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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SUMMARY

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 intramuscular (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 IFNγ-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+ IFNγ-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 IFNγ-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.

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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 60-75years, 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 (F0ΔTM, 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 5×10^{10} viral particles (vp) and 1×10^8 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 6 h 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 1:10,240. 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–Karber 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 were 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 2×10^6 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 $M_{2\text{-}1}$ specific IFNγ -producing T-cells in peripheral blood

Antigen-specific IFNγ-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 $3\times$ 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 metapenumovirus, 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, sTable 3 and sTable 4) with only one serious adverse event (SAE) which was considered unrelated to vaccination (sTable 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
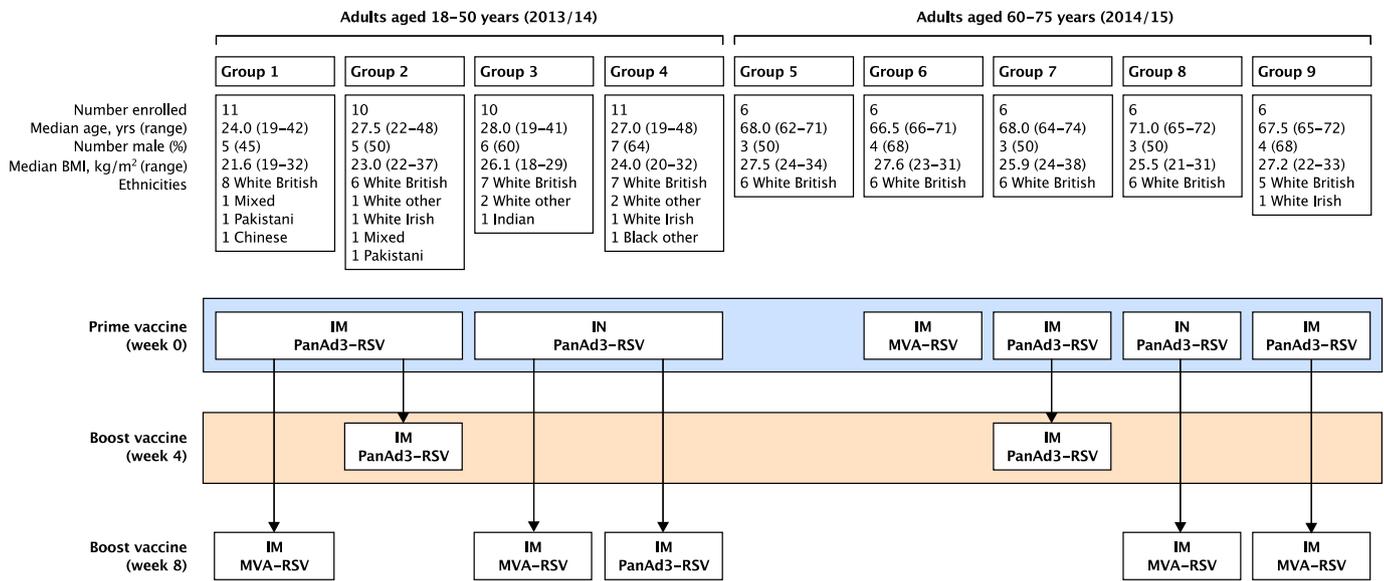
Fig. 1. Study groups defined by prime/boost combination and age, with the baseline physical characteristics of volunteers enrolled into each group.

