TITLE PAGE

Humoral and cellular immunity to RSV in infants, children and adults

CA Green[1], CJ Sande[1], C de Lara[2], AJ Thompson[1], L Silva-Reyes[1], F Napolitano[3], A Pierantoni[3], S Capone[3], A Vitelli[3], P Klenerman[2], AJ Pollard[1].

[1] Oxford Vaccine Group, Department of Paediatrics and the NIHR Oxford Biomedical Research Centre, University of Oxford, Oxford, UK; [2] Experimental Medicine Division, Nuffield Department of Medicine, University of Oxford, Peter Medawar building, Oxford OX1 3SY, UK; [3] ReiThera Srl, Via di Castel Romano 100, 00128 Roma, Italy.

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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

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ABSTRACT

Background. Respiratory syncytial virus (RSV) causes respiratory disease throughout life. Here we report differences in naturally acquired immunity with age and presumed exposure.

Methods. A longitudinal, non-interventional, observational study was performed in healthy adults (20 paediatric healthcare workers and 10 non-healthcare workers), children (10 aged 3-6years) and infants (5 aged 2-4 months and 20 aged 6-12 months). Blood samples were analysed for RSV-neutralising antibody titre, F/Ga/Gb-specific antibody titres, F-specific IgG/IgA memory B-cell frequencies and T-cell production of IFNγ, IL-4, IL-13 and IL-17.

Results. Serum G-specific antibody titres were significantly lower in infants and children than adults. However, serum titres of F-specific and RSV-neutralising antibody and IFNγ-producing T-cell frequencies were low or absent in the infants, but comparable between children and adults. Interestingly, F-specific memory IgA B-cells could not be detected in paediatric samples and in samples from non-healthcare workers, but recordable IgA memory B-cells were found in 9/18 paediatric healthcare workers and 2/8 non-healthcare workers at the end of the RSV season. These responses waned 4-6 months later. By contrast, F-specific IgG memory B-cells were detectable in samples from all adults without significant variation across time points. T-cells producing IL-4, IL-13 and IL-17 responses were not detectable in peripheral blood from a subset of volunteers.

Conclusions. Repeated RSV exposure in early life generates immune responses that are inversely related to frequency of severe disease. Induction of F-specific antibody and cellular immune responses through infant vaccination might help to accelerate the development of protective immune responses at an early age.

Clinicaltrials.gov reference NCT01563692 and NCT01640652.

INTRODUCTION

Human respiratory syncytial virus (RSV) is an enveloped RNA virus that causes respiratory tract infections throughout life. Infection does not confer immunity to reinfection and infants, the elderly and severely immune compromised adults are at particular risk of progression to developing severe lower respiratory tract disease and death. The peak of severe disease is among infants in the first months of life[1-5], when approximately 2-3% of primary infections will require hospital admission[6,7] and where annual epidemics of bronchiolitis accounts for up to 18% of winter paediatric admissions (RSV can be responsible for up to 80% of these)[8-10]. Prematurity, low birth-weight, male sex, broncho-pulmonary dysplasia, congenital heart disease, immunodeficiency, cerebral palsy and Down's syndrome are risk factors for severe RSV-bronchiolitis,[6,11-14] but 50-80% of emergency admissions occur in otherwise healthy infants born at term[12,13]. Worldwide, RSV disease in children under the age of 5-years account for an estimated 33.8 million lower respiratory tract infections, 3.4 million hospitalisations and up to 200,000 deaths annually, and for resource poor areas of the world RSV is second only to malaria in all-cause infant mortality between one and 12-months of age[15,16]. Healthy adults experience a 9-11% annual risk of mild upper respiratory tract infection but senescence of the immune system and comorbid conditions re-establish a risk of severe disease and death in the elderly, and although estimates of the hospital burden and mortality from RSV in the elderly vary it may be comparable to seasonal influenza[17-24].

There is no licenced vaccine for RSV. An incomplete understanding of the complex and different facets of immunity needed for protection in the different populations at risk of severe disease have been formidable obstacles for vaccine development. Here we report our observations on RSV immunity following different degrees of natural exposure. We included healthy infants without presumed exposure, infants after one season of exposure, children after 3-6 seasons of exposure and adult paediatric healthcare workers and non-healthcare workers at the end of the RSV transmission season and again 4-6 months later.

MATERIALS & METHODS

A non-interventional longitudinal observational study was performed in 2012 in healthy adults using blood draws of 50mls timed according to Public Health England epidemiological surveillance data for the end of the 2011/12 RSV season (visit 1, or V1) and again 4-6 months later (V2). Between 2013 and 2014 healthy infants/children attended a single visit, timed for before or after the 2013/14 RSV season, when 5-6mls of blood were obtained.

Study populations.

Adults. Volunteers were self-selected males and females aged 18-60 years working as clinical paediatric healthcare workers and members of the public recruited through advertisement. 20 nurses and doctors caring for RSV infected infants on acute medical paediatric wards and the paediatric intensive care unit at the Children's Hospital, Oxford (Oxford University Hospitals NHS Trust) were recruited as a cohort of presumed healthy RSV-exposed paediatric healthcare workers. An additional 10 presumed healthy individuals were recruited as a 'non-healthcare' group with presumed reduced RSV-exposure for comparative analysis. Volunteers were excluded if they were aware of any history of immune disorder or immunosuppressive medication that could influence the acquisition of RSV responses. Additional exclusion criteria applied to non-healthcare workers included working on any hospital ward or close contact with populations at higher risk of RSV transmission, such as nursery workers, care home workers or those who were parenting children under 5-years of age.

Infants and children. Parents of presumed healthy infants aged from 2-months to children aged 6-years, who were attending the Children's Hospital, Oxford, and whose child required a blood sample or peripheral cannula for another clinical indication were approached to take part in the study. The study groups consisted of 5 infants aged 2-4 months, 20 infants aged 6-12 months and 10 children aged 3-6 years. Potential volunteers were excluded if they had any known/suspected impairment of immune function, concurrent acute/chronic infection, were born <36 weeks' gestation or had any history of palivizumab use.

Laboratory analyses.

Sample processing. Blood samples were collected in preservative free heparin tubes (400µL heparin per 50mLs blood) for adult samples, and EDTA tubes (BD) for paediatric samples. PBMCs were isolated within 6hrs of sample collection from a 1:1 mix of heparinised/EDTA-treated blood with R0 (RPMI with Penicillin/Streptomycin and L-Glutamine, stored 4°C) by density centrifugation through Lymphoprep™ (Alere, UK). PBMCs were cryopreserved (45% foetal calf serum, 45% RPMI and 10% DMSO). Sera were obtained by centrifugation of whole blood collected in clotted tubes (adult samples) or from the plasma fraction of blood before PBMC isolation (paediatric samples), and then cryopreserved.

Serum RSV-neutralising antibody quantitation. Functional serum antibody immunity was measured using a plaque-reduction neutralisation assay (PRNA), as described in more detail elsewhere[25]. A mixture of 50 plaque-forming units of RSV strain A2 were mixed with dilutions of heat-inactivated sera (1:20 to 1:10240) and then incubated for 60-

minutes (37°C, 5% CO_2, 95% humidity) before adding to a confluent layer of HEp-2 cells ($3x10^4$ cells per well). The plates were then incubated for 60-hours before being fixed using cold acetone (80%/20% v/v). RSV plaques were detected by immuno-staining using amino-ethyl-carbazole, with the serum neutralising titre defined as the dilution at which 50% of plaques survive calculated using the Spearman-Karber method[25].

