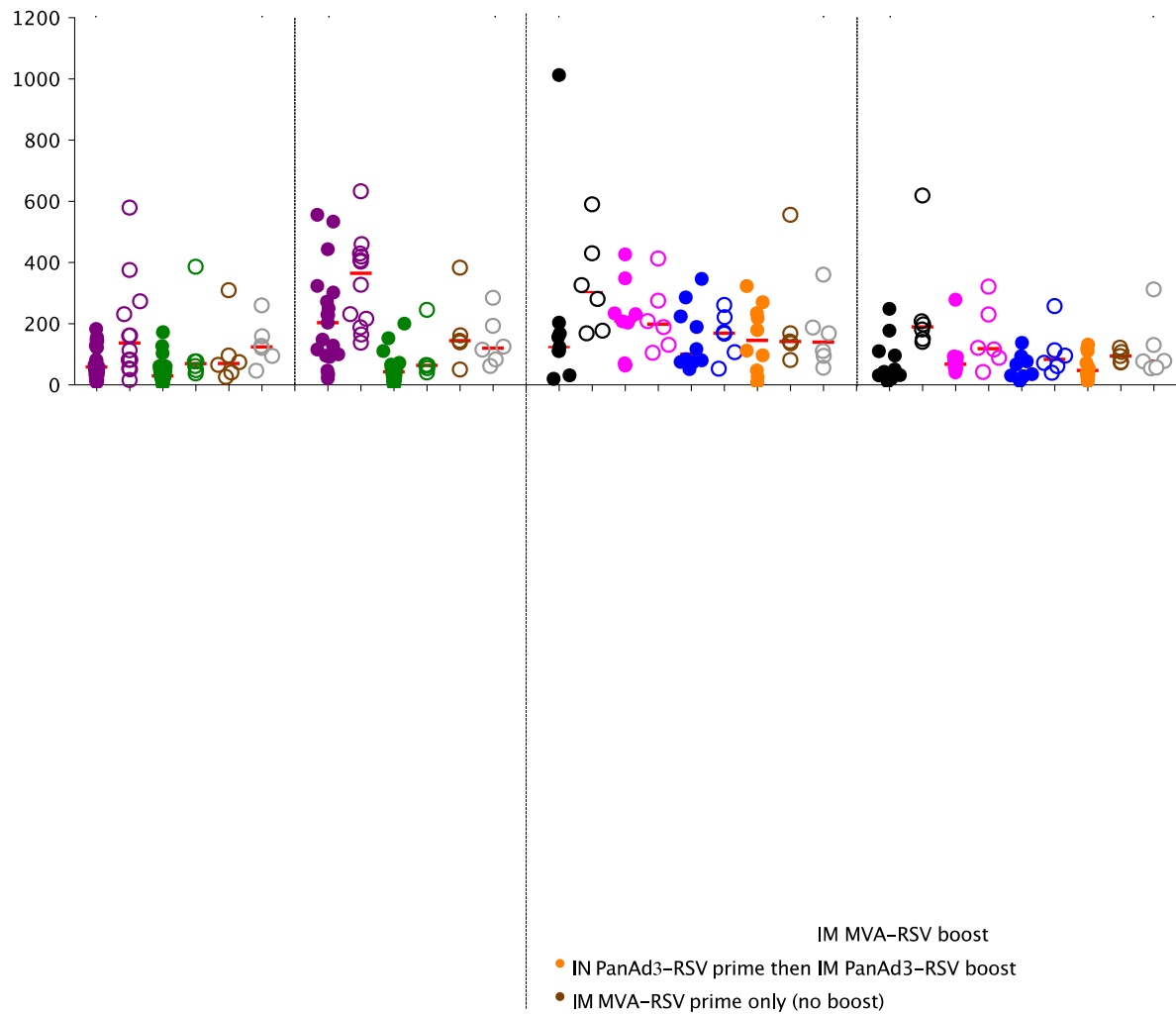
**sFig. 8. F-specific IgG and IgA antibody secreting cell (ASC) response to IM PanAd3-RSV prime in younger and older adults.**

The F-specific IgG (**top panel**) and IgA (**bottom panel**) ASC response to IM PanAd3-RSV prime was measured by dual-colour ex vivo ELISpot 7-days after vaccination, and then correlated to volunteer age. The red bar denotes the median.



