Title: Impaired antibody-mediated protection and defective IgA B cell memory in experimental infection of adults with respiratory syncytial virus

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At a glance commentary:

Scientific knowledge on the subject

A major challenge in RSV research is to understand why infection recurs throughout life, despite a high degree of viral genetic stability. Passive antibody thereapy protects against hospitalization of high-risk infants, but new and better correlates of protection are needed that more accurately predict candidate vaccine efficacy.

What this study adds to the field

We find that virus-specific nasal IgA correlates with protection against experimental RSV challenge in adult volunteers, and replaces serum neutralizing antibody as the best predictor of protection. However, virus-specific IgA memory B cell responses are remarkably absent after RSV infection, possibly explaining susceptibility to re-infection. Ideally, RSV vaccines should induce durable mucosal antiviral IgA and memory B cells thereby overcoming the defective responses seen after infection with live virus.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract:

Rationale: Despite relative antigenic stability, respiratory syncytial virus (RSV) re-infects throughout life. After >40 years of research, no effective human vaccine exists and correlates of protection remain poorly defined. Most current vaccine candidates seek to induce high levels of RSV-specific serum neutralizing antibodies, which are associated with reduced RSV-related hospitalization rates in observational studies but may not actually prevent infection.

Objectives: Characterize correlates of protection from infection and the generation of RSV-specific humoral memory to promote effective vaccine development.

Methods: We inoculated 61 healthy adults with live RSV and studied protection from infection by serum and mucosal antibody. We analyzed RSV-specific peripheral blood plasmablast and memory B cell frequencies and antibody longevity.

Measurements and Main Results: Despite moderately high levels of pre-existing serum antibody, 34 (56%) became infected, of whom 23 (68%) developed symptomatic colds. Prior RSV-specific nasal IgA correlated significantly more strongly with protection from PCR-confirmed infection than serum neutralizing antibody. Increases in virus-specific antibody titers were variable and transient in infected subjects, but correlated with plasmablasts that peaked around day 10. During convalescence, only IgG (and no IgA) RSV-specific memory B cells were detectable in peripheral blood. This contrasted with natural influenza infection, where virus-specific IgA memory B cells were readily recovered.

Conclusions: This observed specific defect in IgA memory may partly explain RSV's ability to cause recurrent symptomatic infections. If so, vaccines able to induce durable RSV-specific IgA responses may be more protective than those generating systemic antibody alone.

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Key words: antibody-secreting cells, mucosal immunity, immunologic memory, respiratory syncytial virus vaccines, nontherapeutic human experimentation

Introduction

Respiratory syncytial virus (RSV) is the leading cause of infant hospitalization worldwide, infecting around 34 million children each year (1). It is also a major under-appreciated contributor to morbidity and mortality in elderly and immunosuppressed adults (2). While RSV infection is essentially confined to the respiratory epithelium, extensive lung inflammation frequently causes life-threatening disease in these at-risk populations (3, 4). It remains one of the few common severe infections for which there is no specific intervention and an effective vaccine represents a major unmet need.

Natural infections with most viruses including influenza and rhinoviruses induce long-lasting protective immunity, with recurrent infections then occurring because of antigenic variation. In contrast, re-infection with identical strains of RSV can occur at as little as 2 month intervals even with a healthy mature immune system (5). Although re-infection with human metapneumovirus and parainfluenza viruses have been observed, only RSV has been categorically shown to induce recurrent symptomatic disease in the absence of antigenic change (6, 7). The mechanisms underlying this phenomenon remain unclear and the lack of durable immunity after natural infection has made it difficult to define precise correlates of protection. With over 51 RSV vaccine candidates currently in development (8), the ability to predict efficacy via immune correlates is urgently required. Although the humanized monoclonal IgG palivizumab can reduce infant hospitalizations with RSV by at least 50% (9), the association between serum antibody levels and protection is imprecise (10, 11). Therefore, the value of serum antibody induction as the primary readout of potential vaccine candidates and in vaccine licensure may be limited.

While animal models of RSV infection provide mechanistic insights, they do not recapitulate all aspects of human disease and remain imprecise guides to the clinical efficacy of vaccines (12). Investigation of hospitalized children and high-risk adults allows natural disease to be observed, but cannot correlate prior immune status with susceptibility to infection. In addition, lack of uniformity in virus inoculum, co-morbidities, medical interventions and atypical disease presentations limit the conclusions that can be reached from these observational studies. Experimental infection of volunteers therefore offers an important complementary approach, allowing intensive sampling and the opportunity to perform detailed investigations of pre-existing and pre-symptomatic responses (13, 14).

To investigate the mechanisms underlying recurrent RSV infection, we analyzed antibody-mediated protection and B cell responses before and after experimental human challenge. We found that serum antibody was loosely correlated with protection, but that there was a tight relationship between pre-existing RSV-specific nasal antibody and resistance to infection. These data allowed us to derive a model that accurately predicts infection risk. In addition, we demonstrate a novel RSV-specific defect in IgA memory that may explain the susceptibility to re-infection with RSV and discuss its implications for RSV vaccine development. Some of the results of this study have been previously reported in the form of abstracts (15–19).

Materials and Methods

Study design

Healthy non-smoking adults aged 18-55 were eligible for inclusion. All were screened to exclude underlying immunodeficiencies including IgA deficiency by serum immunoglobulin class levels. Subjects were inoculated with with 10^4 plaque-forming units (PFU) of RSV A Memphis 37 (RSV M37) by intranasal drops (as defined in previous dose-ranging studies) and quarantined from 1 day prior to inoculation to the 10^{th} day after (13). Nasal washes and blood were taken immediately before inoculation and regularly for the next 10 days (Supplementary Fig. E1A). Infection was defined as RSV detectable by PCR in nasal lavage on ≥2 days between day +2 and day +10 to avoid false positives from detection of the viral inoculum and to align case definitions with previous challenge studies using RSV M37 (13). Subjects completed a diary of upper respiratory tract symptoms (see Online Data Supplement) from day -1 to day +14. All subjects returned for further nasal and blood sampling on day +14, +28 and optionally 6-12 months after inoculation (nominally day +180). All subjects provided written informed consent and the study was approved by the UK National Research Ethics Service (study numbers 10/H0711/94 and 11/LO/1826).

Antibody assays

Serum neutralizing antibody titer was determined by plaque reduction neutralization titer (PRNT) in HEp-2 cells and expressed as mid-point titers (EC50). Sera from 4 hospitalized RSV PCR negative infants hospitalized were included as negative controls and 3 RSV immune reference sera (Wyeth 06937, 06938, 06939) as positive controls (20). Nasal wash IgA endpoint binding titer to RSV lysate and F protein was determined by enzyme-linked immunosorbent assay (ELISA) as the highest titer exhibiting an optical density of >2x the background. End-point

titers were used as mid-point titers could not be calculated in view of the dilute nature of nasal lavage. Observed endpoint titers were corrected for dilution using the ratio of serum to nasal lavage urea before analysis as described (21).

Detection of antibody secreting cells by B cell ELISpot

Antibody secreting cells (ASCs) were detected using enzyme-linked immunospot (ELISpot) as previously described (22), using whole RSV M37 lysate from Hep-2 cells and recombinant F protein based on the RSV A2 strain (see Online Data Supplement for detailed methods). For memory B cells, additional plates were coated with 10μg/mL measles antigen (Meridian Lifesciences), and 5μg/mL HEp-2 antigen, 2.5μg/mL keyhole limpet hemocyanin (KLH, Sigma), and media as negative controls. Total ASCs were expressed as spot-forming cells / 10^6 PBMC and antigen-specific ASCs as % of total Ig-secreting cells.

Polyclonal activation of memory B cells

PBMCs were cultured *in vitro* according to the method of Crotty *et al.* (23). The alternative polyclonal activation mix described by Tengvall *et al.* was used in a subset of samples (24). ASCs were detected by B cell ELISpot as above. Subjects exhibiting a total Ig^+ cell responses the below the 10^{th} centile in either the day 0 or day 28 sample for either IgG or IgA were excluded (n = 9) as inadequate responders to stimulation.

Statistical analysis

All data analyses and graphs were produced using the software R (25, 26). Results are expressed as median and interquartile range. Non-parametric data was compared using Mann-Whitney-Wilcoxon tests with Holm's correction for multiple comparisons. Binary response variables were related to continuous explanatory variables using logistic regression. Odds ratios (OR) and 95%

confidence intervals (CI) of the OR for the explanatory variables were calculated (see Online Data Supplement). For estimation of serum neutralizing antibody titers, weighted (1/y) four-parameter logistic models were fitted to the plaque counts and the 50% neutralizing titer (EC_{50}) was derived from the midpoint of the curve using package 'drc' (27).

Results

Nasal antibody correlates strongly with protection from infection by RSV

Sixty-one healthy non-smoking adult volunteers were enrolled (age 18-50, median 22 years; Table 1) without pre-selection according to anti-RSV antibody levels. All were inoculated with RSV M37 via nasal drops and admitted to a residential quarantine facility for 10 days. Thirty-four (56%) subjects became infected as defined by PCR positivity on ≥2 consecutive days between days 2-10 post-inoculation (Supplementary Fig E1B). Five individuals deemed "uninfected" had a low level of detectable viral shedding on day 1 post-inoculation with no virus detected at any point thereafter, which likely indicated the remains of the virus inoculum and not evidence of productive infection. Two "uninfected" subjects had RSV detected with a high Ct value on a single day during days 2-10. Neither of these had any subsequent change in their RSV-specific immune responses. Median (interquartile range, IQR) cumulative symptom scores were 26.5 (9.6–41.9) for PCR+ "infected" and 9.0 (0.8–20.3) for PCR- "uninfected" subjects (P = 0.004). Daily nasal lavage viral load measurements were made during the quarantine period and cumulative viral load for PCR+ subjects was 4.0 (3.1–5.2) $\log_{10}$ copies/mL. No difference in the number infected was observed for differing age, sex or ethnicity.

Pre-existing PRNTs did not differ significantly between PCR- (median 7.8 log_2) and PCR+ (median 7.6 log_2) subjects (Fig 1A). Similarly, there was no significant difference in anti-RSV or anti-F protein IgG (RSV-IgG 7.8 log_2 vs. 7.6 log_2, F-IgG 7.9 log_2 vs. 7.2 log_2) or IgA (RSV-IgA 9.0 log_2 vs. 8.0 log_2, F-IgA 8.0 log_2 vs. 7.5 log_2) in serum between PCR- and PCR+ individuals (Supplementary Fig. E2A & E2B). By contrast, nasal anti-RSV and anti-F protein IgA titers in individuals who remained uninfected were significantly higher (RSV-IgA 10.1 log_2 vs. 9.4 log_2, F-IgA 10.2 log_2 vs. 9.6 log_2 Fig. 1A). However, serum IgA correlated poorly with nasal IgA (the Spearman's correlation coefficient of 0.25 implying only a weak relationship between responses against RSV lysate in these compartments with a borderline P-value of 0.05), suggesting that it is IgA in the airway that specifically plays a role in protection against RSV infection and that serum IgA may not derive from the same source (Supplementary Fig. E2C).

Serum antibody (PRNT) did not affect the likelihood of infection (odds ratio (OR) = 1.6, 95% confidence interval 0.9–2.9, P = 0.1), but virus- and F protein-specific nasal IgA were again significantly associated with protection (RSV-IgA: OR = 1.9 (1.2–3.4), P = 0.014; F-IgA: OR = 1.7 (1.1–2.9), P = 0.016, Fig. 1B). Thus, serum antibody correlates poorly with protection whereas a 1 log_2 increase in RSV-specific nasal IgA titers renders subjects nearly twice as likely to be protected.

Increased RSV-specific antibody levels after infection are poorly maintained

Following RSV challenge, RSV PCR+ subjects showed a median 3.4–fold increase in PRNT (IQR 2.3–4.6). Similar increments were seen in nasal anti-RSV IgA (2.6 fold, IQR 2.0–5.4) and

nasal anti-F protein IgA (3.0, 1.4–4.5). By contrast, in the group that was inoculated but remained uninfected, no significant rise in the median neutralization titer (1.4, 1.0–2.0), nasal anti-RSV IgA (0.9, 0.6–2.5) or nasal anti-F protein IgA (0.8, 0.6–1.6; Fig. 2A) was seen by Mann-Whitney test. Although two uninfected individuals displayed a 4-fold rise in PRNT and 8 showed approximately 2-fold increases in nasal anti-RSV IgA, none were PCR+ on any day. Repetition of these assays showed no run-to-run variability, suggesting that these changes represented either normal biological variability in serum antibody levels or responses to RSV antigen in the inoculum in the absence of infection.