Serum anti-F and anti-G IgG antibody quantitation by enzyme linked immunosorbent assay (ELISA). Nunc 96-well plates (Nunclon) were coated with 0.5µg/100µL recombinant F and G protein (SinoBiologicals) in 0.05M $NaHCO_3$ buffer. Bound IgG was revealed by anti-human IgG−alkaline phosphatase antibody (Sigma A 9544). Data were expressed as the highest dilution giving an OD_{405} reading greater than the mean+3StDev background wells (no serum).

Anti-F IgG and IgA memory B-cell frequency quantitation by cultured dual-colour Enzyme-Linked Immunosorbent Spot (ELISpot). PBMCs were thawed and cultured for 6-days with CpG (BioScience UK), Pokeweed Mitogen (Sigma) and *Staphylococcus aureus* Cowans Strain (VWR International) in R10 media (RPMI, 10% Foetal Bovine Serum, 2mM L-Glutamine, 50µg/ml Streptomycin, 50U Penicillin) at a concentration of $2x10^6$ cells/mL, and then used in a dual-colour ELISpot assay as described elsewhere[25,26]. In summary, $Multiscreen_{HTS}$ HA plates (Millipore, MSHAN4510) were coated with 5µg/mL F protein antigen (Sino Biological Inc), 10µg/mL Human Serum Albumin (HSA, Sigma), 5µg/mL tetanus toxoid protein (Statens Seruminstitute) and 10µg/mL polyvalent goat anti-human immunoglobulins (Caltag). Plates were blocked (45-minutes with 1% milk) and then cells were incubated overnight (37°C, 5% CO_2, 95% humidity). After washing, the plates were developed using anti-human IgG-FITC (Sigma) and anti-human IgA-Biotin (AbD Serotec), followed by anti-FITC AP (Sigma) and Streptavidin-HRP (AbD Serotec), and finally 3-Amino-9-ethylcarbazole (AEC) substrate kit (Sigma) and Vector Blue substrate (Vector Laboratories). After drying overnight plates were read using Autoimmun Diagnostika (AID version 5.0) and responses measured as the antigen-specific spots per million PBMCs with HSA background subtracted. A positive response was defined as any detection of spots above HSA background.

T-cell responses by Enzyme-Linked Immunospot (ELISpot). The CD4+/CD8+ IFNγ and Th2 associated cytokine producing T-cell frequencies in peripheral circulation was measured using an ELISpot assay as described elsewhere[25]. In summary, plates were coated with mouse anti-human IFNγ and blocked before the addition of peptide pools dissolved in DMSO and covering the sequence of RSV proteins F, N and M2-1 (JPT Peptide Technologies). The peptides were 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) and used in the assay at a final concentration of 3µg/mL of each peptide. DMSO (Sigma) was used as a negative control and CMV cell lysate, FEC (mixed HLA class–I restricted peptides from Flu, EBV and CMV) and ConA (Sigma) acted as positive controls. PBMCs were added to peptide wells (200'000 cells/well) in triplicate and incubated overnight (37°C, 5%CO_2, 95% humidity). Detection was with anti-human IFNγ, biotin conjugate and anti-Biotin AP conjugate with 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride substrate (Pierce). IFNγ producing cells were enumerated using AID software version 5.0, and the mean+3StDev of the DMSO response from all samples identified a cut off whereby individual samples with background DMSO values ≥35 spot forming cells per million PBMCs were excluded from analysis. Samples were also excluded from analysis if no spots were detected in any positive control well. Calculation of triplicate well variance was applied as described elsewhere and a threshold of 10 applied for exclusion from analysis[27].

Statistical analyses.

No sample size calculation was performed and the analyses were intended to be descriptive in nature. Comparative statistics represent post-hoc analyses and, together with graphs, were performed using Prism 7 (GraphPad Inc, USA) with a p value ≤ 0.05 considered statistically significant. All statistical tests were non-parametric tests due to the small sample sizes.

RESULTS

34 adult volunteers were assessed for eligibility, 30 were enrolled and all completed the study. For the paediatric study a total of 66 infants and children were assessed for eligibility and 35 were enrolled (Table 1, sFig. 1 and sFig. 2).

Humoral immunity is largely F-specific antibody that develops after several seasons of exposure and is then robustly maintained in adults. (Fig. 1, sTab. 1 and sTab. 2)

Maternal antibody was detectable from infants aged 2-4 months. By 6-12 months of age, following a single season of RSV exposure, only 6/20 (30%) of infants recorded any titre of serum RSV neutralising antibody, two of which were only marginally above the detection threshold (Fig. 1A). At this time a significant proportion of infants had no maternal or infant serum RSV-neutralising antibody capacity and the ELISA results reflected low measures of antibody to the F and G surface expressed proteins (Fig. 1B-D). The cohort of children aged 3-6 years showed that 3-6 seasons of exposure was associated with titres of serum RSV-neutralising antibody comparable to those seen in adulthood, and there was a positive correlation between these measures of immunity (sFig. 3 and sFig. 4). The development of anti-Ga and anti-Gb IgG antibody appeared to lag behind and required additional exposure later in childhood. From our adult populations there were no differences in any antibody measures between the two groups of presumed differences in exposure at the end of the RSV season and 4-6 months after last presumed exposure. The highest titres of G-specific antibody were from healthcare workers. A positive correlation between F- and G-specific IgG antibody titres was observed in adults but not infants and children (sFig. 3).

F-specific IgA memory B-cells are not detected in infants and children, and poorly induced and maintained in peripheral circulation following recent exposure in adults. (Fig. 2 and sTab. 3)

Beginning with infants before the first season of exposure we found that F-specific IgG memory B-cells were not detectable and by age 6-12 months only 3/6 (50%) infants had developed measurable responses following a single seasonal exposure. After a further 3-6 seasonal exposures all 4/4 children recorded F-specific IgG memory B-cells in circulation with 3/4 with comparable frequencies we observed in adults. In adults, the frequencies of F-specific IgG memory B-cells appeared no different between the two groups of presumed difference in recent RSV exposure at V1 or when measured again 4-6 months later (Fig. 2A). The responses from F-specific IgA memory B-cells were distinctly different from the IgG population (Fig. 2B). Here we failed to detect IgA memory B-cells in circulation from any paediatric samples. This included children aged 3-6 years who had, by now, developed F-specific antibody and IgG memory cells in response to a few seasons of exposure. In adult paediatric healthcare workers and non-healthcare workers we found responses in only 9/18 (50%) and 2/8 (25%) volunteers respectively at the end of the RSV season. From individuals with measurable responses it was clear that the magnitude of this F-specific IgA cell population was much lower than that observed for F-specific IgG memory B-cells. Without further antigenic stimulus the population of F-specific IgA memory cells then disappeared from circulation 4-6 months later in all adult volunteers except 5/20 paediatric healthcare workers. From the total IgA memory B-cell population (all specificities) we were able to detect IgA memory B-cells in the circulation of most (4/6) infants aged 6-12 months and all (4/4) children aged 3-6 years, albeit at a lower frequency than the total

IgG memory B-cell pool (Fig. 2C and Fig. 2D). In adults at the end of winter the size of the total IgA memory B-cell population was broadly comparable to the IgG memory B-cell population in both paediatric healthcare workers and non-healthcare workers. However, measured again 4-6 months later, and consistent with observations from the F-specific IgA memory B-cell population, there was notable contraction in total IgA memory B-cells in both adult study groups with several volunteers failing to record any total IgA memory B-cells in circulation in the summer months.