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.
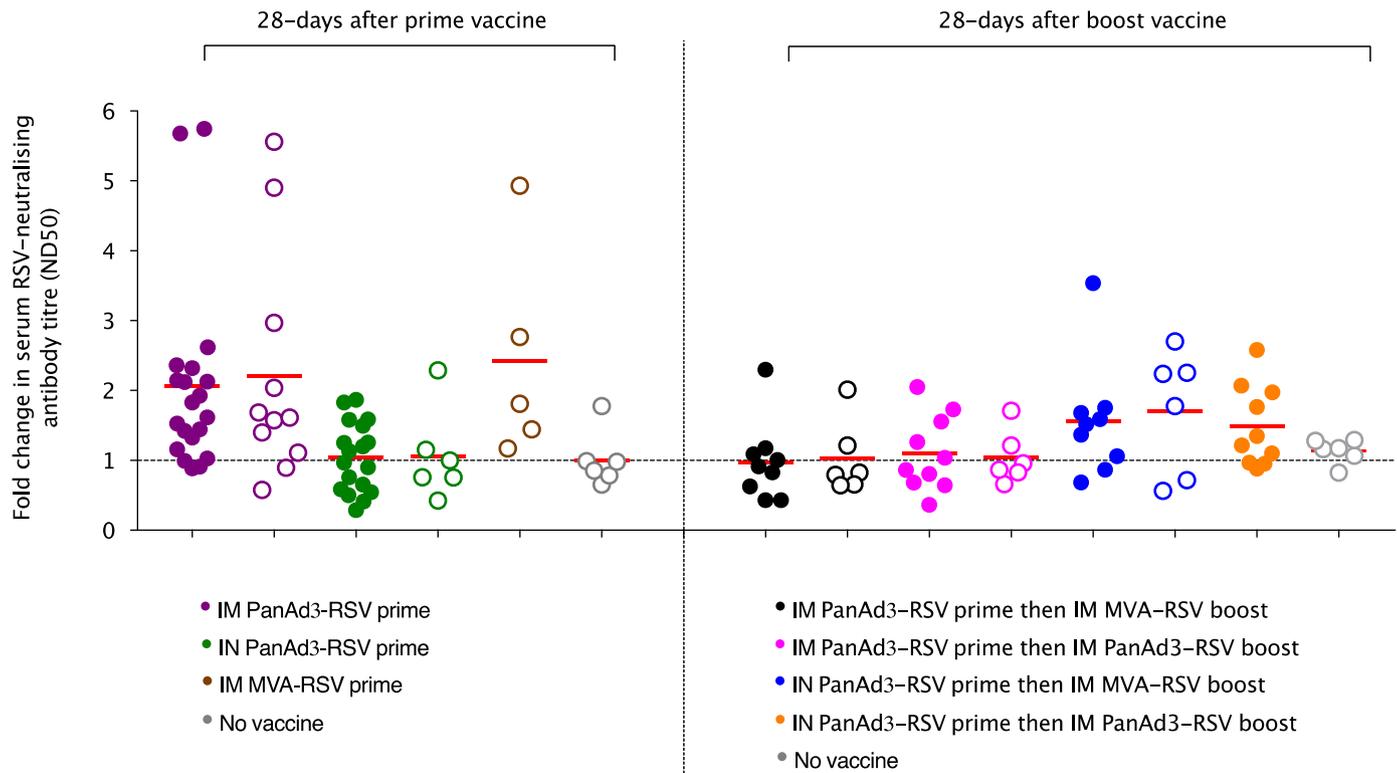

Fig. 2. Serum RSV-neutralising antibody response to vaccination.

The serum RSV-neutralising antibody titre was measured by plaque-reduction neutralisation assay, expressed as the dilution required for 50% plaque survival (ND50). 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.