**sFig. 9. Baseline F-specific IgG and IgA memory B-cell frequencies in younger and older adults.**

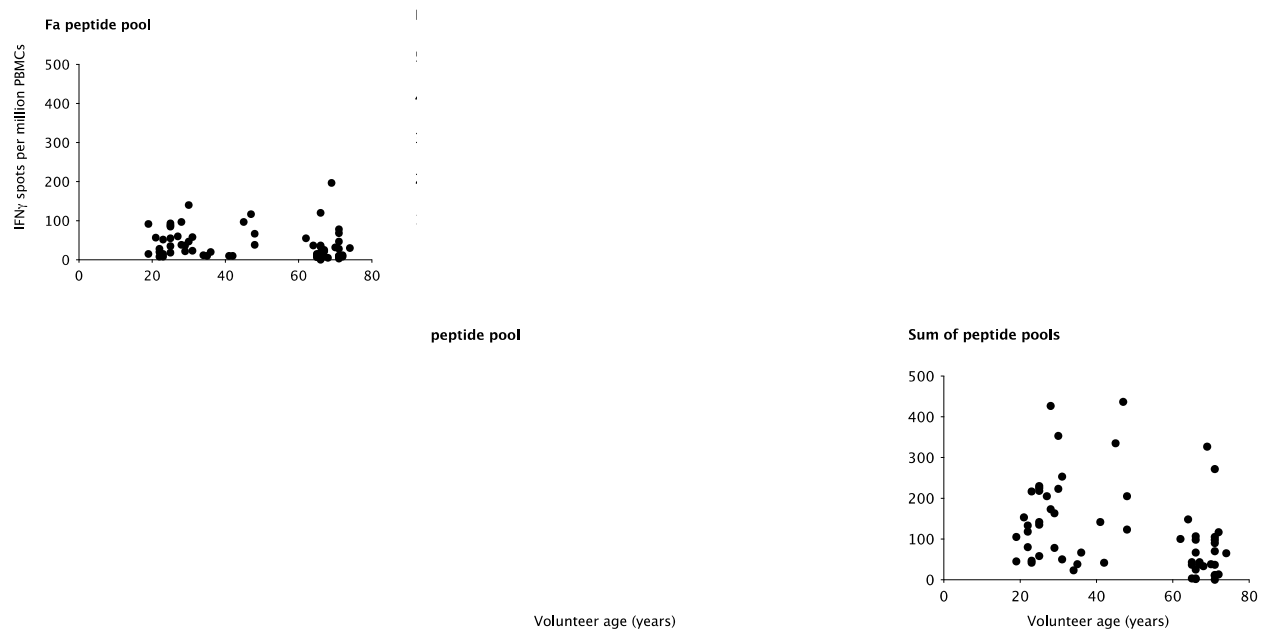
The F-specific IgG and IgA memory B-cell frequencies (**top panel**) and correlated to volunteer age (**bottom panel**).



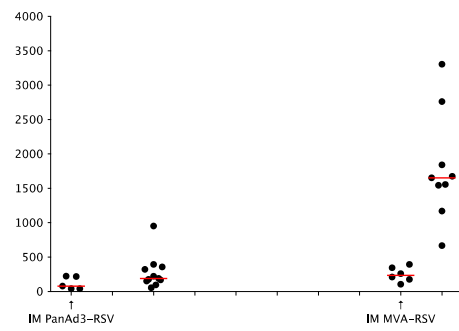
**sFig. 10. F-specific IgG and IgA memory B-cell responses to vaccination in younger and older adults.**

The F-specific IgG (**top panel**) and IgA (**bottom panel**) memory B-cell response to vaccination was measured by dual-colour ex vivo ELISpot at baseline, 28-days after prime, 28-days after boost and 180-days after boost vaccine. The red bar denotes the median.

# T-cell immune responses (IFN $\gamma$ -producing T-cells by ELISpot)



**sFig. 11. RSV Peptide pool specific and total T-cell IFN $\gamma$  baseline responses to natural exposure with age.**  
*Correlation representation between each peptide pool and total T-cell IFN $\gamma$  responses and volunteer age.*



PanAd3-RSV  $\rightarrow$  IM MVA-RSV

**sFig. 12. RSV-specific total T-cell IFN $\gamma$  responses to each prime/boost vaccine in younger and older adults.**

RSV-specific T-cell IFN $\gamma$  responses were measured by ex vivo ELISpot, expressed here as the total response ( $[Fa+Fb+M+N]-[4 \times DMSO]$ ). Each dot represents one volunteer and each increment on the x-axis represent one week. Red bars denote the median and data for group 5 (non-vaccinated older adults) are not shown.



**sFig. 13. RSV peptide-pool specific T-cell IFN $\gamma$  responses to vaccination.**

Peptides included pools covering the vaccine antigens and were designated Fa, Fb, M and N. The dotted line represents the DMSO+4StDev. The post vaccination time points presented are 14- and 7-days post prime and boost respectively.

ICS/FACS analysis of IFN $\gamma$ /IL-2/IL-4 producing T-cells, T-follicular like helper cells and B-cell responses.