IFNγ–producing T-cells appear in circulation after only a few seasons of RSV exposure. (Fig. 3 and sTab. 4)

In the next analysis we sought to characterise the population of IFNγ-producing T-cells, CD4+ and CD8+ without distinction, from each of the study groups to infer the effect of different degrees of RSV exposure on circulating T-cell immunity. Minimal responses were detected from infants aged 2-4 months without RSV exposure. Following just one season of exposure, from infants aged 6-12 months, 7/17 (41%) recorded RSV-specific IFNγ-producing T-cells, although these responses were of a much lower magnitude to the children aged 3-6 years who had had additional exposure. In all adult samples the RSV-specific IFNγ-producing T-cells frequencies were comparable at the end of the RSV season and were maintained 4-6 months later (Fig. 3A). Furthermore, the relative proportions that contributed to the overall RSV-specific IFNγ-producing T-cell response from each peptide pool appeared to be balanced (Fig. 3B-E). Finally, a subset of PBMCs from 10 healthcare workers collected at the end of the RSV season and 29 paediatric samples were stimulated for the detection of Th2-associated cytokines. No significant responses were detected for IL-4, IL-13 and IL-17 (sFig. 5).

DISCUSSION

In this study we found that titres of serum RSV-neutralising antibody in infants were lowest after the waning of maternal antibody, the nadir coinciding with the peak age of RSV-related hospitalisations for severe disease at less than one year[13,28]. The risk of hospitalisation can be significantly reduced with the use of F-specific monoclonal antibody (palivizumab, MedImmune), demonstrating that serum F-specific antibody alone can protect the lower respiratory tract from the development of severe disease[29]. Corroborative evidence has come from observations of a negative association between titres of serum RSV-neutralising antibody and the risk of re-infection in infants and children, and that following primary infection the serum neutralising antibody response was difficult to maintain beyond 3-months with only half of infants with detectable antibody responses by 11-months of age[4,30,31]. The Ga/Gb subtype-specific antibody titres remained relatively low following repeated seasonal exposure and perhaps these differences resulted from the F-protein conservation across RSV subtypes circulating in different years. The low titres of F-specific antibody in early life therefore represent a specific predisposition to developing severe disease and this could be potentially addressed through maternal vaccination. A direct infant vaccination strategy would face considerable obstacles in respect of there being only a brief window to use a single-dose of vaccine before the peak of severe disease, and mandating its use in the presence of maternal antibody where high titres of maternal antibody could impair the development of infant immunity[32,33]. However early infant immunisation might contribute towards desirable antibody protection to overcome the waning of passive immunity from maternal vaccination or from the use of existing or novel extended half-life monoclonal antibody prophylaxis[34] that have now reached advanced stages of clinical evaluation. Although it is clear that F-specific antibody can protect from severe lower respiratory tract disease, it is also clear that despite the attainment of high levels of functional antibody many healthy adults will continue to suffer re-infections throughout life and the elderly suffer a significant burden of severe disease death each year. This would indicate that a more complex explanation of immunity to infection and severe disease is needed.

Our main finding from the analysis of B-cell immunity was that F-specific IgA memory B-cells were not detected in the circulation of infants and children and were found in the circulation of only some adults at the end of the RSV transmission period and were then absent from circulation without further antigenic stimulus in later months. The biological significance of these observations and its relevance to putative roles in protection from re-infection or disease severity are unknown. Under controlled experimental RSV-challenge conditions of healthy adults there was also a noticeable absence of IgA memory B-cells in peripheral blood before and after challenge[35]. Data from separate studies of natural infection, experimental challenge and vaccination have consistently demonstrated an induction of F-specific IgA B-cells in the form of IgA antibody secreting cells, which appeared transiently in peripheral circulation early after stimulation as they were presumably trafficked towards the bone marrow and mucosa[25,37]. The generation of these early IgA B-cell responses does not, as we observed with RSV-specific IgG memory B-cells, result in the persistence of memory cells in peripheral circulation. An interesting possibility concerns the role IgA plays in protection from RSV infection at the mucosa. At this pivotal site of initial contact between virus and the host immunity, the quantity of RSV-specific nasal IgA antibody differentiates the risk of infection on a background of well-developed systemic IgG humoral and cellular immunity[35-37]. Therefore, a better understanding of the complex biology of antigen-specific IgA immune responses in

blood and at the mucosa could help to identify where RSV vaccines candidates could be used to exploit the full repertoire of host protective mechanisms.

Whereas antibody is required to prevent infection and neutralise free virus, there remains a critical role for T-cells in the clearance of infected host cells. MHC class I restricted RSV-specific CD8+ effector T-cell epitopes have been identified on the F protein and internal proteins such as N, M, M2 and NS2[38-42]. From our data, development of RSV-specific IFNγ- T-cell immunity paralleled the kinetics of F-specific antibody with an expansion of IFNγ-producing T-cells to levels comparable with adults after a few seasonal exposures. The relatively low frequencies of RSV-specific IFNγ-producing T-cells in a proportion of infants aged 6-12 months, the rest of whom showed no responses, supported the observations made by others that infant T-cell immunity is variable in early life and when up to 80% of infants develop RSV-specific CD8+ T-cell responses after the first year of exposure[43,44]. The reason for this variation remains unknown. The RSV proteins NS1 and NS2, located at the 3' promotor end of the genome and activated on entry into the host cell, can modulate over 200 host proteins with the purpose of suppressing interferon resistance mechanisms and dendritic cell maturation[45-47]. For infants, where we observed an absence/attenuation of IFNγ-producing T-cells and, in addition to an impairment of direct T-cell mediated viral clearance there may have been further consequences for other pivotal protective roles, such as CD4+ T-follicular helper cells supporting memory B-cells, antibody production and the maintenance of immune responses. An infant RSV vaccine that used a combination of RSV-specific T-cell and antibody epitopes might help to accelerate the development of desirable immunity in early life.

General limitations

The principal limitations were the small group sizes and the lack of information on which volunteers had been infected during the transmission season and when. While it was reasonable to assume that paediatric healthcare workers have repeatedly come into close contact with sick infants shedding virus, we do not know if, at the biological level, this is considerably different from the boosting from RSV exposure in the general population such as in the controls used in this study. Furthermore, given the point of contact with RSV is the respiratory mucosa then responses measured from blood cannot be representative of total immunity to RSV and nasal sampling was not performed.

Conclusions

We found that exposure to RSV through childhood generates antibody and cellular immunity to RSV. Among immune adults, without further antigenic stimulus, there appeared to be a loss of RSV-specific IgA memory B-cells in peripheral circulation in the summer months. An infant RSV vaccine that successfully accelerated humoral and cellular immune responses towards the immunity seen following multiple natural exposures could confer protection in the lower-respiratory tract from severe disease.