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
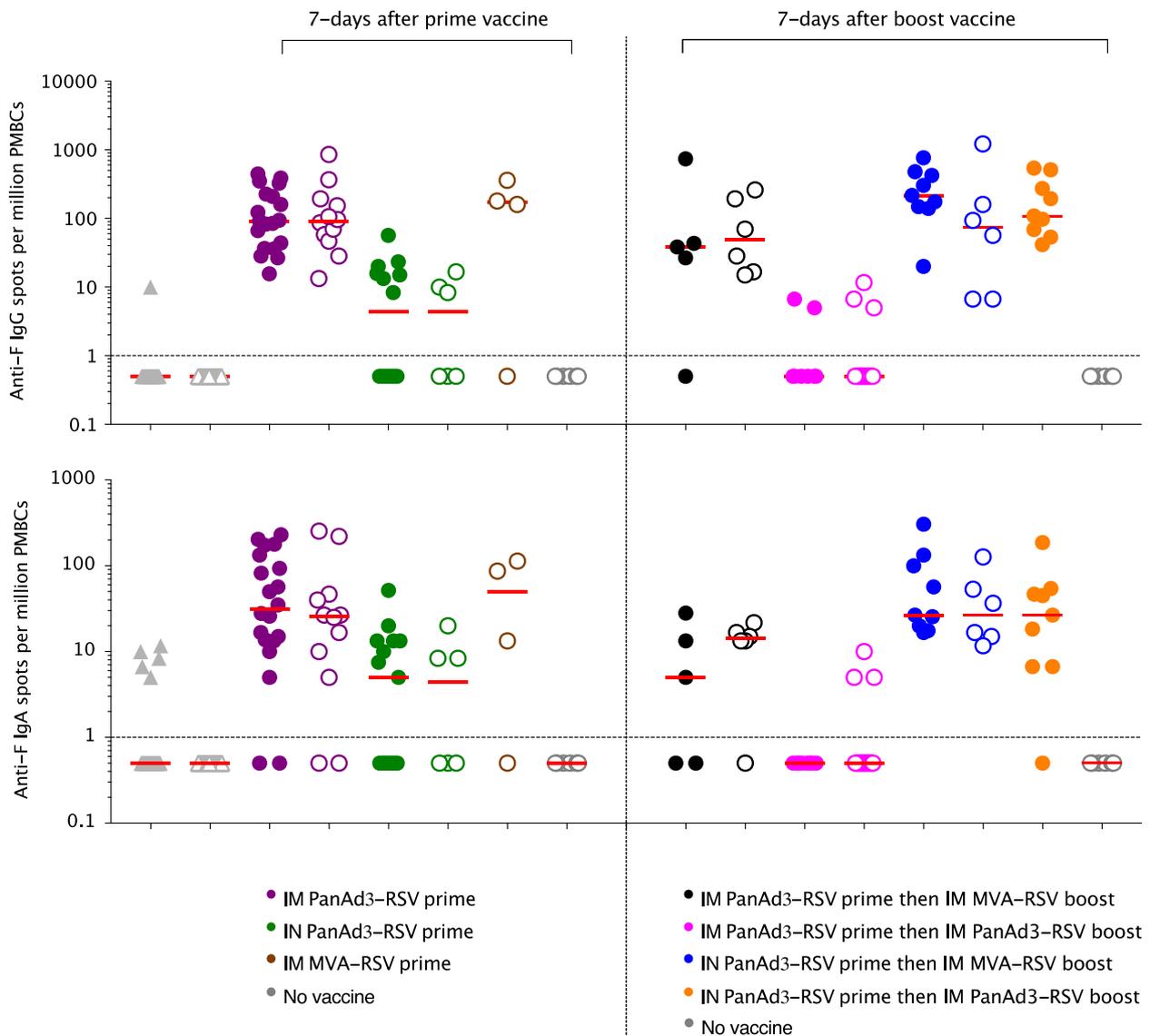
7-days after prime vaccine

7-days after boost vaccine

● IM PanAd3–RSV prime
● IN PanAd3–RSV prime
● IM MVA–RSV prime
● No vaccine

● IM PanAd3–RSV prime then IM MVA–RSV boost
● IM PanAd3–RSV prime then IM PanAd3–RSV boost
● IN PanAd3–RSV prime then IM MVA–RSV boost
● IN PanAd3–RSV prime then IM PanAd3–RSV boost
● No vaccine

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.