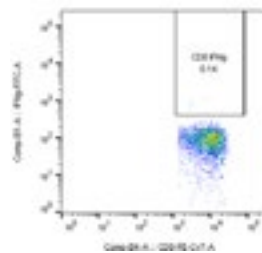
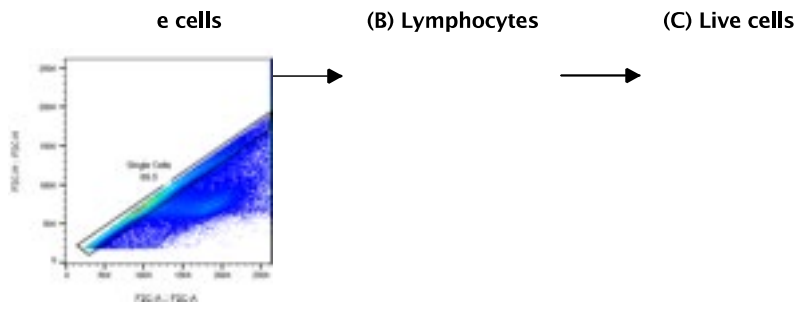
For the intracellular cytokine staining with flow cytometry (ICS/FACS) analysis of IFN $\gamma$ /IL-2/IL-4 producing T-cells, frozen PBMCs were thawed in 10ml complete medium (RPMI with 10% FCS, 1% PenStrep, L-glutamine, NEAA, Sodium pyruvate, bmercaptoethanol) with 20ul Benzonase, re-suspended and rested in an incubator overnight (5% CO<sub>2</sub>, 37°C) in 4mL R10 (RPMI, 10% FBS, 2mM L-Glutamine, 50 $\mu$ g/mL Streptomycin, 50U Penicillin) before plating at a concentration of 1x10<sup>6</sup> cells/well in a 96-well tissue culture plate. DMSO, Fa, Fb, M, N, phorbol 12-myristate 13-acetate (PMA, Sigma)/Ionomycin (Sigma) were added with 1 $\mu$ L to each well brefeldin A (1/100 dilution of 0.5 $\mu$ g/mL stock) and the plate was incubated overnight (5% CO<sub>2</sub>, 37°C). After 1xPBS wash flouochrome-conjugated monoclonal surface staining antibodies L/D-APC-Cy-7 (Life Technologies Limited, UK), 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, San Diego) and re-suspended in permeabilization buffer for 20-minutes. Flouochrome-conjugated monoclonal antibodies added in permeabilization buffer solution included CD3-eflour450 (Affymetrix, eBioscience Inc, San Diego), CD4-Viogreen (Miltenyi Biotec Ltd, UK) and CD8-PE-Cy7 (BD, UK) IFN-gamma-FITC (Miltenyi Biotec Ltd, UK), IL2-PerCP-Cy5.5 (BD) and IL-4-APC (BD). These antibodies and were allowed 25-minutes incubation with cells before washing, and samples were spun at 1500rpm for 5-minutes and re-suspended in 200 $\mu$ L PBS. Responses were background DMSO subtracted and a threshold of 0.02% was applied to define a positive T-cell response.

For the ICS/FACS analysis of T-follicular like helper cells and B-cell responses PMBCs frozen at 10x10<sup>6</sup>/mL in Recovery™ Cell Freezing Medium (10% DMSO, and calf serum, Invitrogen) and were thawed and added to 1mL of thawing medium (9mLs thawing medium with 100 $\mu$ Ls DNAase added). The samples were centrifuged (1600rpm for 6-minutes), the supernatant discarded and the pellet was re-suspended in 1mL R10 for counting using the same method described earlier(16). After centrifugation (1600rpm for 6-minutes) the PBMCs were re-suspended in R10 at a concentration of 5x10<sup>6</sup> cells/mL. 800,000 PBMCs in 160 $\mu$ Ls were added to each well of a 96-well U-bottom plate (Thermo-Fisher), centrifuged (1600rpm for 6-minutes) and then a 50 $\mu$ Ls/well mix of fluorochrome-conjugated monoclonal surface staining antibodies in PBS was added for 30-minutes at 4°C protected from light. Staining antibodies for T-follicular helper cells included CXCR3-PerCPCy5.5 (BD), CXCR5-AF647 (BD), PD-1-PE (BD), ICOS-FITC (Miltenyi), CD4-Pacific Blue (Miltenyi), CD45RA-VioGreen (Miltenyi) and NIR live/dead stain (Invitrogen). Staining antibodies for B-cell responses included CD38-PerCPCy5.5 (BD), IgA-FITC (Miltenyi), IgD-PE (BD), CD27-PECy7 (eBioscience Inc), CD19-Pacific Blue (BD), CD20-Pacific Orange (Invitrogen) and NIR live/dead stain (Invitrogen). Plates were then centrifuged (1600rpm for 6-minutes), the