TABLES

	Adult study groups		Paediatric study groups		
	Paediatric healthcare workers	Non-healthcare workers	Infants aged 2-4 months	Infants aged 6-12 months	Children aged 3-6 years
Group size (N)	20	10	5	20	10
Median age (range)	27.9 years (20.6-52.9)	34.7 years (24.8-58.4)	3 months (2.5-4)	8.3 months (6.2-12)	4.8 years (3.8-5.6)
N male (%)	2 (10)	4 (40)	n/a	n/a	n/a
V1 window, days	35	21	n/a	n/a	n/a
V2 window, days	45	36	n/a	n/a	n/a
V1/V2 interval, days (range)	124 (112-174)	144 (125-147)	n/a	n/a	n/a

Table 1. Baseline characteristics of study volunteers and blood sampling periods.

V1 and V2 are Visits 1 and 2 timed for the end of the 2011/12 RSV season and 4-6 months later respectively (adult healthcare workers study only). For the adult study the first blood sample, V1, was within a 35-day period between March and April 2012 and the second sample, V2, within a 45-day period between July and August 2012. For the infants and children study all samples were obtained either between July and the first week of December 2013, or between February and May 2014 to avoid the 2013/14 RSV season. n/a, not applicable.

FIGURES

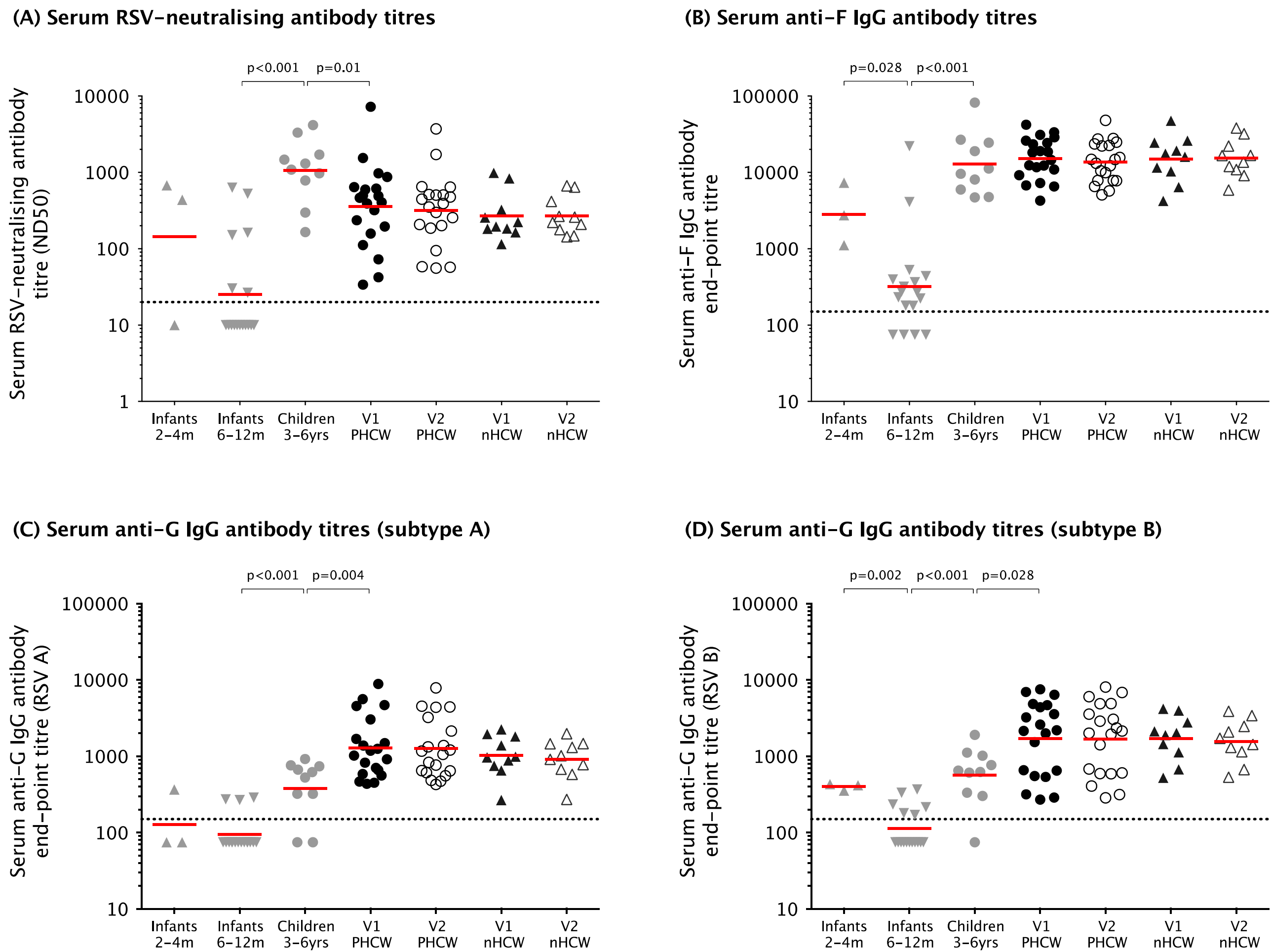


Fig. 1. The serum antibody response to natural RSV exposure.

*Samples from paediatric and adult study groups were assayed in different years. Red bars denote the geometric mean and dotted line the lower limit of detection. Comparative tests were non-parametric paired/unpaired tests (Mann-Whitney or Wilcoxon paired tests) as appropriate. (**A**) Titres of serum RSV-neutralising antibody measured by plaque-reduction neutralising antibody assay (PRNA); adult and paediatric samples were assayed in different year. End-point titres for serum IgG (**B**) F-specific, (**C**) G subtype A-specific, (**D**) G subtype B-specific antibody measured by ELISA. V1 denotes the sampling period at the end of the RSV season, and V2 the sampling period 4-6 months later. PHCW, adult paediatric healthcare workers. nHCW, adult non-healthcare workers. 3/5 samples were available for analysis from infants aged 2-4 months, 17/20 samples were available for analysis from infants aged 6-12 months and 10/10 samples from children aged 3-6 years were available for analysis. 60/60 adult samples were analysed. Missing data arose from insufficient blood volume. Paired V1/V2 results are shown in sFig. 6.*