Whilst prior nasal IgA levels correlated closely with protection, only 21% of individuals judged as infected reached post-infection nasal anti-RSV IgA titers predicted to give ≥80% protection (Supplementary Table E1). Even fewer subjects demonstrated an equivalently protective rise in IgA specific to purified RSV F protein (9%), suggesting that a proportion of antibody boosted by natural infection might be against strain-specific variable sites on surface glycoproteins. Therefore, even the boosted levels of antibody seen shortly after natural infection are unlikely to provide complete immunity in most individuals.

Previous studies have suggested that antibodies induced by RSV infection are poorly maintained in both adults (28) and children (29). We therefore measured serum and nasal anti-RSV antibodies in infected subjects approximately 6-12 months post-inoculation. Even in infected subjects showing the strongest antibody responses in serum and/or nasal secretions at one month, antibody levels were not maintained at later follow-up. Instead, levels returned in many to almost exactly the pre-infection level for that individual (Fig. 2B).

Induction of higher plasmablast frequencies correlates with increased antibody levels

To better understand why RSV-specific antibodies neither reach consistently protective titers nor persist beyond 6 months, we studied B cell responses in peripheral blood by flow cytometry. RSV-induced plasmablasts ($CD3^-/CD19^+/CD38^{hi}/CD27^{hi}$) peaked around 10 days post-inoculation (Supplementary Fig. E3A). Similarly, using B cell ELISpots, antigen-specific antibody-secreting cells (ASCs) against RSV were shown to arise after day +7 and peak around day +10 (Fig. 3A). Peak virus-specific ASC frequencies ranged from 5-50% of total ASCs, comparable to that after influenza or yellow fever vaccination (Fig. 3B) (30–33). Thus, the virus-specific acute ASC responses to RSV infection appeared quantitatively intact.

By day +14, RSV-specific ASCs had declined and by day +28 were completely undetectable. IgM-producing ASCs were largely absent throughout (as expected of a recall response). However, IgG- and IgA-producing RSV-specific ASCs both appeared at similar frequencies, consistent with generation at a mucosal site of infection (Fig. 3B). Furthermore, the frequency of RSV- and F-specific IgG^+ ASCs correlated strongly with fold-change in serum neutralizing antibody (Fig. 3C and Supplementary Fig. E3B) and virus-specific IgA^+ ASCs correlated strongly with the increment in nasal IgA (Fig. 3D and Supplementary Fig. E3B). However, pre-existing serum neutralizing antibody levels negatively correlated with peak ASC frequencies, suggesting that even though antibodies in this compartment did not provide sterilizing immunity, they might still have blunted responses against homologous antigens in the challenge agent (Fig. 3E and Supplementary Fig. E3C). Further, cumulative viral load and symptom scores (by trapezoidal area under the curve (AUC)) were not associated with peak ASC frequencies,

indicating that once infection was established the frequency of antigen-specific B cells had no effect on virus control or disease severity (Supplementary Fig. E4A & E4B). Therefore, the peak frequency of ASCs depended on pre-existing antibodies inasmuch as antibody can prevent the establishment of infection. Once infection occurred, ASCs proliferated rapidly; this correlated with the transient production of anti-RSV IgG and IgA, but otherwise had little impact on disease course or outcome.

IgG^+ memory B cell frequencies prior to infection do not influence the outcome of viral challenge

Circulating memory B cells (MBCs) rapidly differentiate into ASCs and plasma cells on re-encounter with cognate antigen and are essential for rapid humoral response to recurrent infection. To assess whether pre-infection RSV-specific MBCs correlate with protection we analyzed RSV-specific MBC frequencies and compared with those against measles virus, a related pathogen to which protection following natural infection or live-attenuated vaccination is known to be long-lasting (34).

Identifying ASCs by B cell ELISpot (23), there were no significant differences in IgG^+ MBC frequencies against whole RSV, F-protein or measles virus prior to RSV challenge or between pre-infection MBC frequencies in subsequently infected or uninfected subjects (Fig. 4A). Pre-infection MBC frequencies neither correlated with later symptom score nor viral load in infected subjects (Supplementary Fig. E5A & E5B), nor with PRNT or nasal IgA antibody levels (Supplementary Fig. E5C, E5D, & E5E). This confirmed that pre-existing RSV-specific MBCs

per se neither influence infection rates nor contribute to control of disease or viral replication once infection is established.

Following infection, subjects demonstrated a median (IQR) increase in IgG^+ MBC frequencies of 0.4 (0.2−0.5) % against whole RSV and 0.1 (0−0.2) % against F protein (Fig. 4A). These equated to a median 3.8 (3.1−5.1) –fold increase in RSV-specific MBCs and 1.8 (1.3−4.4) –fold increase in F-specific MBCs compared with pre-infection frequencies (Fig. 4B). By contrast, measles-specific IgG^+ MBC frequencies remained unchanged (Fig. 4A & 4B). The fold-change in both virus- and F-protein specific IgG^+ MBCs correlated with the size of ASC burst specific for the homologous antigens, suggesting that these populations were generated by associated mechanisms (Fig. 4C). IgG^+ MBCs therefore incremented appropriately following RSV infection but did not affect the course of acute disease.

RSV infection fails to induce virus-specific IgA memory B cells

To investigate the mechanisms underlying the decline in virus-specific nasal IgA, we studied RSV-specific IgA^+ MBCs. In contrast to IgG^+ MBCs, RSV- and F-specific IgA-producing MBCs (and measles-specific IgA^+ MBCs) were undetectable pre-infection (Fig. 5A). Strikingly, anti-RSV IgA^+ MBCs then demonstrated no detectable increase in frequency even shortly after RSV infection; these remained undetectable at convalescence in the same way as measles (Fig. 5B).

IgA^+ and IgG^+ MBCs differ in their requirements for optimal *in vitro* activation (24). To ensure that the apparent absence of RSV-specific IgA^+ MBCs was not simply due to inadequate activation, we repeated the *in vitro* activation of PBMC from 6 selected subjects who had previously demonstrated a robust RSV-specific IgG^+ MBC response with an alternative protocol using CpG, l and IL-15 to enhance IgA^+ MBC stimulation (24). However, this did not improve the polyclonal stimulation of IgA^+ MBCs, which were induced at similar frequencies to the original method, while total IgG^+ MBCs were less well induced (Supplementary Fig. E6A & E6B). Again, RSV-specific IgA^+ MBCs were undetectable both at baseline and convalescence using either stimulation method (Supplementary Fig. E6C & E6D).

To determine whether poor IgA^+ MBC generation was RSV-specific, we compared these responses with those after natural influenza A(H1N1)pdm09 infection. Convalescent PBMCs were obtained as part of the Mechanisms of Severe Acute Influenza Consortium (MOSAIC) study from 8 patients hospitalized with virologically-confirmed pdmH1N1 infection during the 2009/10 and 2010/11 influenza seasons. All were previously healthy with no known immunosuppressive disease. Antigen-specific MBCs were polyclonally stimulated under identical conditions and identified by B cell ELISpot. As expected, the virus-specific IgG^+ MBCs induced following both pdmH1N1 and RSV were found at similar frequencies (Fig. 5C). However, PBMCs from patients naturally infected with influenza showed no defect in IgA^+ MBC response, with significantly higher numbers of virus-specific IgA^+ MBCs than in those infected with RSV (Fig. 5D). In influenza, virus-specific IgA^+ and IgG^+ MBC numbers were therefore comparable and IgA^+ MBCs were generally abundant in those who had recovered from infection, contrasting with the paucity of IgA^+ MBCs after RSV infection.

Discussion

In this study, we show that mucosal IgA titers are predictive of susceptibility to RSV infection (in contrast to serum antibody levels which are not), and identify a previously unrecognized defect in IgA memory B cell responses to RSV. The few previous studies of mucosal IgA in RSV disease have been restricted in their applicability by study design and other technical limitations (35–37). Our investigations now show the power of the experimental human infection model, which for the first time has allowed development of a tightly predictive model of RSV infection risk in seropositive individuals.

A previous study examining the protective role of RSV-specific antibodies during experimental human RSV challenge (38) showed that serum neutralization and IgG titers against F protein were both significantly higher in those remaining uninfected, whilst no difference was seen in nasal IgA titers. The reasons for the discrepancy between those results and our present findings may be methodological: in the earlier study, a different method of nasal lavage was used, resulting in a more dilute sample and average 2-3 fold lower nasal IgA titer. The normalization according to total protein content may also have led to underestimation of true IgA level. In addition, the laboratory-adapted RSV A2 strain used in the previous study induced little symptomatic disease in infected subjects and may have had different host-virus dynamics compared with RSV M37, a more recent, non-attenuated, clinically derived strain. Finally, the previous study included fewer subjects (a total of 28 inoculated individuals), some of whom were pre-stratified on the basis of serum antibody levels, which may have affected the analyses.

Interestingly, consistently protective levels of anti-RSV IgA were only seen in a small minority of individuals, even during early convalescence . Furthermore, while antibodies were temporarily boosted by infection, titers waned to pre-infection steady-state levels within 6-12 months. This contrasts sharply with known responses to viruses such as influenza or rhinovirus, in which strain-specific serum IgG can persist life-long and nasal IgA remain significantly raised for a year or more (39, 40). Antibody responses to RSV infection are therefore remarkably transient, accounting for the frequency of adult re-infection. This study now sets a new goal for vaccine-induced immunity: mucosal IgA responses in the respiratory mucosa beyond those induced naturally. Indeed, if mucosal IgA could be induced in the major at-risk population of young children, there would be advantages not only in reducing the incidence of RSV infection but also by limiting the extent of virus transmission, although extrapolation of these data to children should be undertaken with caution.

Importantly, we found that short-lived RSV-specific IgG^+ and IgA^+ B cells were generated robustly at comparable frequencies to those observed following other challenges including influenza infection or vaccination (30, 32). With acute production of short-lived ASCs apparently intact, a vaccine capable of inducing higher frequencies of these cells should also induce greater antibody responses and therefore enhanced protection. Previous studies in natural RSV infection of adults indicated that this might be further modulated by duration of antigenic stimulation, with prolonged viral shedding driving ASC responses (41). However, anti-RSV antibody titers were poorly maintained, implying a defect in long-lived plasma cells (LLPCs) that arise from the ASC population and which normally migrate to survival niches including

bone marrow and respiratory mucosa (42, 43). Future studies to address this contention will require direct sampling of these compartments, which may be difficult.

In animal models, IgA-producing B cells are abundant at mucosal sites (44) and appear rapidly in mucosal tissues under the influence of mediators such as BAFF (B cell activating factor) and APRIL (A proliferation inducing ligand) (45–47). However, to generate high affinity antibodies and long-term memory responses, antigen-presenting cells, B cells and T follicular helper (Tfh) cells must interact in germinal centers (GCs) (48, 49). T cell help allows not only class-switching to IgA but also the promotion of ASC and MBC formation (50, 51). MBCs require many of the same differentiation signals as LLPCs and we therefore hypothesized that MBC responses would also be sub-optimal. Our analysis of IgG^+ MBCs pre- and post-infection found no evident defect, but remarkably, we were unable to detect any RSV-specific IgA^+ MBCs prior to infection and no increment in IgA^+ MBC frequency afterwards. Since the acute ASC response was dominated by RSV-specific IgA^+ ASCs, which are likely to have arisen from pre-existing IgA^+ MBCs, the finding that RSV-specific IgA^+ MBCs were so infrequent both pre- and post- infection was highly unexpected. Despite this, moderate levels of serum and nasal IgA against RSV could be detected in all volunteers. Thus, while incremental boosting of IgA^+ MBCs might be poor, over multiple re-infections LLPCs in bone marrow or respiratory tract are likely to have developed that are capable of producing RSV-specific IgA long-term but whose population size or antibody-producing capabilities are minimally augmented following re-challenge.