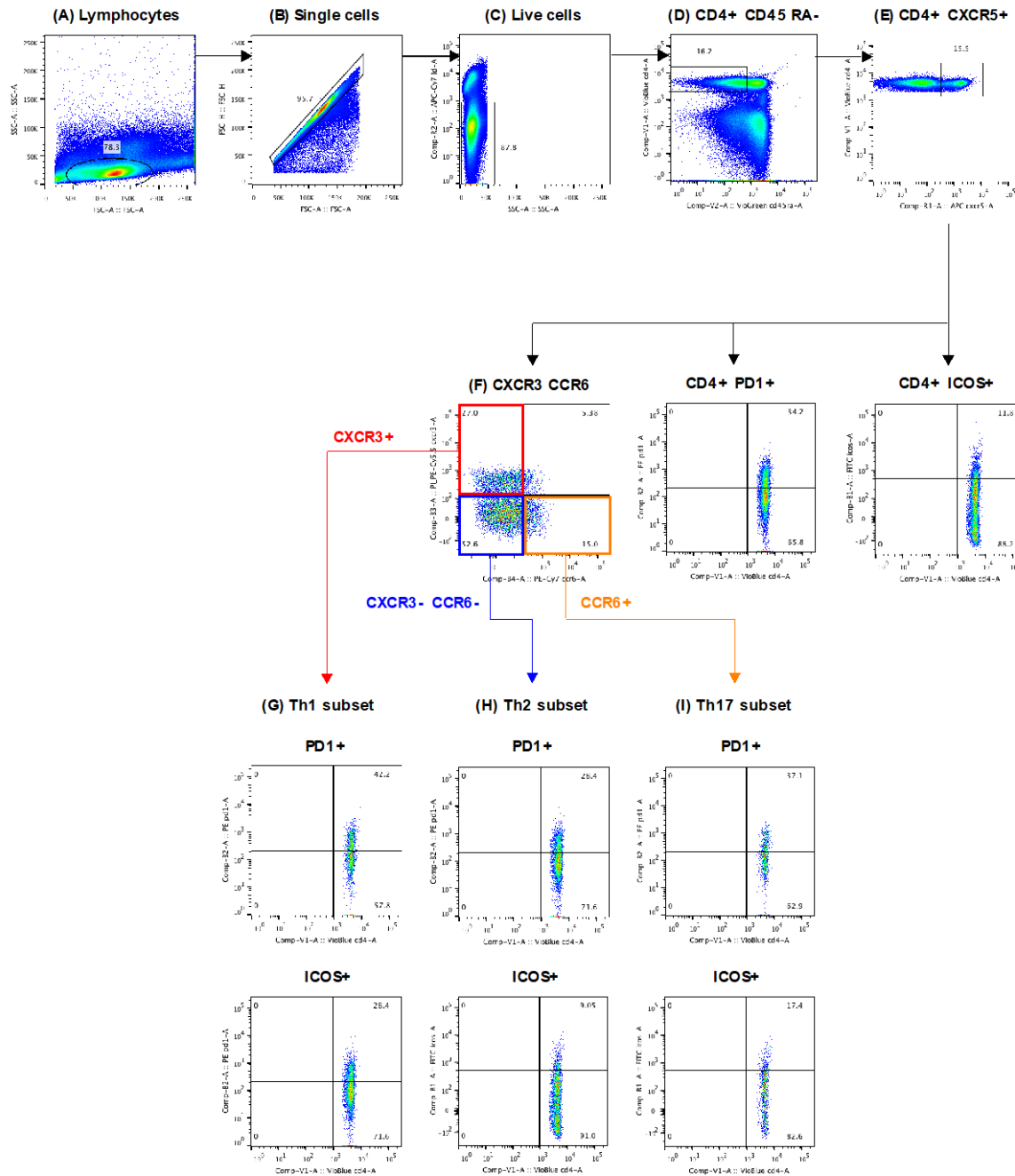
supernatants discarded, and PBMCs were re-suspended in 200 $\mu$ Ls/well PBS. Samples used in both assays were analysed by FACS using a MACSQuant flow cytometer (Miltenyi Biotec) and FlowJo software (version X0.7 for Mac). The gating strategies are shown below.

We also used ICS/FACS analyses to further explore whether IgA memory B-cell responses had occurred 7-days after IM PanAd3-RSV prime and boost vaccination (IN primed volunteers were not analysed). The results showed a modest expansion in the memory B-cell population after vaccination that corroborated the stimulated IgG memory B-cell ELISpot data as well an expansion of plasmablasts 7-days after vaccination that corroborated the detection of IgG/IgA ASCs in circulation by fresh *ex-vivo* ELISpot. Notably the assay also failed to demonstrate any perturbation in the IgA memory B-cell population at this time point. We next sought to characterise the T-follicular-like (Tfh) helper cell population in a separate ICS/FACS assay. Tfh cells are essential in the development of B-cell effector and memory responses within lymphoid follicles and were characterised these by the surface expression of CXCR5 (CD185), the receptor for CXCL13. Tfh cells (CD4+ CXCR5+ CD45RA-) were further subdivided into the Tfh subsets Th1 (CXCR3+, CCR6-), Th2 (CXCR3- CCR6-) and Th17 (CXCR3- CCR6+). The un-stimulated frozen PBMCs from the peripheral blood of volunteers who received IM PanAd3-RSV prime vaccine were analysed for Tfh cell frequencies and expression of PD1 and ICOS activation markers before vaccination and one-week post prime/boost (sFig. 18 and sFig. 19). The baseline Tfh population derived from natural exposure did not appear different between younger and older adults, yet in all CXCR5+ cell populations the ICOS and PD1 expression was lower in older adults. IM PanAd3-RSV prime appeared to be associated with a contraction of the Tfh cell population 7-days after vaccination in older adults (and not younger adults), which recovered and was maintained without further disturbance following boost. PD1 expression appeared unchanged after vaccination and ICOS expression more variable and without any apparent relationship to vaccination or volunteer age. The Tfh2 and Tfh17 subsets also contracted 7-days after prime in the younger and older adults respectively.