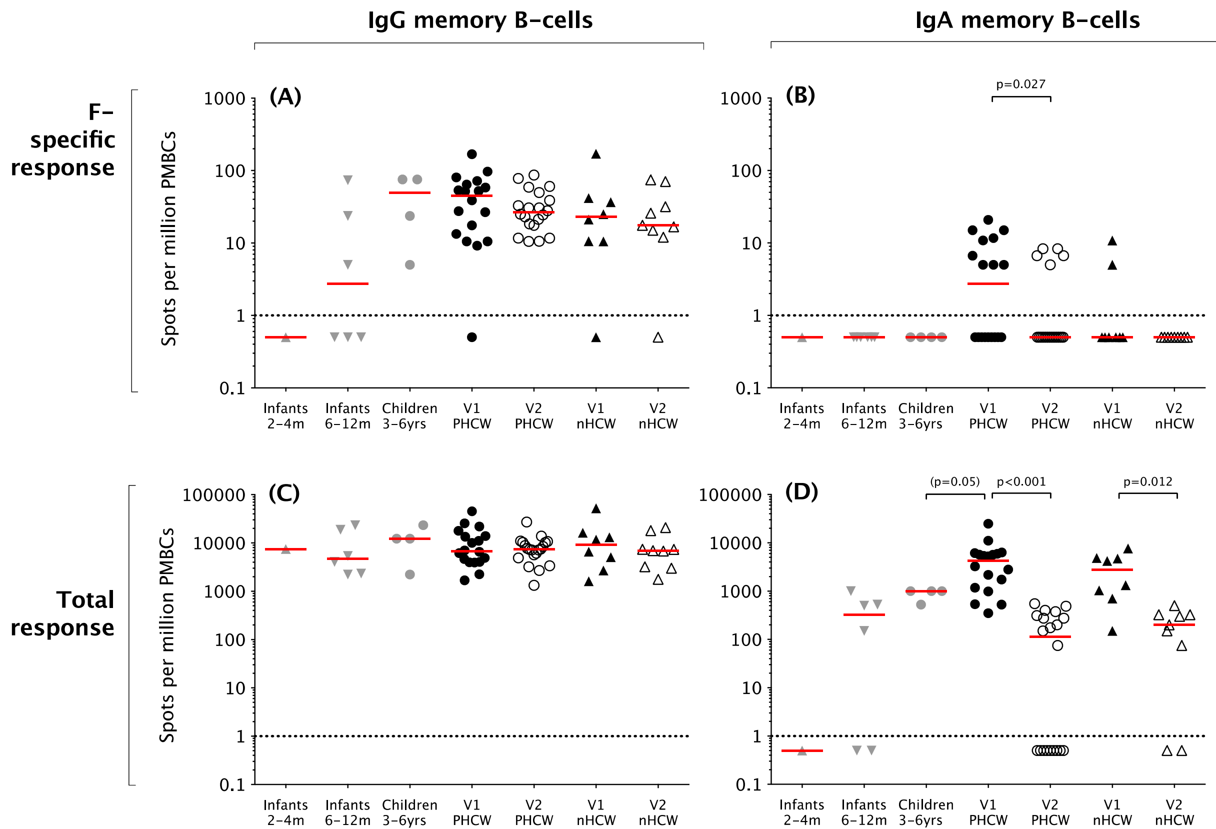


Fig. 2. The RSV F-specific and total IgG and IgA memory B-cell responses to natural exposure.

*Dual-colour memory B-cells measured by ELISpot. The red lines denote the median value and the lower limit of detection for the assay is marked by the horizontal dotted line (1 spot per million PBMCs). Non-parametric Mann-Whitney or Wilcoxon paired tests, as appropriate, generated the annotated p-values. The F-specific IgG (**A**) and IgA (**B**) memory B-cell responses, on a background total IgG (**C**) and IgA (**D**) cell population frequencies. V1 denotes the sampling period at the end of the RSV season, and V2 the sampling period 4-6 months later. PHCW, adult paediatric healthcare workers. nHCW, adult non-healthcare workers. For the F-specific memory B-cell ELISpot assay, only 1/5 samples were available from infants aged 2-4 months, 6/20 samples from infants aged 6-12 months, 4/10 samples from children aged 3-6 years and 55/60 adult samples. Missing data arose from insufficient blood volume. Paired V1/V2 results are shown in sFig. 6.*

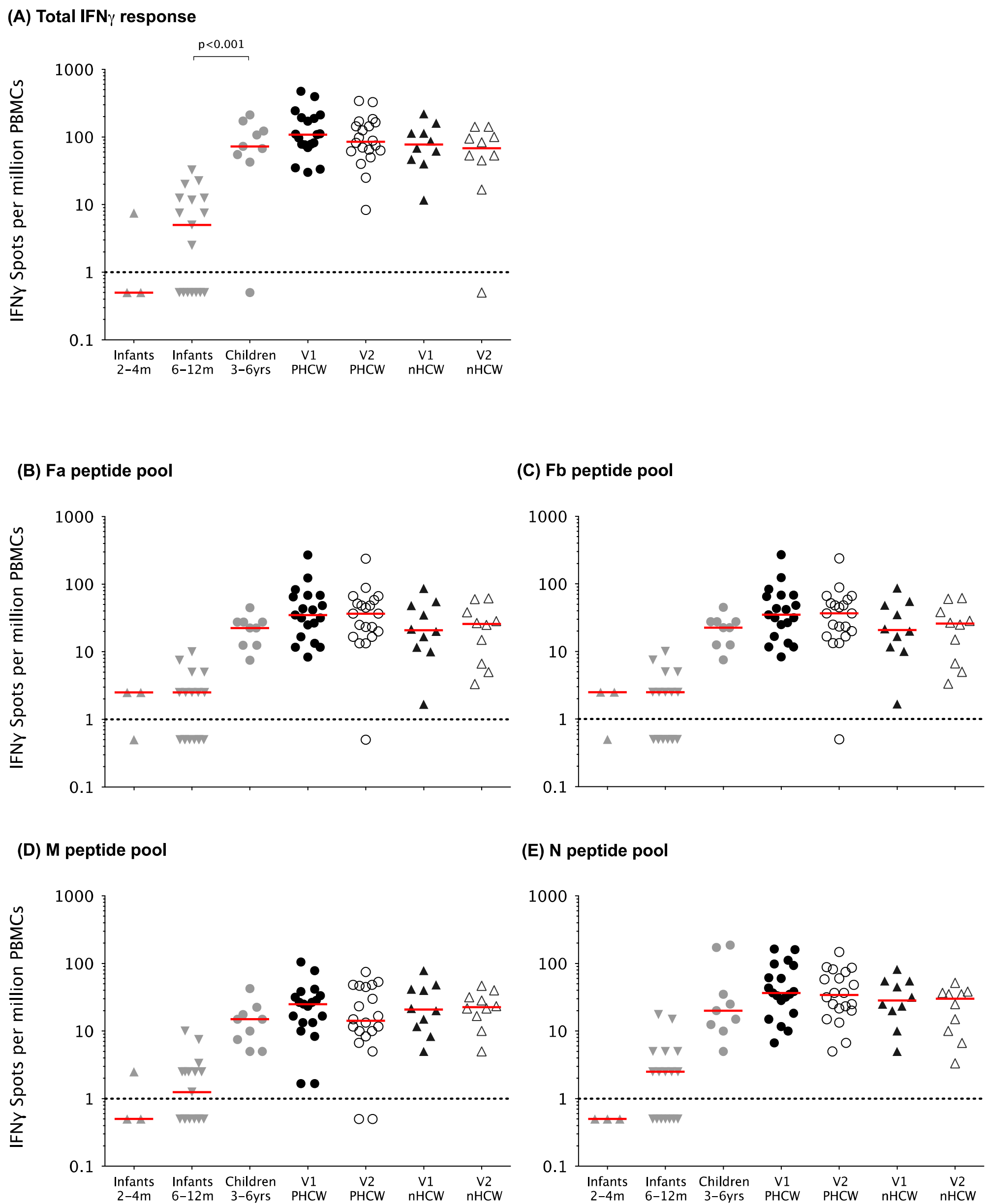
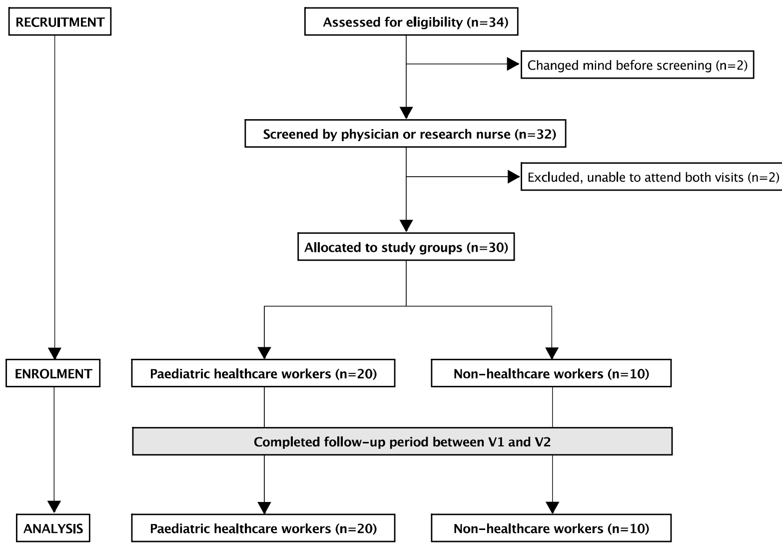