RSV is known to express several immunomodulatory proteins and we therefore suggest that this defect in IgA memory represents a viral immune evasion strategy. However, some alternative

explanations should be considered. It is possible that IgA^+ MBCs following respiratory infections in general are so infrequent that these assays cannot detect them. Alternatively, IgA^+ MBCs might be preferentially retained in mucosal sites and therefore not detectable in the circulation. However, if either of these were the case, we would also not have expected to find influenza-specific IgA^+ MBCs in convalescent PBMCs. Furthermore, studies of oral cholera vaccination show that vaccine antigen-specific IgA^+ MBCs do appear in the circulation of vaccinees (52). It could be argued that the more vigorous inflammatory response to influenza is responsible for induction of a greater overall humoral response, thus explaining the longer persistence of mucosal IgA and higher frequencies of influenza-specific MBCs. However, there was no difference in the frequency of IgG^+ MBCs between the two infections, suggesting that generalized deficiency of inflammatory signaling was unlikely to have been the cause of reduced anti-RSV IgA^+ MBCs. By contrast, intranasal infection with attenuated influenza strains and rhinoviruses are still capable of inducing persistent humoral responses despite causing less inflammation than RSV (40, 53).

We therefore propose the hypothesis that RSV infection is unusual in limiting the induction of B cell memory responses in the respiratory tract. This would explain why the defect is specifically in IgA^+ MBCs (which are preferentially generated at mucosal sites) and does not affect IgG^+ MBCs. Recent studies have suggested that while BAFF is elevated in BAL and bronchial brushings from children with RSV bronchiolitis, APRIL is not (54). In addition, defects in RSV antigen-presentation and T cell memory generation have been observed in several animal models, which may impact on Tfh cell activity (55). Thus, both T cell-independent and dependent mechanisms may be influenced by RSV to specifically impair local immunity. If few

IgA^+ MBCs are induced following infection, this may explain why antibody-mediated immunity plateaus despite recurrent virus exposure. Further mechanistic investigation to support this interpretation will require invasive respiratory tract sampling of infected individuals, since no animal model experiences recurrent symptomatic RSV infections. It also remains to be seen whether similar mechanisms exist in human metapneumovirus or other respiratory viruses where recurrent infections occur.

The ability to predict infection risk using nasal IgA titers and discovery of a specific defect in IgA memory have important implications for RSV vaccine development. Although findings from experimental infection of young adults may not be directly applied to infants and elderly individuals, they can be extrapolated to women of child-bearing age in whom mucosal vaccination may reduce RSV transmission to their children and confer a "cocooning" effect. Community cohort studies will support the applicability of these data in high-risk populations, but our results suggest that vaccines inducing mucosal RSV-specific IgA are likely to be more effective than those stimulating systemic antibodies alone. For vaccines that induce local virus-specific IgA, we can also now accurately predict their protective efficacy prior to commitment to phase II/III clinical trials and for licensing purposes. However, the unique absence of IgA^+ MBCs following RSV infection will necessitate re-consideration of which types of vaccine to pursue. Live-attenuated RSV vaccines, for example, may also be subject to this same defect and be incapable of inducing sufficient long-lived IgA to provide solid protection (56). Even novel vaccine constructs that might bypass this defect will encounter this limitation, with the paucity of pre-existing IgA^+ MBCs necessitating multiple-boost regimens.

In conclusion, we demonstrate that nasal antibody strongly predicts protection from RSV infection in adults. However, antibody levels wane much more rapidly than reported in other respiratory viral infections and are almost always below those needed for protection within months. Unexpectedly, we found that RSV-specific IgA^+ memory B cells were not significantly induced by infection, implying that effective RSV vaccines may need to overcome this defect to provide durable immunity.

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References

1. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *The Lancet* 2010;375:1545–1555.
2. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory Syncytial Virus Infection in Elderly and High-Risk Adults. *N Engl J Med* 2005;352:1749–1759.
3. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory Syncytial Virus Infection of Human Airway Epithelial Cells Is Polarized, Specific to Ciliated Cells, and without Obvious Cytopathology. *J Virol* 2002;76:5654–5666.
4. Gardner PS, McQuillin J, Court SDM. Speculation on Pathogenesis in Death from Respiratory Syncytial Virus Infection. *Br Med J* 1970;1:327–330.
5. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. *J Infect Dis* 1991;163:693–698.
6. Okamoto M, Sugawara K, Takashita E, Muraki Y, Hongo S, Nishimura H, Matsuzaki Y. Longitudinal course of human metapneumovirus antibody titers and reinfection in healthy adults. *J Med Virol* 2010;82:2092–2096.
7. Glezen WP, Frank AL, Taber LH, Kasel JA. Parainfluenza virus type 3: seasonality and risk of infection and reinfection in young children. *J Infect Dis* 1984;150:851–857.

6. PATH. 2014. Respiratory syncytial virus: Vaccine development against a major cause of childhood respiratory illness. [Online]. [Accessed 16th December 2014]. Available from: http://sites.path.org/vaccinedevelopment/respiratory-syncytial-virus-rsv/.

9. The IMpact-RSV Study Group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. *Pediatrics* 1998;102:531–537.
10. Falsey AR, Walsh EE. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. *J Infect Dis* 1998;177:463–466.
11. Piedra PA, Jewell AM, Cron SG, Atmar RL, Glezen WP. Correlates of immunity to respiratory syncytial virus (RSV) associated-hospitalization: establishment of minimum protective threshold levels of serum neutralizing antibodies. *Vaccine* 2003;21:3479–3482.
12. Bem RA, Domachowske JB, Rosenberg HF. Animal models of human respiratory syncytial virus disease. *Am J Physiol Lung Cell Mol Physiol* 2011;301:L148–156.
13. DeVincenzo JP, Wilkinson T, Vaishnaw A, Cehelsky J, Meyers R, Nochur S, Harrison L, Meeking P, Mann A, Moane E, Oxford J, Pareek R, Moore R, Walsh E, Studholme R, Dorsett P, Alvarez R, Lambkin-Williams R. Viral load drives disease in humans experimentally infected with respiratory syncytial virus. *Am J Respir Crit Care Med* 2010;182:1305–1314.
14. Pollard AJ, Savulescu J, Oxford J, Hill AV, Levine MM, Lewis DJ, Read RC, Graham DY, Sun W, Openshaw P, Gordon SB. Human microbial challenge: the ultimate animal model. *Lancet Infect Dis* 2012;12:903–905.
15. Chiu C, Habibi MS, Jozwik A, Paras A, Makris S, Openshaw PJM. B cell responses following experimental RSV challenge in adult volunteers. Porto, Portugal: 2013.
16. Habibi MS, Jozwik A, Makris S, Paras A, Openshaw P, Chiu C. Human B cell responses following experimental respiratory syncytial virus challenge. Jesus College, Cambridge, UK: 2013.

17. Habibi MS, Guvenel AK, Jozwik A, Paras A, Chiu C, Openshaw PJM. Immunity to Respiratory Syncytial Virus (RSV) infection. *Pathog Respir Viruses J5* Keystone, Colorado, USA: 2014.
18. Habibi MS, Jozwik A, Makris S, Dunning J, Paras A, Wrammert J, Openshaw PJM, Chiu C. Defective IgA B cell memory generation following experimental human respiratory syncytial virus infection impairs protective immunity. Stellenbosch, South Africa: 2014.
19. Medrano González L, Habibi MS, Jozwik A, Openshaw PJM, Chiu, Christopher. Quality of antigen-specific B cell responses as a correlate of protection against RSV. *Immunology* 2014;143:75.
20. Yang DP, Zielinska E, Quiroz J, Madore D, Rappaport R. Preparation of a respiratory syncytial virus human reference serum for use in the quantitation of neutralization antibody. *Biol J Int Assoc Biol Stand* 2007;35:183–187.
21. Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, Crystal RG. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J Appl Physiol Bethesda Md 1985* 1986;60:532–538.
22. Saletti G, Çuburu N, Yang JS, Dey A, Czerkinsky C. Enzyme-linked immunospot assays for direct ex vivo measurement of vaccine-induced human humoral immune responses in blood. *Nat Protoc* 2013;8:1073–1087.
23. Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *J Immunol Methods* 2004;286:111–122.
24. Tengvall S, Lundgren A, Quiding-Järbrink M, Svennerholm A-M. BAFF, stimulatory DNA and IL-15 stimulates IgA+ memory B cells and provides a novel approach for analysis of memory responses to mucosal vaccines. *Vaccine* 2010;28:5445–5450.

25. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2013. at <http://www.R-project.org/>.
26. Sarkar D. *Lattice: Multivariate Data Visualization with R*. New York: Springer; 2008. at <http://lmdvr.r-forge.r-project.org>.
27. Ritz C, Streibig JC. Bioassay Analysis using R. *J Stat Softw* 2005;12:
28. Falsey AR, Singh HK, Walsh EE. Serum antibody decay in adults following natural respiratory syncytial virus infection. *J Med Virol* 2006;78:1493–1497.
29. Sande CJ, Mutunga MN, Okiro EA, Medley GF, Cane PA, Nokes DJ. Kinetics of the neutralizing antibody response to respiratory syncytial virus infections in a birth cohort. *J Med Virol* 2013;85:2020–2025.
30. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng N-Y, Mays I, Garman L, Helms C, James J, Air GM, Capra JD, Ahmed R, Wilson PC. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* 2008;453:667–671.
31. Wrammert J, Koutsonanos D, Li G-M, Edupuganti S, Sui J, Morrissey M, McCausland M, Skountzou I, Hornig M, Lipkin WI, Mehta A, Razavi B, Del Rio C, Zheng N-Y, Lee J-H, Huang M, Ali Z, Kaur K, Andrews S, Amara RR, Wang Y, Das SR, O'Donnell CD, Yewdell JW, Subbarao K, Marasco WA, Mulligan MJ, Compans R, Ahmed R, Wilson PC. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. *J Exp Med* 2011;208:181–193.
32. Li G-M, Chiu C, Wrammert J, McCausland M, Andrews SF, Zheng N-Y, Lee J-H, Huang M, Qu X, Edupuganti S, Mulligan M, Das SR, Yewdell JW, Mehta AK, Wilson PC, Ahmed

R. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. *Proc Natl Acad Sci U S A* 2012;109:9047–9052.

33. Wrammert J, Onlamoon N, Akondy RS, Perng GC, Polsrila K, Chandele A, Kwissa M, Pulendran B, Wilson PC, Wittawatmongkol O, Yoksan S, Angkasekwinai N, Pattanapanyasat K, Chokephaibulkit K, Ahmed R. Rapid and Massive Virus-Specific Plasmablast Responses during Acute Dengue Virus Infection in Humans. *J Virol* 2012;86:2911–2918.
34. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. *N Engl J Med* 2007;357:1903–1915.
35. Mills V J, Van Kirk JE, Wright PF, Chanock RM, Fishburne IE. Experimental Respiratory Syncytial Virus Infection of Adults: Possible Mechanisms of Resistance to Infection and Illness. *J Immunol* 1971;107:123–130.
36. Watt PJ, Robinson BS, Pringle CR, Tyrrel DAJ. Determinants of susceptibility to challenge and the antibody response of adult volunteers given experimental respiratory syncytial virus vaccines. *Vaccine* 1990;8:231–236.
37. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *J Infect Dis* 2004;190:373–378.
38. Lee FE-H, Walsh EE, Falsey AR, Betts RF, Treanor JJ. Experimental infection of humans with A2 respiratory syncytial virus. *Antiviral Res* 2004;63:191–196.
39. Fujimoto C, Takeda N, Matsunaga A, Sawada A, Tanaka T, Kimoto T, Shinahara W, Sawabuchi T, Yamaguchi M, Hayama M, Yanagawa H, Yano M, Kido H. Induction and maintenance of anti-influenza antigen-specific nasal secretory IgA levels and serum IgG levels after influenza infection in adults. *Influenza Other Respir Viruses* 2012;6:396–403.