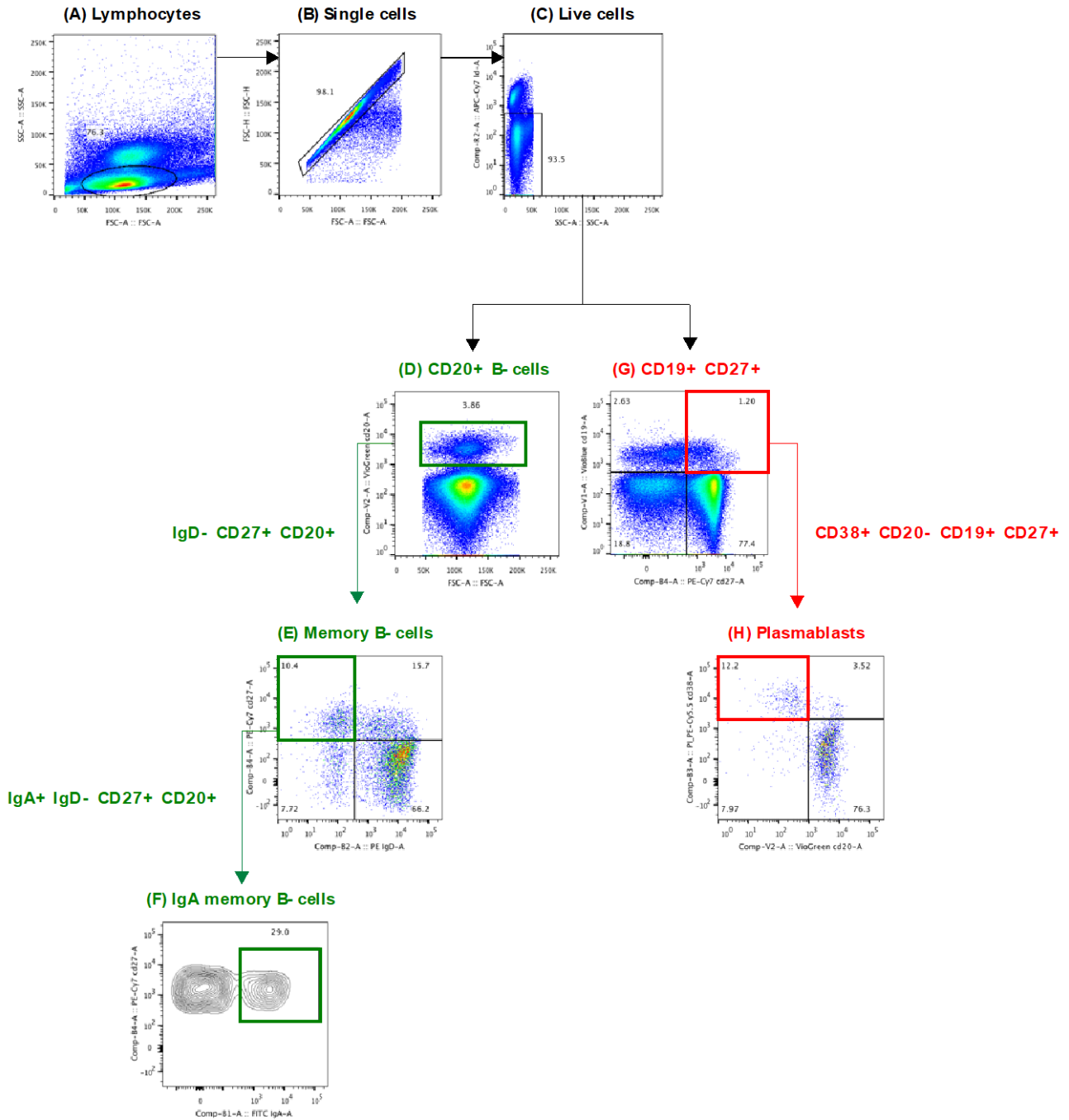
**sFig. 14. Gating strategy for the detection and quantitation of T-cells producing IFN $\gamma$ , IL-2 and IL-4 after vaccination.**

The example used here is from an un-immunised blood donor. PBMCs were un-stimulated and gated from (A) single cells, (B) lymphocytes, (C) live cells, (D) CD3+ IFN $\gamma$ + cells, (E) CD4+ or CD8+ T cells, (F) CD4+ T cells producing IFN $\gamma$  (G) CD4+ T cells producing IL4 (H) CD4+ T cells producing IL2, (I) CD8+ T cells producing IFN $\gamma$  (J) CD8+ T cells producing IL4 (K) CD8+ T cells producing IL2.