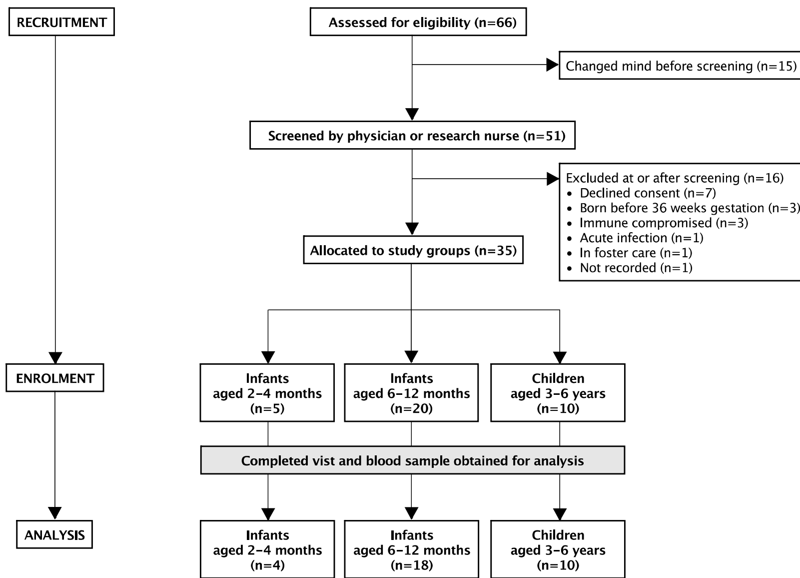
Fig. 3. RSV-specific IFNγ-producing (CD4+/CD8+) T-cells frequencies in peripheral circulation derived from natural exposure.

*Samples from paediatric and adult study groups were assayed in different years. Total and peptide-pool specific T-cell IFNγ response to natural RSV exposure measured by ELISpot. The red lines denote the median response. PHCW, adult paediatric healthcare workers. nHCW, adult non-healthcare workers. Non-parametric Mann-Whitney or Wilcoxon paired tests, as appropriate, generated the annotated p-values. (**A**) Total IFNγ response to natural exposure, measured as the sum of peptide pool responses – 4xDMSO background. (**B-E**) The peptide pool specific IFNγ response to natural exposure. For the total T-cell IFNγ response analysis, 3/5 samples were available from infants aged 2-4 months, 17/20 samples from infants aged 6-12 months, 9/10 samples from children aged 3-6 years and 59/60 adult samples. Missing data arose from insufficient blood volume. Paired V1/V2 results are shown in sFig. 6.*

SUPPLEMENTARY FIGURES



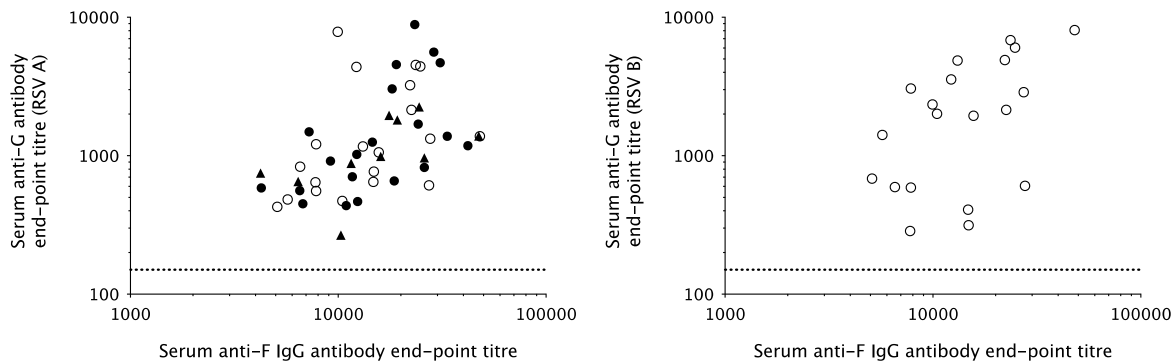
sFig. 1. CONSORT map for the recruitment, retention and sample analysis for adult study groups.



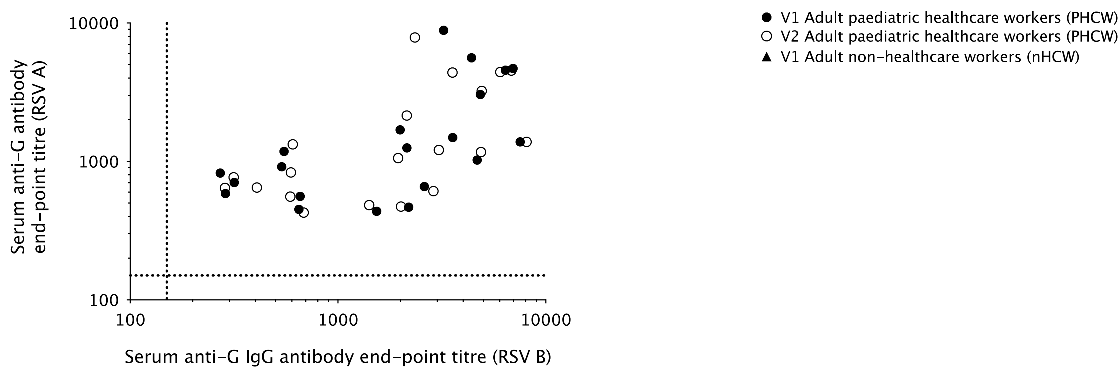
sFig. 2 CONSORT map for the recruitment, retention and sample analysis for paediatric study groups.

A total of 3 infants were unable to provide a blood sample after enrolment.