40. Barclay WS, al-Nakib W, Higgins PG, Tyrrell DA. The time course of the humoral immune response to rhinovirus infection. *Epidemiol Infect* 1989;103:659–669.
41. Lee FE-H, Falsey AR, Halliley JL, Sanz I, Walsh EE. Circulating antibody-secreting cells during acute respiratory syncytial virus infection in adults. *J Infect Dis* 2010;202:1659–1666.
42. Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral Immunity Due to Long-Lived Plasma Cells. *Immunity* 1998;8:363–372.
43. Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. *Annu Rev Immunol* 2005;23:367–386.
44. Mora JR, von Andrian UH. Differentiation and homing of IgA-secreting cells. *Mucosal Immunol* 2008;1:96–109.
45. Mora JR, Iwata M, Eksteen B, Song S-Y, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* 2006;314:1157–1160.
46. He B, Xu W, Santini PA, Polydorides AD, Chiu A, Estrella J, Shan M, Chadburn A, Villanacci V, Plebani A, Knowles DM, Rescigno M, Cerutti A. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity* 2007;26:812–826.
47. Cerutti A, Chen K, Chorny A. Immunoglobulin Responses at the Mucosal Interface. *Annu Rev Immunol* 2011;29:273–293.
48. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 2007;25:5467–5484.
49. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;29:621–663.

50. Cazac BB, Roes J. TGF-beta receptor controls B cell responsiveness and induction of IgA in vivo. *Immunity* 2000;13:443–451.
51. Borsutzky S, Cazac BB, Roes J, Guzmán CA. TGF-beta receptor signaling is critical for mucosal IgA responses. *J Immunol Baltim Md 1950* 2004;173:3305–3309.
52. Harris AM, Bhuiyan MS, Chowdhury F, Khan AI, Hossain A, Kendall EA, Rahman A, LaRocque RC, Wrammert J, Ryan ET, Qadri F, Calderwood SB, Harris JB. Antigen-specific memory B-cell responses to Vibrio cholerae O1 infection in Bangladesh. *Infect Immun* 2009;77:3850–3856.
53. Clements ML, Murphy BR. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. *J Clin Microbiol* 1986;23:66–72.
54. McNamara PS, Fonceca AM, Howarth D, Correia JB, Slupsky JR, Trinick RE, Al Turaiki W, Smyth RL, Flanagan BF. Respiratory syncytial virus infection of airway epithelial cells, in vivo and in vitro, supports pulmonary antibody responses by inducing expression of the B cell differentiation factor BAFF. *Thorax* 2013;68:76–81.
55. Openshaw PJ, Chiu C. Protective and dysregulated T cell immunity in RSV infection. *Curr Opin Virol* 2013;3:468–474.
56. Karron RA, Buchholz UJ, Collins PL. Live-attenuated respiratory syncytial virus vaccines. *Curr Top Microbiol Immunol* 2013;372:259–284.

Figure Legends

Fig. 1. RSV specific nasal IgA is a superior correlate of protection to serum neutralization titer.

(**A**) Serum neutralizing antibody was determined by plaque reduction neutralization and nasal IgA by ELISA. Baseline serum neutralizing (top), nasal IgA against whole RSV (middle), and nasal anti-RSV fusion (F) protein (bottom) antibody levels in uninfected (○) vs. infected (●) subjects are shown. Serum neutralization includes 4 infant samples (Δ) and 3 reference serum standards (■ Wyeth 06937, 06938, 06939. Horizontal bars indicate the median. (**B**) Logistic regression of probability of protection with baseline serum neutralizing (top), nasal whole RSV IgA (middle), and nasal RSV fusion (F) protein IgA (bottom) antibody levels with predicted probability (solid line) and point-wise 95% confidence band (shaded) are shown. OR = odds ratio (and 95% confidence interval), P-values for unpaired Mann-Whitney-Wilcoxon (U) test or two-tailed *z*-test as appropriate are shown (ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Fig. 2. Serum and nasal antibody increase following infection but are not maintained.

Healthy adult volunteers were challenged intranasally with RSV A M37. Serum and nasal lavage was taken at baseline and 28 days post-inoculation. A subset of subjects returned for further blood and nasal sampling 6-12 months later (nominally day +180). Serum neutralizing antibody was determined by plaque reduction neutralization and nasal IgA by ELISA. (**A**) Fold-change vs. baseline of serum in serum PRNT (top), nasal IgA against whole RSV (middle), and

nasal anti-RSV fusion (F) protein (bottom) antibody levels in uninfected (○) vs. infected (●) subjects. Horizontal bars indicate the median. (**B**) Individual plots showing trend in antibody levels at baseline, day +28, and +180. P-values for unpaired Mann-Whitney-Wilcoxon (U) test are shown (ns = P > 0.05, * = P < 0.05, ** = P < 0.01, *** = P < 0.001).

Fig 3. RSV induced plasmablast expansion correlates with IgG and IgA increment.

(**A**) Antigen-specific antibody secreting cells (ASCs) were enumerated using B cell ELISpot with whole RSV lysate where sufficient PBMCs were available. Uninfected (PCR-) subjects (n = 12) are shown in blue, infected (PCR+) subjects (n = 16) in red. (**B**) Peak antigen-specific ASC frequencies as % of total Ig^+ cells for infected subjects. Horizontal bars indicate the median. (**C**) Correlation of fold-change in serum neutralizing antibody with peak IgG^+ ASC frequency. (**D**) Correlation of fold-change in nasal IgA against RSV with peak IgA^+ ASC frequency (data from 27 subjects; nasal IgA in one subject could not be measured). (**E**) Correlation of peak ASC frequency with pre-infection baseline serum neutralizing antibody titers. ASC frequencies are expressed as $\log_{10}$ per million PBMC. P values for unpaired Mann-Whitney-Wilcoxon (U) tests and or Spearman's rank correlation coefficient (Rs) are shown as appropriate. (○) Uninfected subjects, (●) infected subjects.

Fig 4. Antigen-specific IgG^+ memory B cells are induced by RSV infection.

PBMCs were obtained on days 0 and 28 post-inoculation and memory B cells (MBCs) polyclonally activated. Total and antigen-specific immunoglobulin-producing cells were then

enumerated using ELISpot. (**A**) Antigen-specific IgG^+ MBC frequencies for uninfected (○) vs. infected (●) subjects at baseline and day 28 post-inoculation (infected only). $P > 0.05$ for all comparisons except where shown. MBC frequencies are expressed as % of total Ig^+ cells. Horizontal bars indicate the median. (**B**) Fold-change (log_2) of IgG^+ MBCs post-infection compared with baseline for infected subjects (n = 17). Horizontal bars indicate the median. (**C**) Correlation between peak acute ASC frequency at day 10 and fold-change over baseline (log_2) of RSV specific IgG^+ MBC. Spearman's rank correlation coefficient (Rs) or P-values for unpaired Mann-Whitney-Wilcoxon (U) test are shown as appropriate. RSV: whole RSV, MV: measles virus, FP: purified RSV fusion protein.

Fig 5. RSV infection fails to induce IgA+ memory B cells.

PBMCs were obtained on days 0 and 28 post-inoculation and memory B cells (MBCs) activated polyclonally *in vitro*. Total and antigen-specific immunoglobulin-producing cells were then enumerated using B-cell ELISpot as before. Data for infected subjects exhibiting adequate PBMC responses to *in vitro* stimulation only (n = 17) is shown. (**A**) Frequency of IgA^+ MBCs at baseline (pre-infection) and (**B**) convalescence (day +28). Subjects experimentally challenged with RSV A M37 were compared with patients naturally infected with pandemic influenza A/H1N1/09 (7 exhibited adequate response to stimulation and were analyzed). (**C**) IgG^+ MBC frequencies and (**D**) IgA^+ MBC frequencies at day 28 post-inoculation (RSV) and day 28-42 post-hospitalization (H1N1) are shown. Horizontal bars indicate the median. P-values for unpaired Mann-Whitney-Wilcoxon (U) test are shown as appropriate. RSV: whole RSV, MV: measles virus, FP: purified RSV fusion protein.

	Uninfected (n = 27)	**Infected (n = 34)**	**P**
Median (range) age (years)	23 (18-39)	21 (18-50)	ns
Gender (M:F)	17:10	18:16	ns
Ethnicity			
White	21	28	ns
Black	4	1	ns
Asian	0	2	ns
Mixed	2	1	ns
Other	0	2	ns
Median (IQR) cumulative† symptom score	9 (0.8 – 20.3)	26.5 (9.6 – 41.9)	P = 0.004
Median (IQR) cumulative† viral load (log_{10} copies / mL)	0	4.0 (3.1 – 5.2)	NA

Table 1. Demographic, clinical and virological data from 61 individuals experimentally inoculated with RSV A Memphis 37.

IQR = interquartile range. † = trapezoidal area under the curve. P-values for unpaired Mann-Whitney-Wilcoxon (U) test or χ^2 test for comparison of uninfected vs. infected are shown.

Figures:

Figure 1

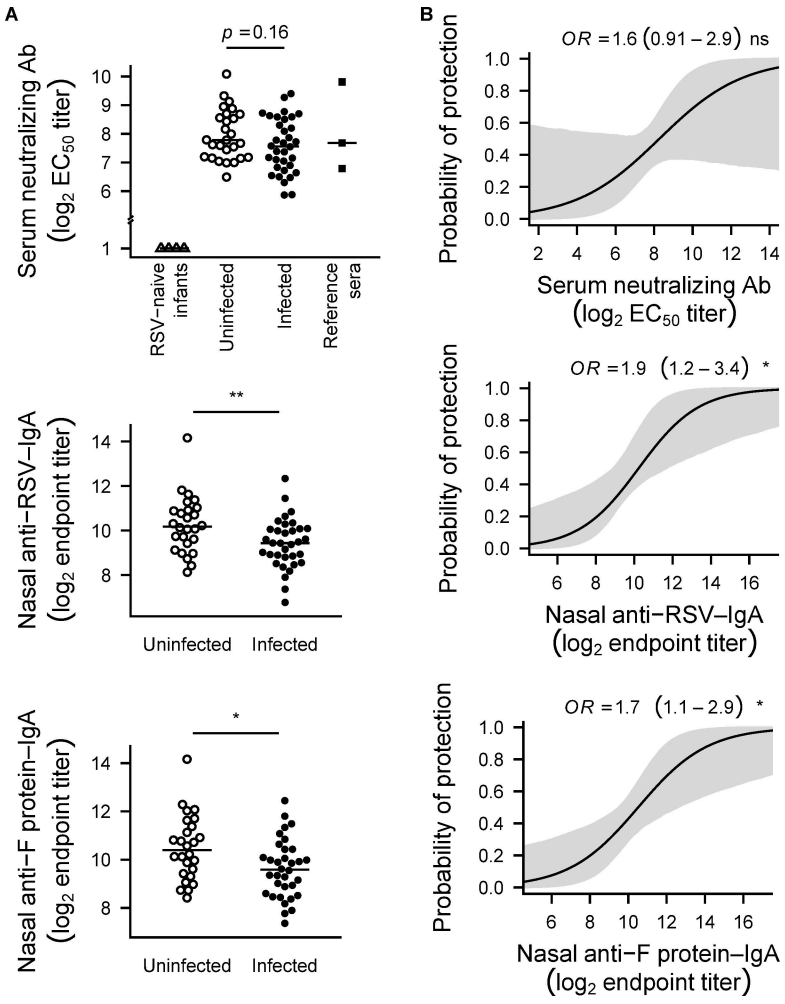


Figure 2

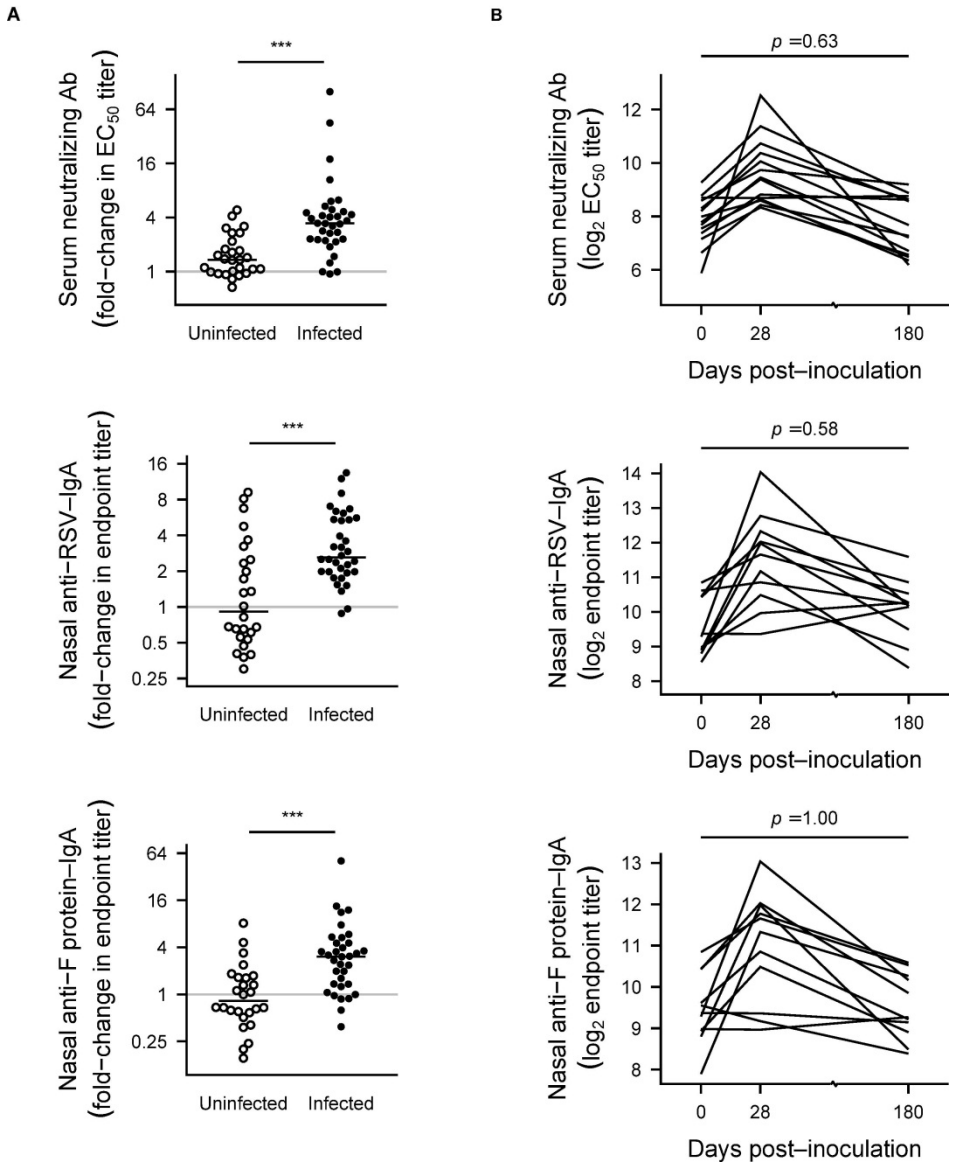

A
B
***
Serum neutralizing Ab (fold−change in EC50 titer)
64
16
4
1
Uninfected
Infected
Nasal anti−RSV−IgA (fold−change in endpoint titer)
16
8
4
2
1
0.5
0.25
Nasal anti−F protein−IgA (fold−change in endpoint titer)
p =0.63
Serum neutralizing Ab (log2 EC50 titer)
12
10
8
6
0
28
180
Days post−inoculation
p =0.58
Nasal anti−RSV−IgA (log2 endpoint titer)
14
13
12
11
10
9
8
p =1.00
Nasal anti−F protein−IgA (log2 endpoint titer)

Figure 3

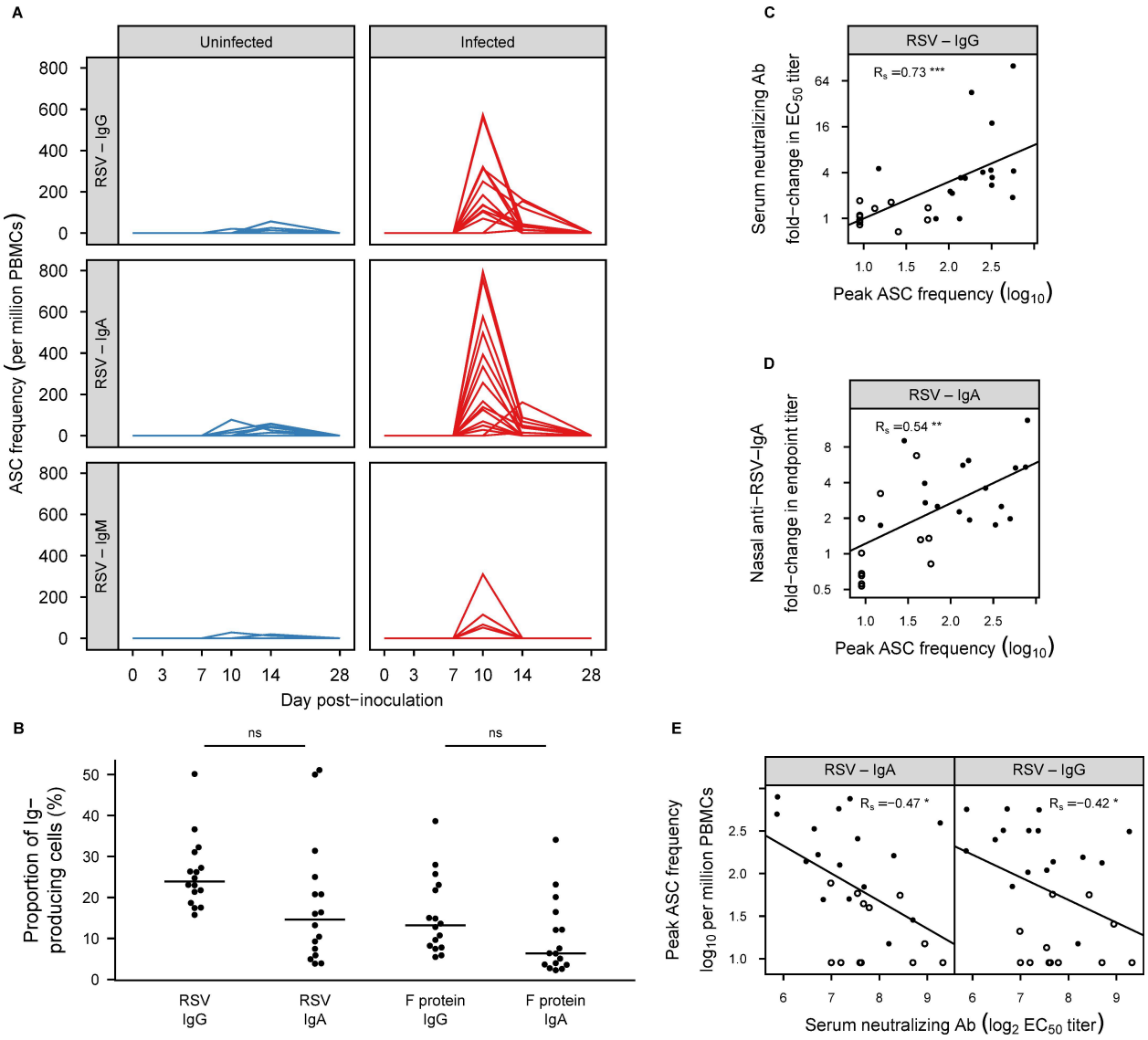

A
Uninfected
Infected
RSV – IgG
RSV – IgA
RSV – IgM
ASC frequency (per million PBMCs)
Day post−inoculation
B
ns
ns
Proportion of Ig−producing cells (%)
RSV IgG
RSV IgA
F protein IgG
F protein IgA
C
RSV – IgG
R_s =0.73 ***
Serum neutralizing Ab fold−change in EC_{50} titer
Peak ASC frequency (log_{10})
D
RSV – IgA
R_s =0.54 **
Nasal anti−RSV−IgA fold−change in endpoint titer
Peak ASC frequency (log_{10})
E
RSV – IgA
RSV – IgG
R_s =−0.47 *
R_s =−0.42 *
Peak ASC frequency log_{10} per million PBMCs
Serum neutralizing Ab (log_2 EC_{50} titer)

Figure 4

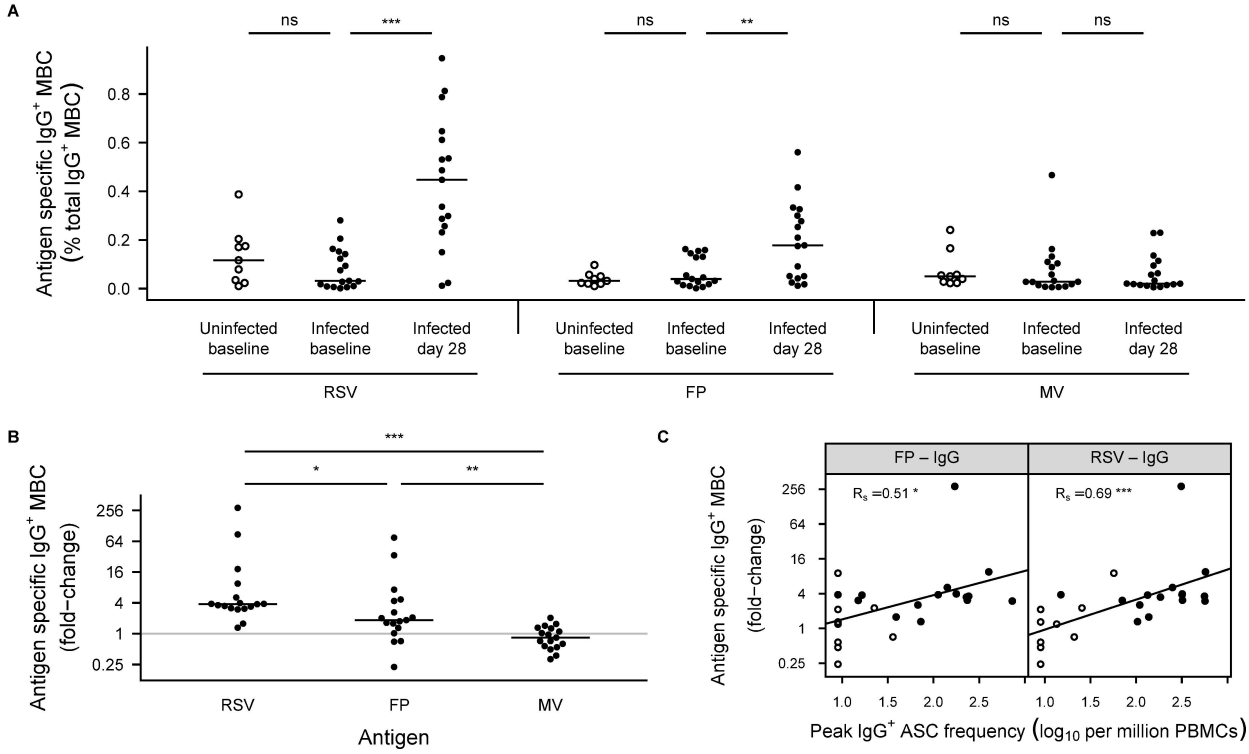
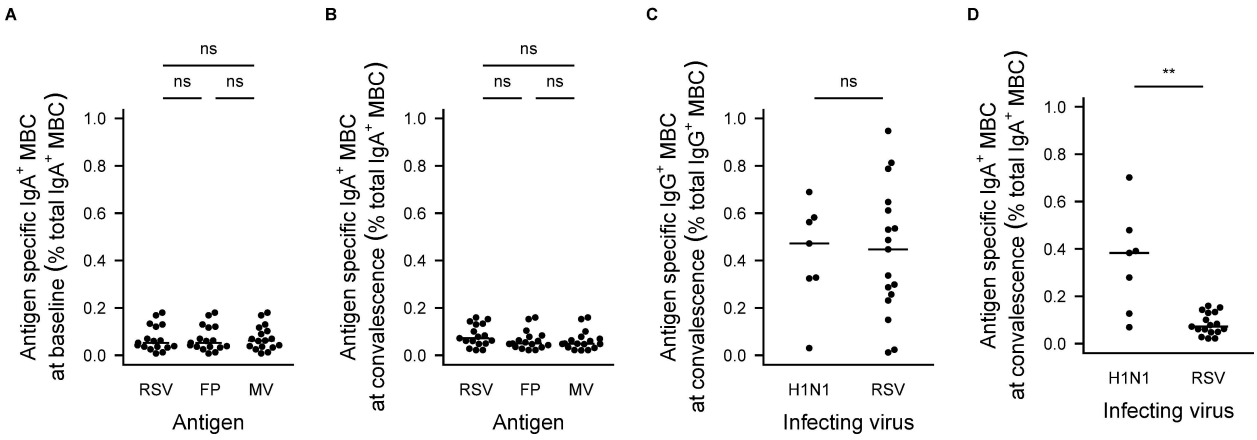


Figure 5



Online data supplement

Title: Defective IgA B cell memory impairs local antibody-mediated immunity to human respiratory syncytial virus

Authors: Maximillian S. Habibi, Agnieszka Jozwik, Spyridon Makris, Jake Dunning, Allan Paras, The MOSAIC Investigators, John P. DeVincenzo, Cornelis A.M. de Haan, Jens Wrammert, Peter J.M. Openshaw and Christopher Chiu

Supplementary methods

Symptom scores and clinical cold

Eight symptoms were evaluated in the symptom diary: nasal discharge, nasal congestion, sneezing, cough, sore throat, headache, feverishness and fatigue. Subjects were asked to score each symptom 0 (absent), 1 (mild), 2 (moderate), 3 (severe). A clinical cold was defined as 2 out of 3 of: a cumulative 14-day symptom score of ≥14; a subjective feeling of a cold; nasal discharge for ≥3 days. Symptoms were evaluated over a longer period than in Jackson's original description to account for an anticipated slower onset and longer duration of illness with RSV compared with rhinovirus (E1).