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 see 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-$\log_{10}$ range in F-specific IgG memory B-cell frequencies and responses where 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
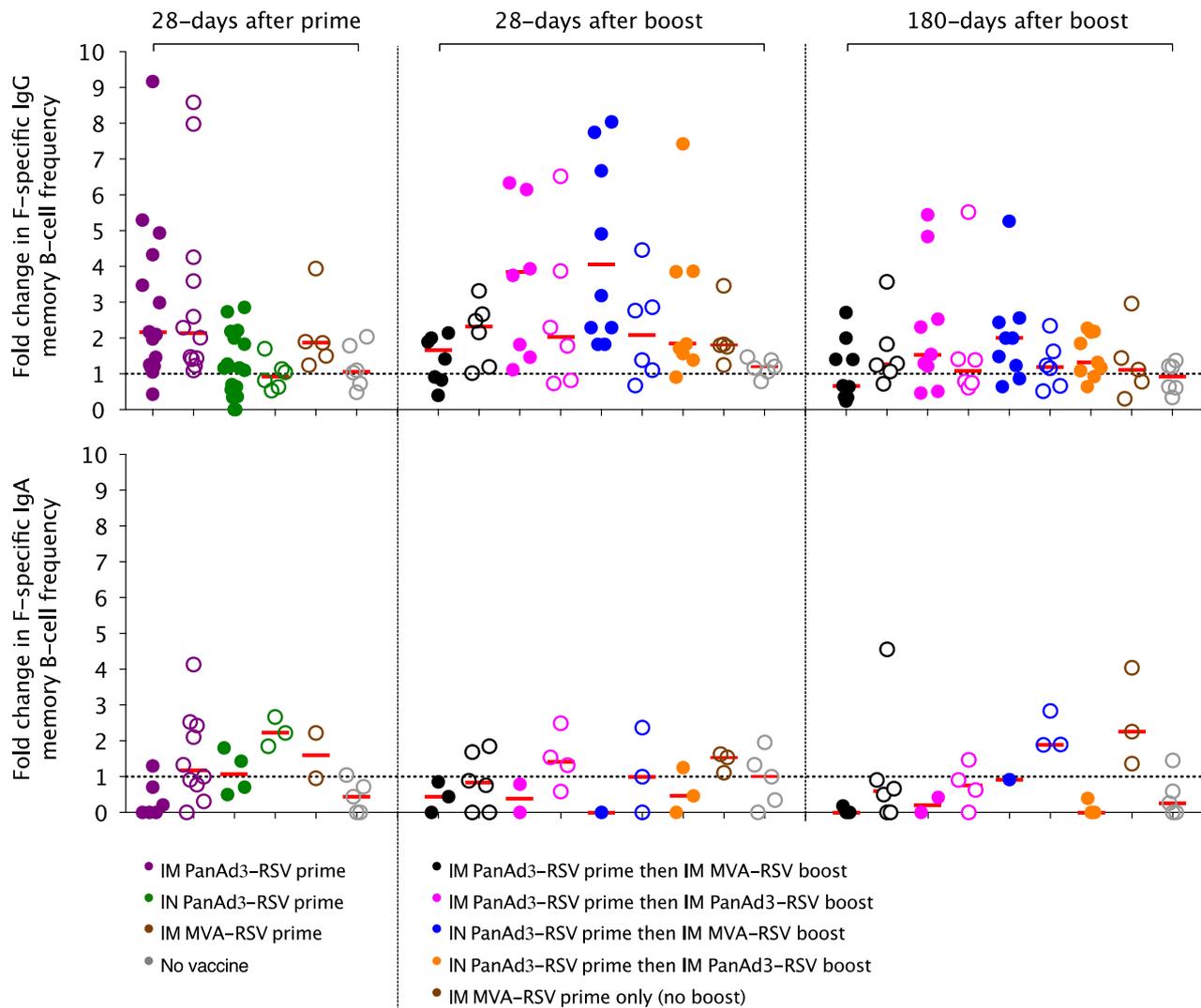
Fig. 4. The F-specific IgG and IgA memory B-cell response to vaccination.
The F-specific IgG (*top figure*) and IgA (*bottom figure*) memory B-cell response to vaccination expressed a fold change in spots per million PBMCs from baseline. F-specific IgG and IgA memory B-cell frequencies were measured 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.