Correlations between F-specific and G-specfic serum IgG titres

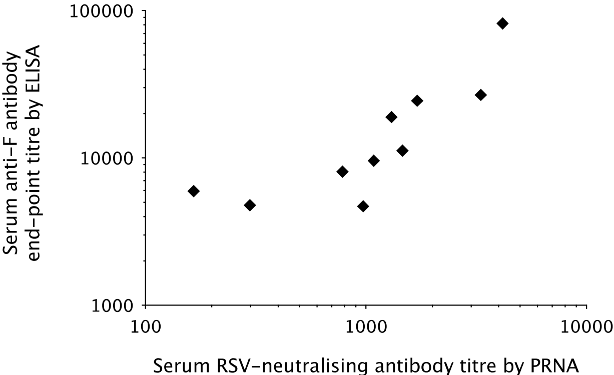


Correlations within subtype A and subtype B G-specific serum IgG titres



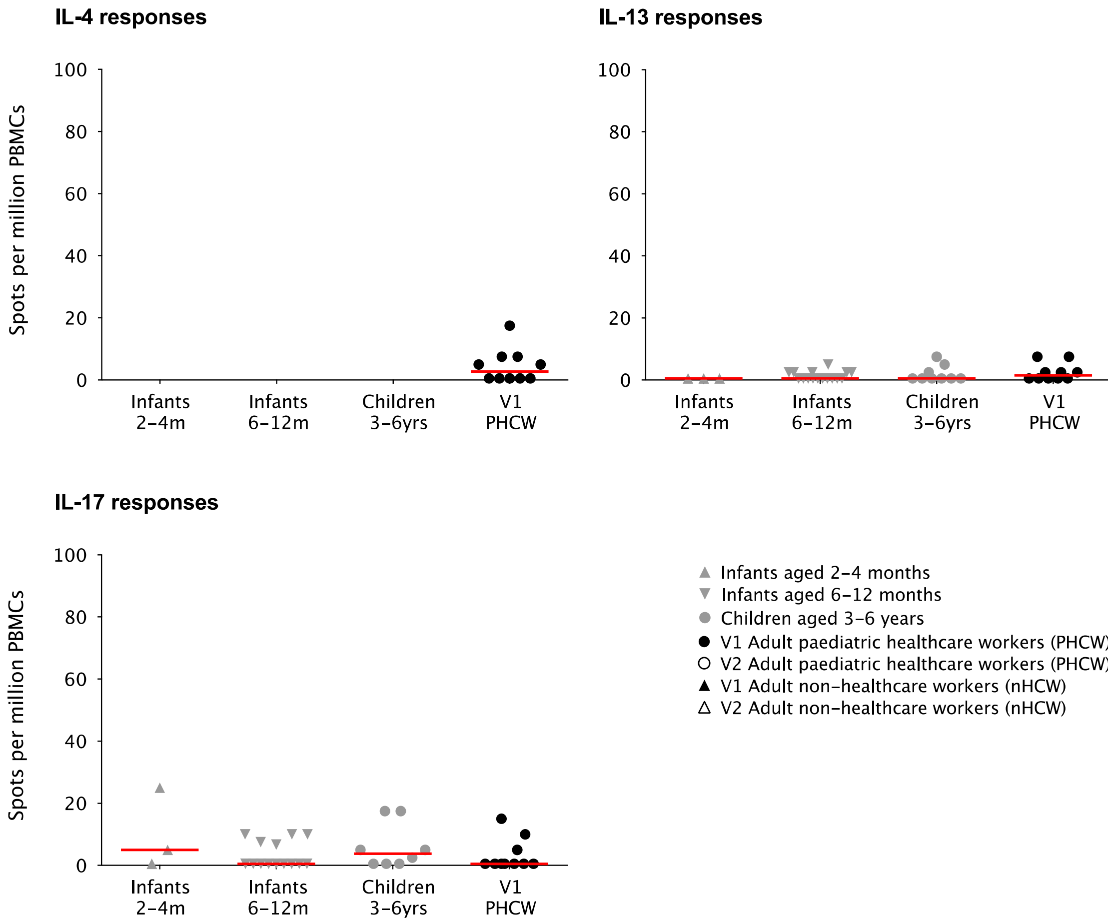
sFig. 3. Correlations between F- and G-specific serum IgG antibody titres.

*End-point titres for serum F-specific and G-specific IgG antibody measured by ELISA. Correlation analyses used a two-tailed Spearman's test. The lower limit of detection of each assay is marked by the dotted lines. (**Top left**) Statistical significance was observed with F- and G (subtype A)-specific titres from paediatric healthcare workers at the end of the RSV season (V1, p=0.004, r=0.62) and 4-6 months later (V2, p=0.020, r=0.52), and non-healthcare workers at the end of the RSV season (V1, p=0.027, r=0.71). (**Top right**) Statistical significance was observed with anti-F and anti-G (subtype B) antibody titres from paediatric healthcare workers from blood obtained in the summer months (V2, p=0.024, r=0.50). (**Bottom left**). Between subtypes A and B of the anti-G IgG titres, statistical significance was observed with paediatric healthcare workers at the end of the RSV season (V1, p=0.003, r=0.63) and 4-6 months later (V2, p=0.003, r=0.64). Non-healthcare workers recorded a borderline significant association (p=0.05, r=0.64, data not shown).*



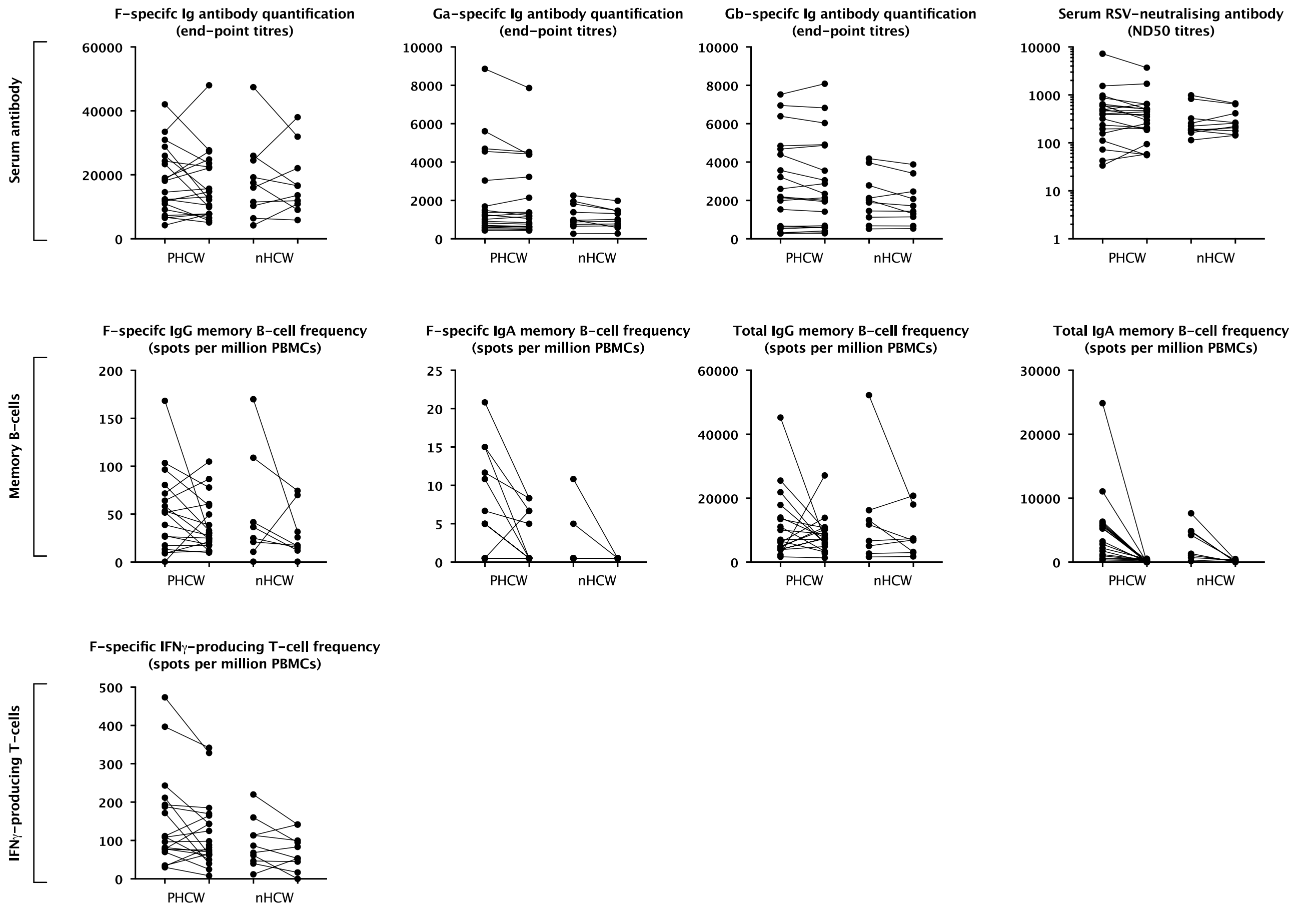
sFig. 4.Correlations between serum F-specific IgG antibody titres and serum RSV-neutralising antibody titres.