Viruses and viral antigens

RSV A Memphis 37 (Meridian Lifesciences, Memphis, Tennessee, US) was used for challenge (E2, E3). Doses of 10^3 to 10^6 PFU had previously been equally effective at producing symptomatic infection in healthy adults. RSV antigen lysates were produced by three passages in HEp-2 cells followed by lysis in 1% Triton-X (Sigma). Uninfected cells were used to produce HEp-2 control antigen. Protein concentrations of the RSV and HEp-2 lysates were 7.7mg / mL and 12.0mg / mL respectively. RSV antigen titer was estimated by ELISA to be 1:16,000. UV-inactivated influenza A/H1N1/England/195 was a gift from Wendy Barclay, Imperial College London.

Purified RSV F protein was made from a cDNA clone encoding the RSV F ectodomain (residues 26 to 513; Genbank accession number AAS93654.1) using human-preferred codons (GenScript USA Inc.), cloned into a pCD5 expression vector containing a triple Strep-tag II (IBA,

Germany). This was transfected into HEK293T cells (ATCC CRL-11268) as described. F protein was purified using Strep-tactin Sepharose beads (IBA, Germany).

Sample collection

Nasal washes were collected by instillation of 0.9% saline and washing the nose 10 times. Aliquots were snap frozen for later RNA extraction and remaining supernatants stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll Paque PLUS (GE Healthcare) according to the manufacturer's instructions and re-suspended in fetal calf serum (FCS)/10% dimethylsulphoxide for storage in liquid nitrogen. Serum was stored at −80°C.

RSV detection

RSV was detected in nasal lavage by real-time polymerase chain reaction (RT-PCR) for nucleoprotein (N) gene as previously described (E4) except that random hexamer primers (Qiagen) were used for cDNA synthesis. RSV N-gene copy number was estimated using a DNA plasmid standard curve. All subjects additionally had nasal lavage tested for a panel of respiratory viruses by multiplex PCR at day 0 and +7.

Antibody assays

Serum neutralizing antibody titer was determined by plaque reduction in HEp-2 cells. Serum samples were heat-inactivated and triplicate 2-fold serum dilutions were made in Dulbecco's modified Eagle's medium (DMEM, Sigma). These were subsequently added to confluent HEp-2 monolayers. Media alone was used as a non-neutralizing negative control and palivizumab (Synagis, Medimmune) as a positive neutralization control. Plates were incubated for 48 hours and monolayers fixed with methanol+2% H_2O_2. RSV was detected by immunoperoxidase

staining as described (E5). Plaques were counted using the AID iSpot Reader (Oxford Biosystems Cadama, UK) with manual verification.

Nasal IgA titer was determined by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates (Nunc Maxisorb, Thermofisher Scientific) were coated with 5 μg/mL RSV antigen or 0.1 μg/mL F protein. Two-fold dilutions of nasal lavage supernatant were made in PBS/1% bovine serum albumin (BSA). A serum sample with a previously determined titer of RSV IgA was used as a positive control. Diluted lavage supernatant was added to each well and incubated overnight at room temperature. Plates were developed using peroxidase-conjugated anti-human IgA (Sigma A0295) followed by tetramethylbenzidine (Sigma). Absorption at 450nm was measured on the SpectraMAX 384 PLUS device (Molecular Devices). Endpoint titers were calculated using the highest titer exhibiting an optical density of >2x the plate background. Observed endpoint titers were corrected for dilution using the ratio of serum to nasal lavage urea before analysis as described (E6), except urea was measured using the Urea Assay Kit (Abcam ab83362).

Detection of antibody secreting cells by B cell ELISpot

Antibody secreting cells (ASCs) were detected using enzyme-linked immunospot (ELISpot). Briefly, 96-well plates (MSHAN4B50 Millipore, UK) were coated anti-human IgG, IgA, IgM (Jackson Immunoresearch), RSV antigen, F protein, or UV-inactivated influenza virus. Fifty-thousand PBMCs were added to duplicate wells, serial 3-fold dilutions were made in Roswell Park Memorial Institute (RPMI) medium/10% FCS and plates were incubated for 16 hours. For memory B cells, additional plates were coated with 10μg/mL measles antigen (Meridian Lifesciences), and 5μg/mL HEp-2 antigen, 2.5μg/mL keyhole limpet hemocyanin (KLH, Sigma), and media as negative controls. Duplicate serial 5-fold dilutions were made and cells were

incubated for 4 hours at 37°C. ASCs were detected using biotinylated anti-human IgG (Jackson Immunoresearch), IgA, or IgM (both Life Technologies), horseradish peroxidase-avidin D (Vector Labs) and 3-amino-9-ethylcarbazole (Sigma). Spots were counted on the AID iSpot Reader. Total ASCs were expressed as spot-forming cells / 106 PBMC and antigen-specific ASCs as % of total Ig-secreting cells.

Polyclonal activation of memory B cells

PBMCs were cultured in vitro according to the method of Crotty *et al* (E7). Briefly, PBMCs were incubated for 5 days with a mitogen mix containing, pokeweed mitogen extract (kindly donated by Rafi Ahmed, Emory Vaccine Center), CpG ODN 2006 (Qiagen) and S.aureus Cowan protein A (Sigma). The alternative polyclonal activation mix described by Tengvall et al. was used in a subset of samples (E8). ASCs were detected by B cell ELISpot as above. Subjects exhibiting a total Ig+ cell responses the below the 10th centile in either the day 0 or day 28 sample for either IgG or IgA were excluded (n = 9) as inadequate responders to stimulation.

Logistic regression model of protective effect of antibody

Binary response variables (infected vs. uninfected) were related to continuous explanatory variables ($\log_2$ antibody titer) by a generalized linear model (GLM) using a binomial (logit) link function (logistic regression). Odds ratios (OR) and 95% confidence intervals (CI) of the OR for the explanatory variables were calculated. The GLM was used to predict the probability of protection (i.e. being uninfected) for a given antibody titer. A grid of 120 points of antibody titer across the range of theoretical values from lowest to highest observed titer was used to produce the prediction curves (solid lines of Fig. 1B). Point-wise 95% confidence intervals for the predicted response curves were derived by a bootstrap percentile method. Briefly, 5,000 bootstrap replicates of the data were generated by random sampling with replacement, a GLM

was fitted to each replicate, and the predicted response values for the 120-point antibody titer grid derived, producing a 120 by 5,000 matrix of predicted response values. The 2.5^{th} and 97.5^{th} centiles for each row of the matrix were extracted yielding the lower and upper bound of the estimated 95% CI for the predicted response at that level of antibody titer (shaded region of Fig. 1B).

Supplementary references

E1. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the Common Cold to Volunteers Under Controlled Conditions: I. The Common Cold as a Clinical Entity. *Arch Intern Med* 1958;101:267–278.

E2. DeVincenzo JP, Wilkinson T, Vaishnaw A, Cehelsky J, Meyers R, Nochur S, Harrison L, Meeking P, Mann A, Moane E, Oxford J, Pareek R, Moore R, Walsh E, Studholme R, Dorsett P, Alvarez R, Lambkin-Williams R. Viral load drives disease in humans experimentally infected with respiratory syncytial virus. *Am J Respir Crit Care Med* 2010;182:1305–1314.

E3. Kim Y-I, DeVincenzo JP, Jones BG, Rudraraju R, Harrison L, Meyers R, Cehelsky J, Alvarez R, Hurwitz JL. Respiratory Syncytial Virus Human Experimental Infection Model: Provenance, Production, and Sequence of Low-Passaged Memphis-37 Challenge Virus. *PloS One* 2014;9:e113100.

E4. Perkins SM, Webb DL, Torrance SA, El Saleeby C, Harrison LM, Aitken JA, Patel A, DeVincenzo JP. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. *J Clin Microbiol* 2005;43:2356–2362.

E5. Cannon MJ. Microplaque immunoperoxidase detection of infectious respiratory syncytial virus in the lungs of infected mice. *J Virol Methods* 1987;16:293–301.

E6. Rennard SI, Basset G, Lecossier D, O'Donnell KM, Pinkston P, Martin PG, Crystal RG. Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J Appl Physiol Bethesda Md 1985* 1986;60:532–538.

E7. Crotty S, Aubert RD, Glidewell J, Ahmed R. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. *J Immunol Methods* 2004;286:111–122.

E8. Tengvall S, Lundgren A, Quiding-Järbrink M, Svennerholm A-M. BAFF, stimulatory DNA and IL-15 stimulates IgA+ memory B cells and provides a novel approach for analysis of memory responses to mucosal vaccines. *Vaccine* 2010;28:5445–5450.

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Supplementary Tables

Predicted probability of protection (%)	**% of subjects achieving at least this level at convalescence**	
	Anti-RSV-IgA	**Anti-F-IgA**
100	0	0
80	21	9
60	56	41
40	79	76
20	100	97
0	100	100

Supplementary Table E1. Proportions of individuals achieving levels of nasal anti-RSV IgA post-infection associated with stratified probability of protection.

Supplementary Figure Legends

Supplementary Fig. E1. Study design and infection rate

(**A**) Volunteers were inoculated with RSV A M37 and followed up with blood and nasal sampling up to day +180. (**B**) RSV was detected in nasal washes by quantitative PCR. Common-cold symptoms were determined using symptom questionnaires.

Supplementary Fig. E2. Virus-specific serum IgG and IgA do not correlate with protection from RSV infection.

Serum and was taken at baseline (7-14 days pre-inoculation) and 28 days post-inoculation. Serum anti-RSV IgG and IgA and anti-fusion (F)-protein IgG and IgG antibodies were determined by ELISA. (**A**) Baseline serum IgG against whole RSV (left), and F-protein (right) antibody levels in uninfected (○) vs. infected (●) subjects. (**B**) Baseline serum IgA against whole RSV (left), and F-protein (right) antibody levels in uninfected (○) vs. infected (●) subjects. (**C**) Correlation of serum IgA and nasal IgA against whole RSV (left) and F-protein (right). P-values for unpaired Mann-Whitney-Wilcoxon (U) test or Spearman's rank correlation coefficient (Rs) are shown (ns = $P > 0.05$).

Supplementary Fig. E3. Acute plasmablasts measured by flow cytometry and correlation of RSV F-protein-specific response.