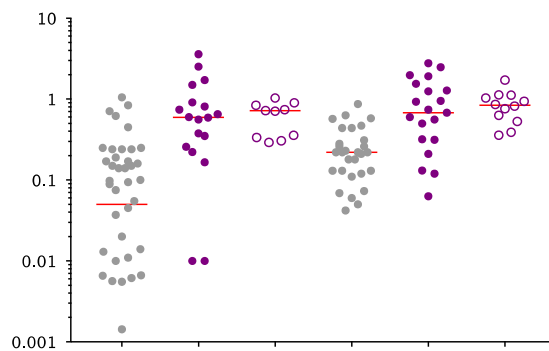
**sFig. 15. Gating strategy for the detection and quantitation of T-follicular helper (Tfh) cells after vaccination.**

The example used here is from a volunteer 7-days after high-dose IM PanAd3-RSV prime. PBMCs were un-stimulated and gated from (A) lymphocytes, (B) single cells, (C) live cells, (D) CD4+ CD45RA- memory T-cells, (E) CD4+ CXCR5+ Tfh cells, (F). Tfh cells gated on CCR6 into (G) Th1 subset (CD4+ CD45RA- CXCR5+ CXCR3+), (H) Th2 subset (CD4+ CD45RA- CXCR5+ CXCR- CCR6-) and (I) Th17 subset (CD4+ CD45RA- CXCR5+ CCR6+). CXCR5+ Th1/Th2/Th17 subsets were further gated on PD1+ and ICOS+.



**sFig. 16. Gating strategy for the detection and quantitation of memory B-cells and plasmablasts after vaccination.**

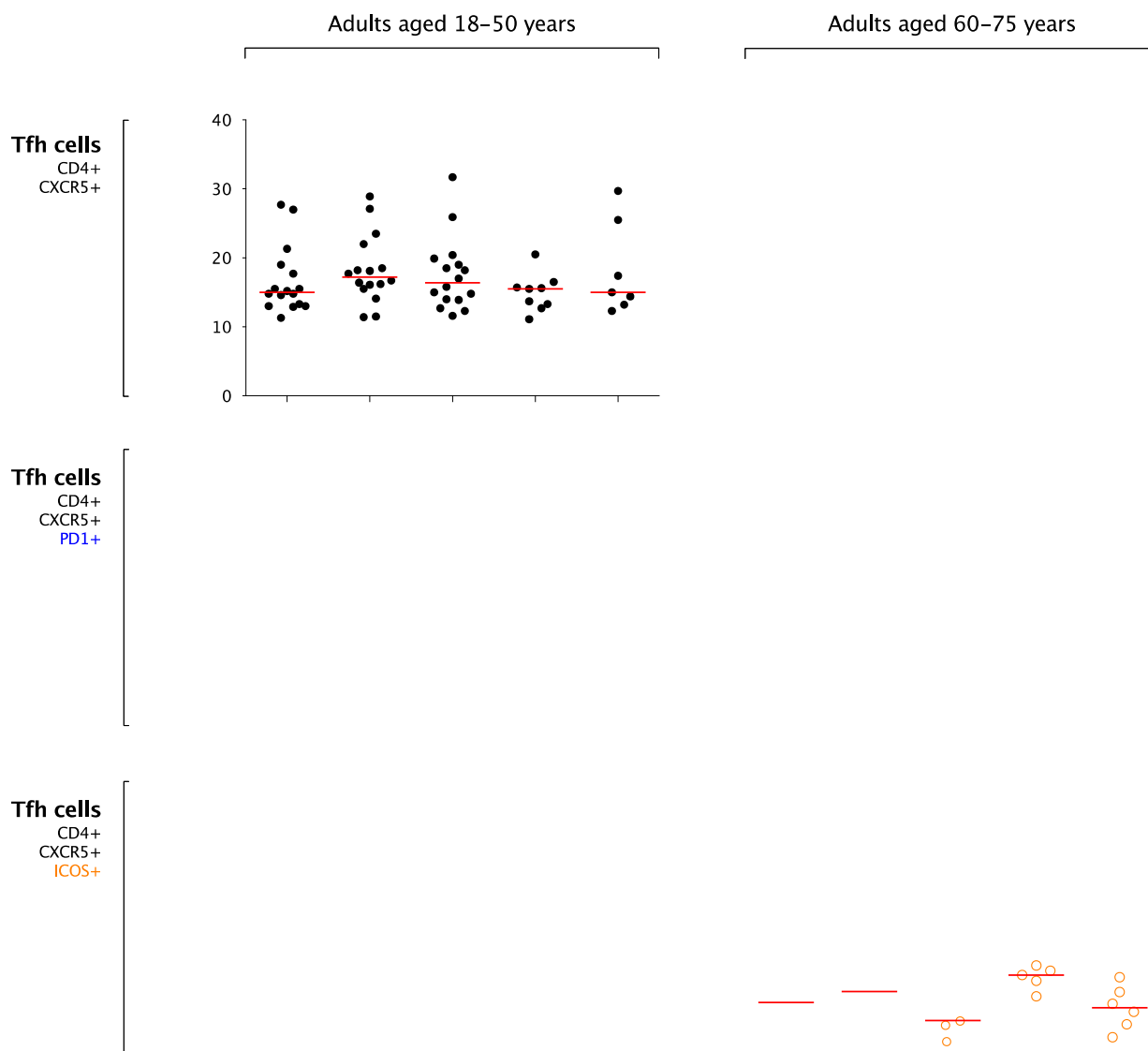
The example used here is from a volunteer 7-days after high-dose IM PanAd3-RSV prime. PBMCs were un-stimulated and gated from (A) lymphocytes, (B) single cells and (C) live cells. For IgA memory, (D) CD20+ B-cells, (E) IgD- CD27+ CD20+ memory B-cells, (F) IgA+ IgD- CD27+ CD20+ memory B-cells. After (C), cells were also gated by (G) CD19+ CD27+ B-cells and (H) CD38+CD20- CD19+ CD27+ plasmablasts.