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 IFNγ-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 IFNγ-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 IFNγ-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
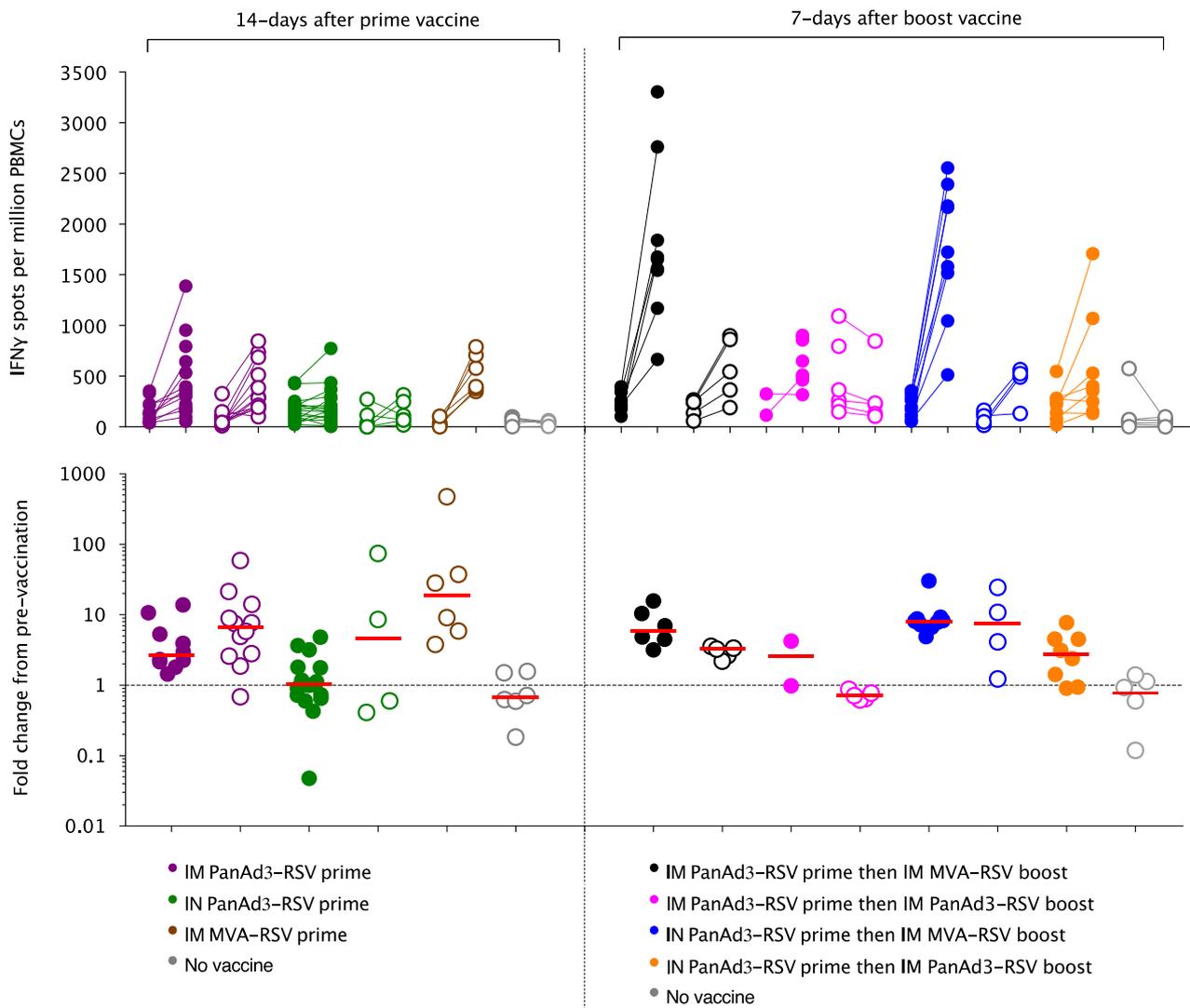
Fig. 5. The total T-cell IFNγ response to vaccination.

The total T-cell IFNγ response to vaccination was by ex vivo ELISpot (sum of Fa, Fb, M and N peptide pools with 4×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*) IFNγ responses before vaccination (pre-prime or pre-boost) paired to the post-vaccination response from each individual14-days after prime and 7-days after boost. (*Bottom panel*) The same data expressed as a fold-change in IFNγ 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).