(**A**) Flow cytometric analysis of plasmablasts in whole blood following RSV M37 infection. One representative subject is shown. Plots were gated on $CD3^-/CD20^-/CD19^{int/+}$ lymphocytes and plasmablasts identified as $CD27^{hi}/CD38^{hi}$. Plasmablast frequencies are expressed as % of $CD19^{int/+}$ lymphocytes. (**B**) Correlation of fold-change in serum neutralizing antibody with peak

F-protein-specific IgG^+ ASC frequency. (**C**) Correlation of fold-change in nasal IgA against F-protein with peak IgA^+ ASC frequency (data from 27 subjects; nasal IgA in one subject could not be measured). (**D**) Correlation of peak F-protein-specific ASC frequency with pre-infection baseline serum neutralizing antibody titers. ASC frequencies are expressed as $\log_{10}$ per million PBMC. P values for unpaired Mann-Whitney-Wilcoxon (U) tests and or Spearman's rank correlation coefficient (Rs) are shown as appropriate. (○) Uninfected subjects, (●) infected subjects. FP: purified RSV fusion protein.

Supplementary Fig. E4. Peak plasmablast frequencies following RSV infection are not associated with viral load or severity of symptoms.

Antigen-specific ASCs were identified ex vivo by B cell ELISpot and frequencies are expressed as % total Ig^+ cells. (**A**) Correlation of peak ASC frequency with cumulative daily viral load (trapezoidal area under curve). (**B**) Correlation of peak ASC frequency with cumulative daily upper respiratory tract (URT) symptom score (trapezoidal area under curve). Spearman's rank correlation coefficient (Rs) is shown. All correlations non-significant.

Supplementary Fig. E5. Pre-existing RSV specific memory B cells have no impact on symptoms, viral load or antibody titers.

PBMCs from days 0 and 28 post-inoculation were polyclonally activated in vitro. Total and antigen-specific immunoglobulin-producing cells were revealed using B-cell ELISpot. MBC frequencies are expressed as % total Ig^+ cells. Correlation between baseline antigen-specific IgG^+ MBC frequencies and (**A**) cumulative symptoms scores, (**B**) cumulative daily viral load, (**C**)

serum neutralizing antibody, (**D**) nasal anti-RSV IgA, and (**E**) nasal anti-F IgA at baseline. Spearman's rank correlation coefficient (Rs) is shown. FP: purified RSV fusion protein.

Supplementary Fig. E6 No difference is seen in RSV specific memory B cells when polyclonally stimulating with an alternative mitogen mix.

PBMCs from 6 subjects demonstrating robust total and antigen-specific IgG^+ MBC activation following the method of Crotty *et al.* (SAC + PWM) were selected for alternative in vitro stimulation using recombinant human BAFF, IL-15, and CpG ODN 2006. Total (**A**) IgA^+ and (**B**) IgG^+ MBCs (day 0 and day 28 combined) following stimulation with both methods. (**C**) RSV specific IgA^+ MBCs at day 0 and day 28 post-infection. (**D**) RSV specific IgA MBC for day 0 and day 28 combined comparing two MBC activation methods. P-values for unpaired Mann-Whitney-Wilcoxon (U) test are shown as appropriate (ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). CpG: CpG oligodeoxynucleotide 2006. rBAFF: recombinant human B cell activating factor. rIL-15: recombinant human interleukin 15. SAC: protein A from Staphylococcus aureus Cowan. PWM: pokeweed mitogen. Both MBC activation methods received CpG ODN 2006 at the same concentration.

Supplementary Figures

Figure E1

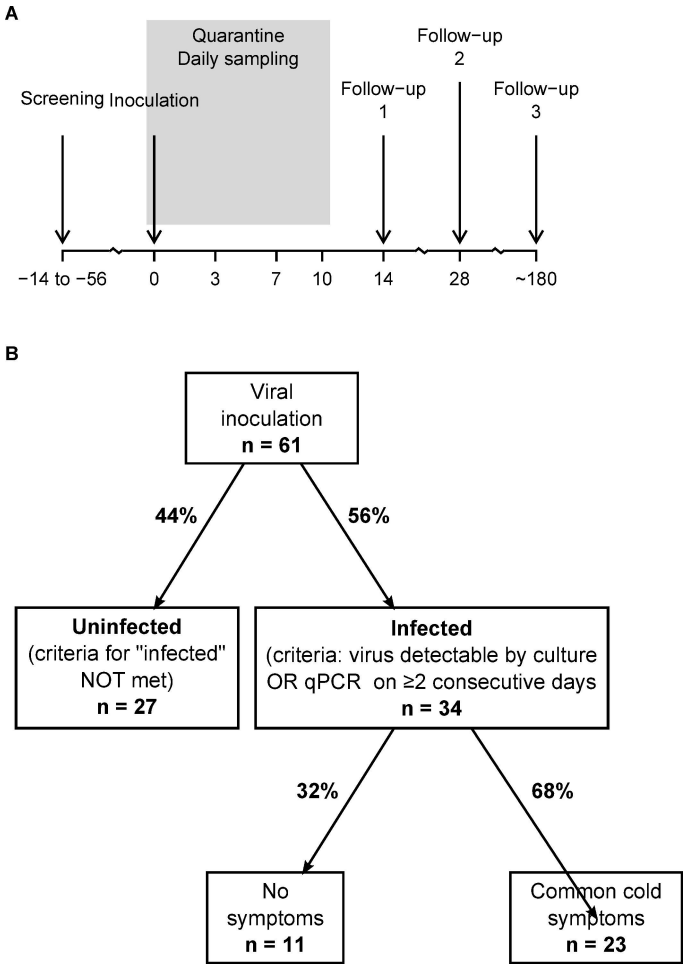


Figure E2

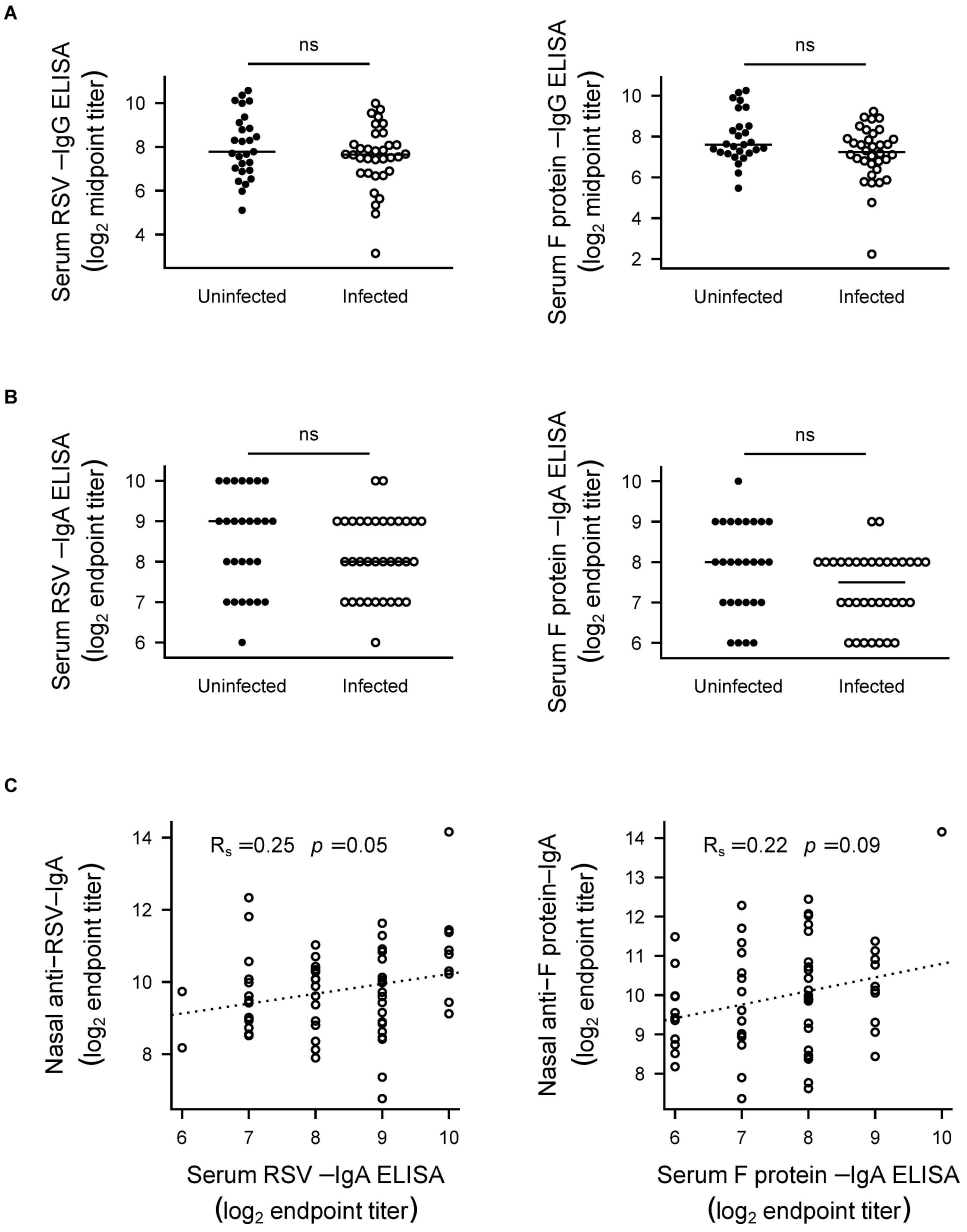

A
Serum RSV –IgG ELISA
(log2 midpoint titer)
ns
Uninfected
Infected
Serum F protein –IgG ELISA
(log2 midpoint titer)
ns
Uninfected
Infected
B
Serum RSV –IgA ELISA
(log2 endpoint titer)
ns
Uninfected
Infected
Serum F protein –IgA ELISA
(log2 endpoint titer)
ns
Uninfected
Infected
C
Nasal anti−RSV−IgA
(log2 endpoint titer)
Rs =0.25 p =0.05
Serum RSV –IgA ELISA
(log2 endpoint titer)
Nasal anti−F protein−IgA
(log2 endpoint titer)
Rs =0.22 p =0.09
Serum F protein –IgA ELISA
(log2 endpoint titer)

Figure E3

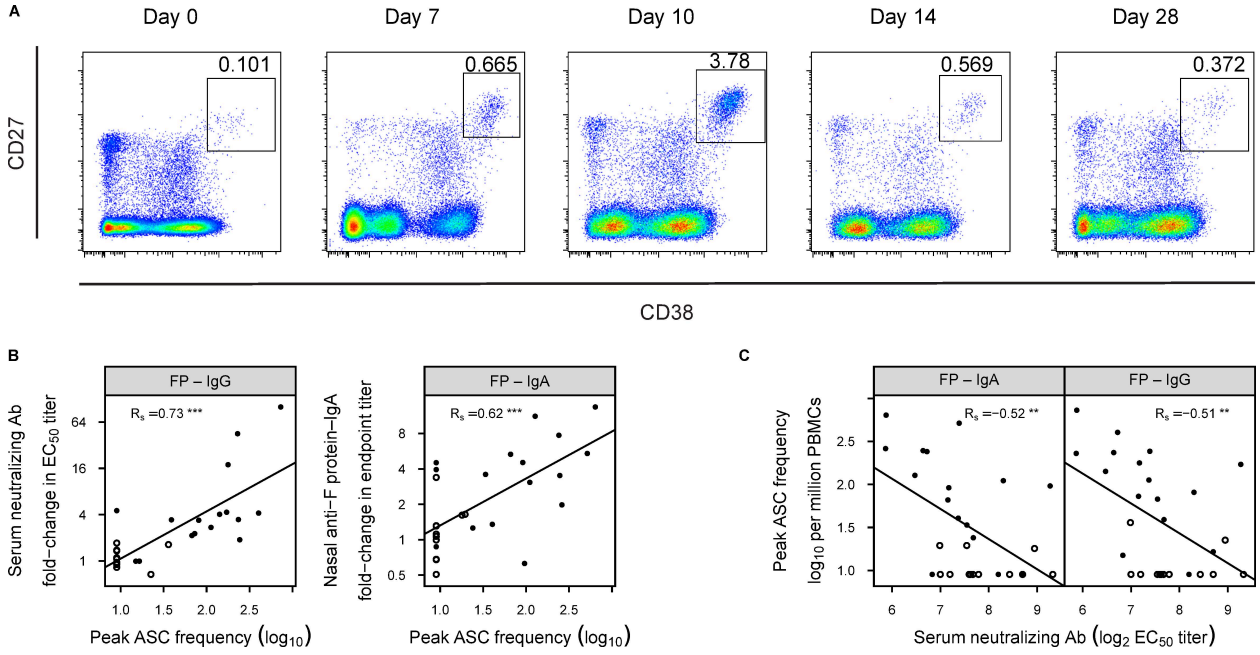

A
Day 0
Day 7
Day 10
Day 14
Day 28
0.101
0.665
3.78
0.569
0.372
CD27
CD38
B
FP – IgG
Rs =0.73 ***
Serum neutralizing Ab fold−change in EC50 titer
Peak ASC frequency (log10)
FP – IgA
Rs =0.62 ***
Nasal anti−F protein−IgA fold−change in endpoint titer
Peak ASC frequency (log10)
C
FP – IgA
Rs =−0.52 **
FP – IgG
Rs =−0.51 **
Peak ASC frequency log10 per million PBMCs
Serum neutralizing Ab (log2 EC50 titer)