4-producing  
PBMCs

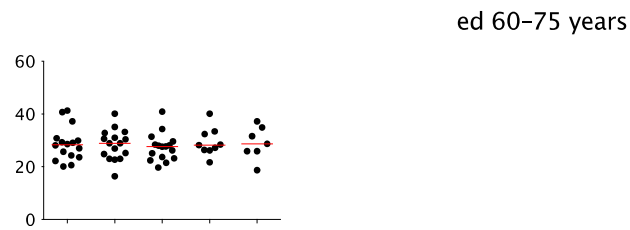
**sFig. 17. ICS/FACS analysis of IFN $\gamma$ , IL2 and IL4-producing CD4+ and CD8+ T-cells after IM PanAd3-RSV prime in younger and older adults.**

*The red bars denote the median.*



**sFig. 18. The T-follicular helper cell (CD4+ CXCR5+ CD45-) cell frequency response to IM PanAd3-RSV prime and IM PanAd3-RSV/IM MVA-RSV boost.**

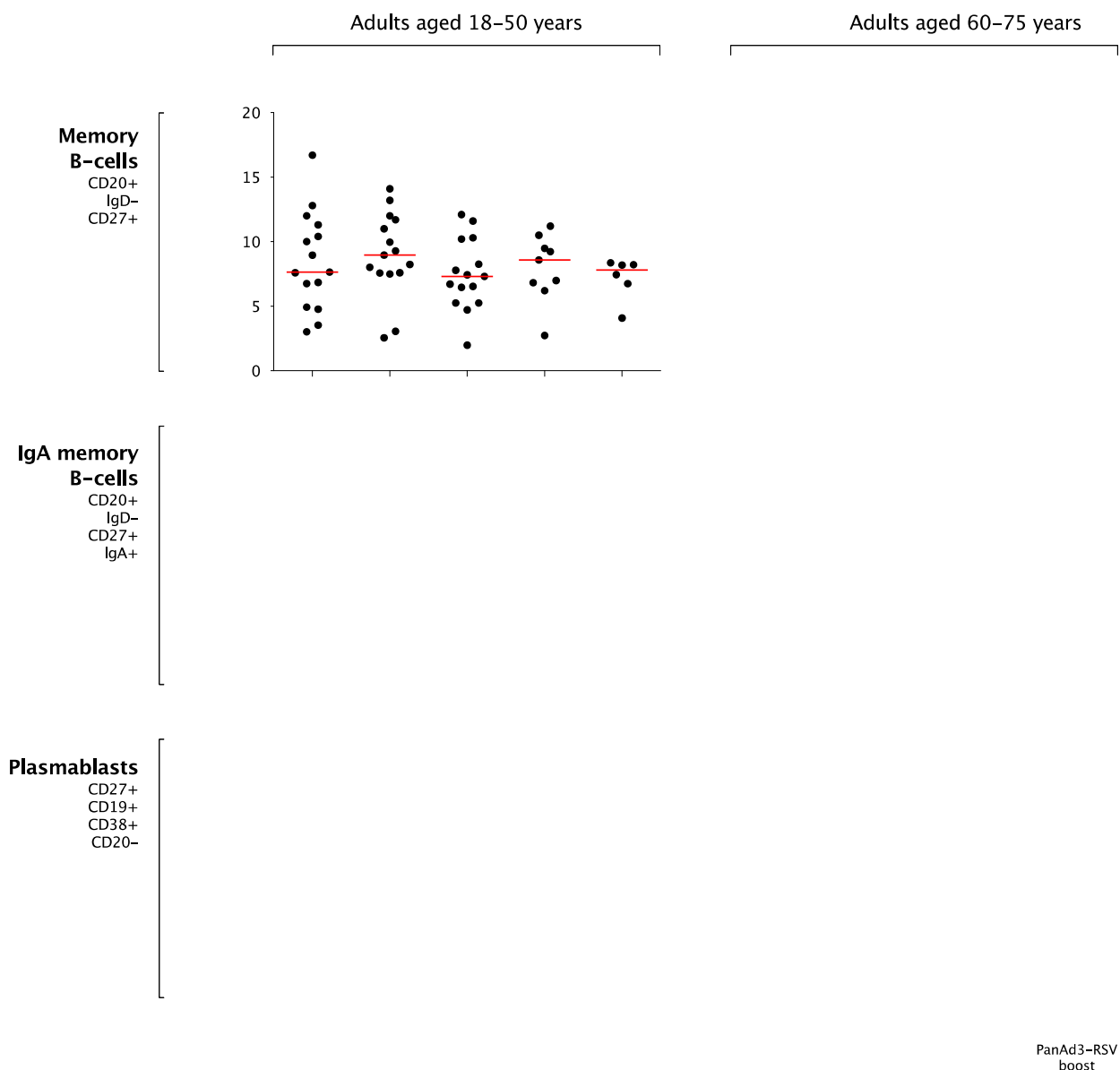
FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4- weeks post prime for groups 2 and 7; 8- weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. (**Top row**) The overall T-follicular helper (Tfh) cell populations in younger and older adults and (**middle row**) with PD1 co-expression and (**bottom row**) ICOS co-expression.