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 IFNγ-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 IFNγ-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 IFNγ-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+ and CD8+ responses for IFNγ, 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
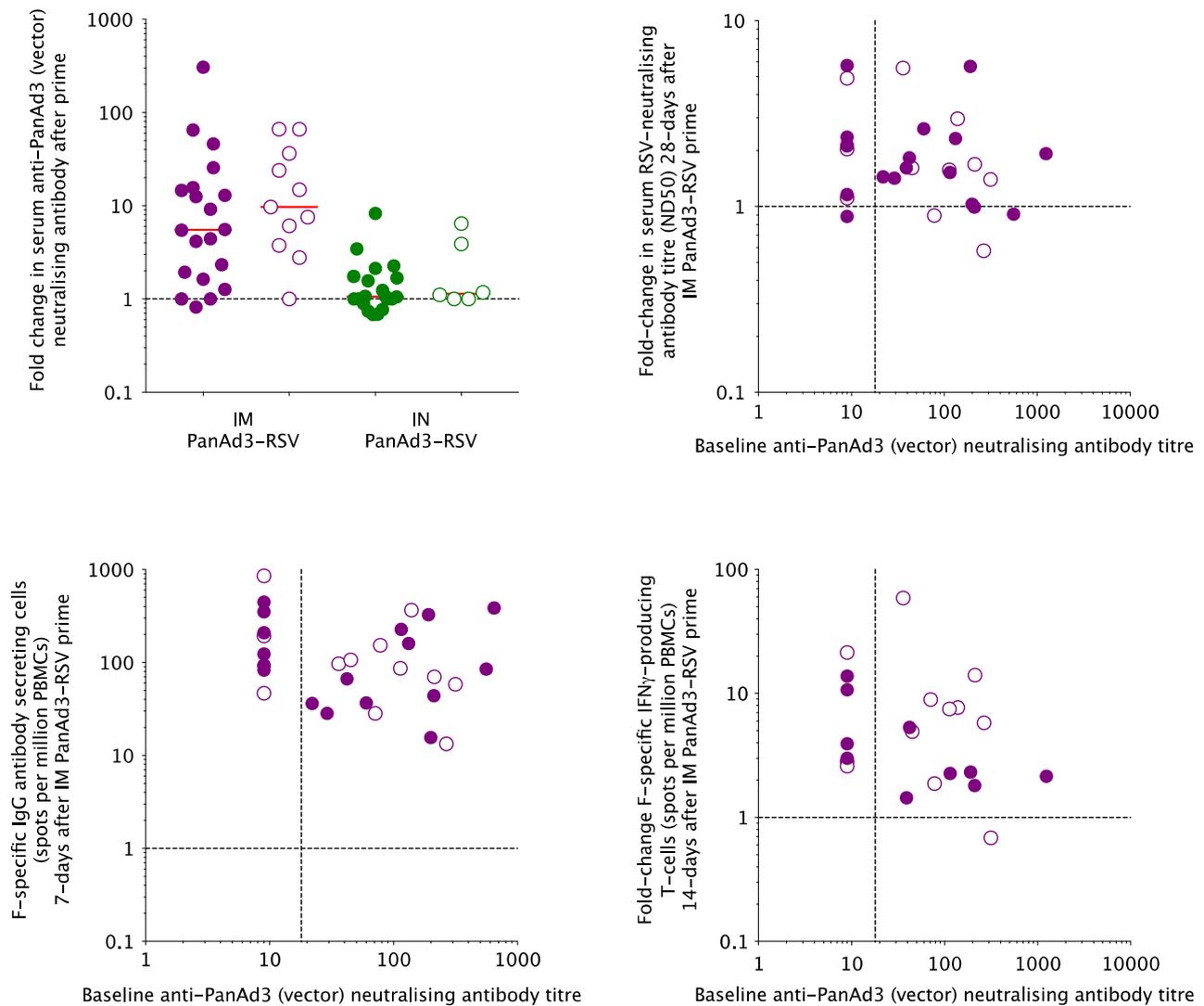
Fig. 6. Serum anti-PanAd3 (vector) neutralising antibody response to IM/IN PanAd3-RSV prime and the effect on immune responses to prime.
Serum PanAd3-vector neutralising antibody titres were measured by SEAP assay before prime and before boost. The vertical dotted line denotes the lower limit of detection 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 IFNγ–producing T-cells 14-days after prime (*bottom right*).

F-specific IgG antibody secreting cell response and the fold-change expansion of IFNγ-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 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, MedImmue).[20–23] High RSV-specific neutralising antibody titres from natural exposure persist into later life but relatively lower titres 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 (IFNγ–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 IFNγ–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 IFNγ-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+ IFNγ 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 IFNγ 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, and 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∆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.

Conflict of Interest

The remaining authors declare they have no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.02.003.

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