Figure E4

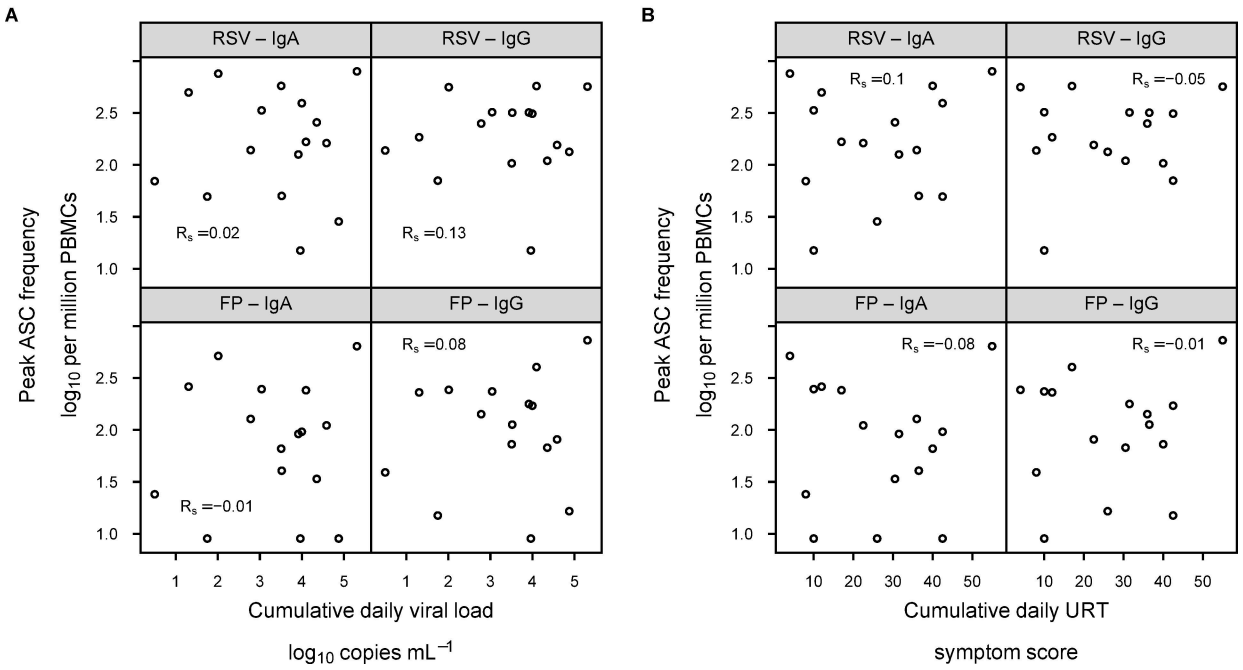

A
RSV – IgA
RSV – IgG
FP – IgA
FP – IgG
Peak ASC frequency
log10 per million PBMCs
Rs =0.02
Rs =0.13
Rs =0.08
Rs =−0.01
Cumulative daily viral load
log10 copies mL−1
B
Rs =0.1
Rs =−0.05
Rs =−0.08
Rs =−0.01
Cumulative daily URT
symptom score

Figure E5

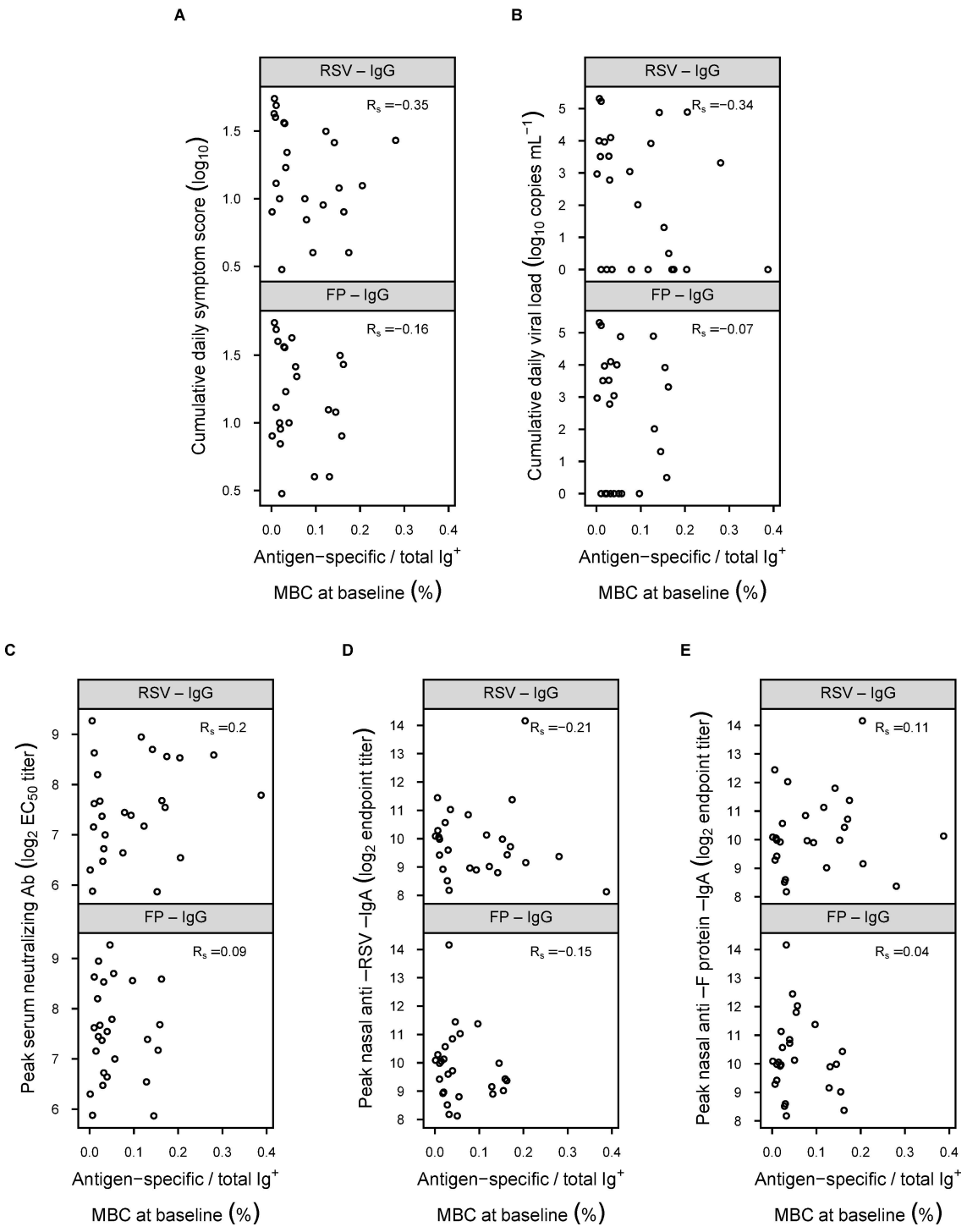
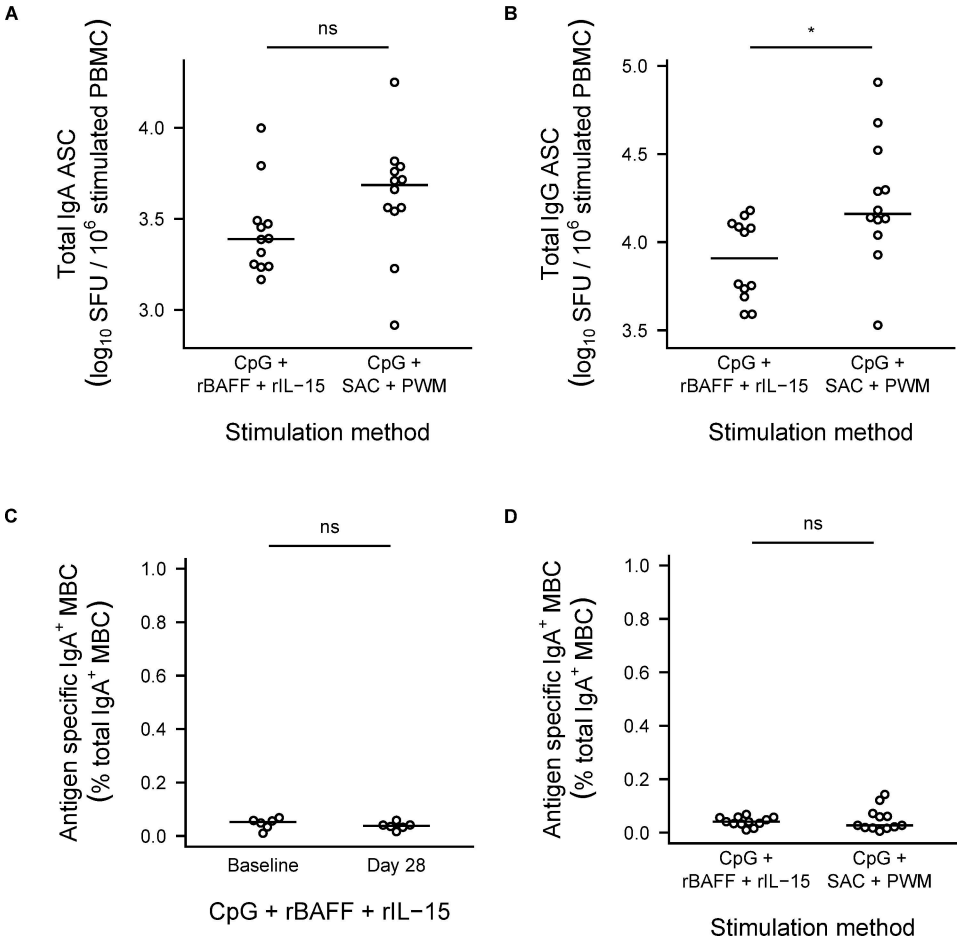

A
B
C
D
E
RSV – IgG
FP – IgG
Cumulative daily symptom score (log10)
Cumulative daily viral load (log10 copies mL−1)
Peak serum neutralizing Ab (log2 EC50 titer)
Peak nasal anti –RSV –IgA (log2 endpoint titer)
Peak nasal anti –F protein –IgA (log2 endpoint titer)
Rs =−0.35
Rs =−0.16
Rs =−0.34
Rs =−0.07
Rs =0.2
Rs =0.09
Rs =−0.21
Rs =−0.15
Rs =0.11
Rs =0.04
Antigen−specific / total Ig+
MBC at baseline (%)

Figure E6


A
ns
Total IgA ASC
(log10 SFU / 10^6 stimulated PBMC)
4.0
3.5
3.0
CpG + rBAFF + rIL−15
CpG + SAC + PWM
Stimulation method
B
*
Total IgG ASC
(log10 SFU / 10^6 stimulated PBMC)
5.0
4.5
4.0
3.5
CpG + rBAFF + rIL−15
CpG + SAC + PWM
Stimulation method
C
ns
Antigen specific IgA+ MBC
(% total IgA+ MBC)
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
Day 28
CpG + rBAFF + rIL−15
D
ns
Antigen specific IgA+ MBC
(% total IgA+ MBC)
1.0
0.8
0.6
0.4
0.2
0.0
CpG + rBAFF + rIL−15
CpG + SAC + PWM
Stimulation method