**sFig. 19. Analysis of the Tfh Th1/Th2/Th17 subset responses to IM PanAd3-RSV prime and IM PanAd3-RSV/IM MVA-RSV boost vaccination.**

FACS data from un-stimulated PBMCs from IM PanAd3-RSV primed volunteers (study groups 1, 2, 7 and 9). All cell frequencies are presented as the % of parent population with red lines used to mark the median value. Time points included were (1) baseline, (2) 7-days after IM PanAd3-RSV prime, (3) before boost (4-weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and (4) 7-days after boost for IM MVA-RSV and (5) IM PanAd3-RSV respectively.

(1) (2) (3) (4) (5)                      (1) (2) (3) (4) (5)

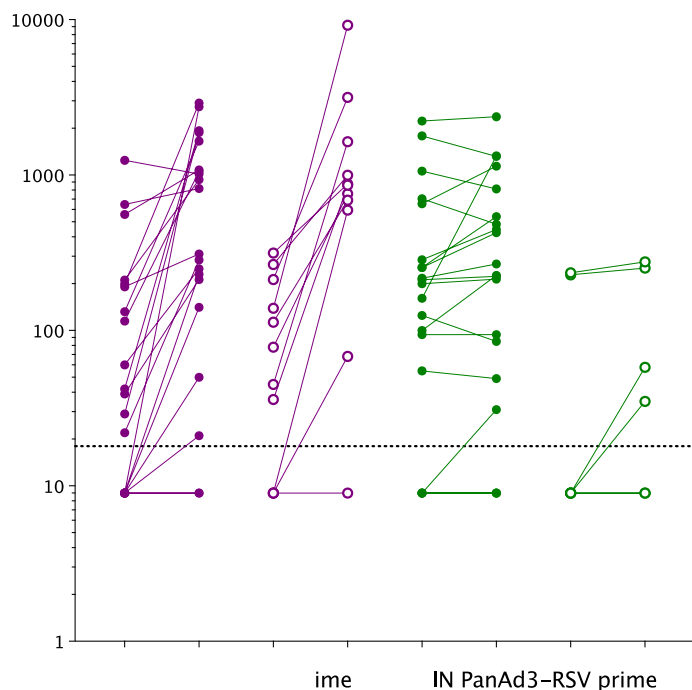


**sFig. 20. Changes in frequencies of memory B-cells, IgA memory B-cells and plasmablasts after vaccination.**

Data from IM PanAd3-RSV primed volunteers only. Cell frequencies are presented as the % of parent population with the red lines used to mark the median value. Data from younger and older adults side-by-side. Time points included were baseline, 7-days after IM PanAd3-RSV prime, before boost (4-weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9) and 7-days after boost for IM MVA-RSV and IM PanAd3-RSV respectively. (**Top row**) Memory B-cells, (**middle row**) IgA memory B-cells and (**bottom row**) plasmablasts.



# Serum PanAd3 (vector) neutralising antibody



**sFig. 21. The serum anti-PanAd3 (vector) neutralising antibody titres before and after prime.**

Antibody titres were measured by SEAP assay and presented here as the titres linked by individual before prime and before boost (4- weeks post prime for groups 2 and 7; 8-weeks post prime for groups 1 and 9). Coloured and empty circles denote younger and older adults respectively. The horizontal dotted line marks the lower limit of detection for the assay.

## SUPPORTING STATEMENTS

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### Ethical approvals and study registrations.

RSV001 was registered with clinicaltrials.gov and EudraCT (references NCT01805921 and 2011-003589-34 respectively). Clinical Trial Authorisation was granted by the United Kingdom Medicines and Healthcare Products Regulatory Agency (MHRA ref 35082/0003/001-0001). Ethical approval and amendments were granted by NRES Berkshire (NRES ref 13/SC/0023). Each vaccine was granted use under GMO (Contained Use) Regulations 2000 by the Oxford University Hospitals NHS Trust Genetic Modification Safety Committee (reference GM462.11.64). 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. 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.

#### Author contributions.

CAG was the lead physician; CJS was the lead scientist; 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, CdL, CH, MDS, TC, 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, CS, 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.

#### Competing interests.

AJP has previously conducted studies on behalf of Oxford University funded by vaccine manufacturers, but currently does not undertake industry funded clinical trials. AJP chairs the UK Department of Health's (DH) Joint Committee on Vaccination and Immunisation (JCVI) and is a member of the World Health Organization's (WHO) Strategic Advisory Group of Experts. The views expressed in this manuscript are those of the authors and do not necessarily reflect the views of the JCVI, the DH, or the WHO. AV 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.

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