Data from children aged 3-6 years only. A statistically significant correlation was observed (p=0.001, r=0.90, two-tailed Spearman's test).



sFig. 5. IL-4, IL-13 and IL-17 responses to natural RSV exposure.

Peripheral T-cell responses measured by ELISpot assay. The red lines denote the median. The dotted line represents the lower limit of detection for the assay (1 spot per million PBMCs; values below this were reverted to 0.5) The summed IL-4, IL-13 and IL-17 responses are the summed response to the separate RSV peptide pools Fa, Fb, M and N with 4xDMSO background subtraction.



sFig. 6. Paired individual V1/V2 results from adult paediatric healthcare workers and non-healthcare workers.

*Each dot in each figure represents one individual response linked between the V1 sample taken at the end of the RSV season and the V2 sample taken 4-6 months later (left and right linked dots respectively). (**Top row**) Serum antibody responses, (**middle row**), memory B-cell frequencies in peripheral circulation and (**bottom row**) the total F-specific IFNγ-producing T-cell frequencies in peripheral circulation. PHCW, adult paediatric healthcare workers. nHCW, adult non-healthcare workers.*

SUPPLEMENTARY TABLES

	Geometric mean antibody titre (with 95% CI)		
	Anti-F	Anti-G (subtype A)	Anti-G (subtype B)
Infants aged 2-4 months	2814 (-)	127 (-)	400 (-)
Infants aged 6-12 months	319 (151-677)	94 (73-123)	113 (83-154)
Children aged 3-6 years	12798 (6679-24526)	382 (197-740)	561 (297-1063)
Paediatric healthcare workers (V1)	15181 (11325-20349)	1296 (845-1986)	1708 (1009-2891)
Paediatric healthcare workers (V2)	13716 (10297-18271)	1268 (838-1919)	1692 (1025-2792)
Non-healthcare workers (V1)	14866 (8968-24644)	1020 (649-1603)	1711 (1044-2806)
Non-healthcare workers (V2)	15291 (10187-22952)	917 (608-1384)	1566 (986-2486)

sTab. 1. Geometric mean titres (GMTs) of serum RSV F- and G-protein specific IgG antibody.

	Geometric mean antibody titre (with 95% CI)
Infants aged 2-4 months	144 (-)
Infants aged 6-12 months	25 (12-54)
Children aged 3-6 years	1063 (526-2148)
Paediatric healthcare workers (V1)	356 (64-1525)
Paediatric healthcare workers (V2)	317 (180-953)
Non-healthcare workers (V1)	268 (129-563)
Non-healthcare workers (V2)	271 (175-454)

sTab. 2. Geometric mean titres (GMTs) of serum RSV-neutralising antibody.

	Geometric mean spots per million PBMCs (95% CI)			
	F-specific IgG	Total IgG	F-specific IgA	Total IgA
Infants aged 2-4 months	0.5 (-)	7500 (-)	0.5 (-)	0.5 (-)
Infants aged 6-12 months	3.2 (-13 – 48)	6063 (-377 – 19119)	0.5 (0.5 – 0.5)	46.3 (-46 – 772)
Children aged 3-6 years	28.6 (-13 – 102)	9399 (-1224-26274)	0.5 (0.5 – 0.5)	851.2 (503 – 1259)
Paediatric healthcare workers (V1)	28.7 (27 – 68)	7910 (5939 – 16751)	2.2 (2 – 9)	2912 (2148 – 7833)
Paediatric healthcare workers (V2)	27.4 (23 – 44)	6834 (5680-10779)	0.9 (0.7 – 4)	15.6 (77 – 251)
Non-healthcare workers (V1)	17.2 (-6 – 85)	8123 (-69 – 27398)	0.9 (-0.7 – 6)	1794 (862 – 5305)
Non-healthcare workers (V2)	16.9 (9 – 49)	6329 (3257-13466)	0.5 (0.5 – 0.5)	59.2 (79 – 338)

sTab. 3. Geometric mean memory B-cell responses to RSV F-protein and total responses.

	Geometric mean spots per million PBMCs (95% CI)
Infants aged 2-4 months	1.2 (-)
Infants aged 6-12 months	3.0 (1.3 – 7.1)
Children aged 3-6 years	52.0 (12.8 – 210.4)
Paediatric healthcare workers (V1)	111.0 (76.5 – 161.1)
Paediatric healthcare workers (V2)	86.8 (58.0 – 129.8)
Non-healthcare workers (V1)	71.7 (39.6 – 129.9)
Non-healthcare workers (V2)	42.0 (12.6 – 139.7)

sTab. 4. Geometric mean total IFNγ responses to RSV peptide pools.

NOTES

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Ethical approvals and study registrations.

Ethical approval and amendments for the paediatric healthcare workers study and the infants and children study were granted by NRES Berkshire (reference 12/SC/0023) and NRES Central Bristol (reference 13/SW/0130), and each study was registered with clinicaltrials.gov (reference NCT 01563692 and 01640652 respectively). Written informed consent was obtained from all volunteers prior to study procedures. Study procedures were conducted at the Children's Hospital, Oxford University Hospitals NHS Trust, and at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), Oxford. Written informed consent was obtained from all volunteers, parents or legal guardians before study any procedures were performed. Procedures were performed in accordance with ICH Good Clinical Practice (GCP) and local standard operating procedures (SOPs) and were monitored by the Clinical Trials Research Governance (CTRG) office, University of Oxford.

Author contributions.

CAG was the lead physician for both studies, analysed the data and wrote the manuscript. All authors have reviewed and had input into the manuscript prior to submission. AJP, CAG, PK, AV and SC instigated and designed both studies. Sample processing was performed by CAG, CdeL and AT. ELISA assays were performed by AP and FN. PRNA assays were performed by AP, CS and CdeL. T-cell assays were performed by CdeL and CAG, and B memory ELISpots by AT and CAG.

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.

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