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**Immune correlates of protection against RSV following Ad26.RSV.preF–RSV preF
vaccination of adults aged >60 years**

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ABSTRACT

Respiratory syncytial virus (RSV) is a leading cause of respiratory illness and there is no universally accepted serological correlate of protection (CoP). We evaluated serum pre-fusion immunoglobulin G binding (preF-IgG) and RSV-A2 neutralizing antibodies (RSV-nAb) in adults aged >60 years following vaccination with investigational Ad26.RSV.preF–RSV preF vaccine. PreF-IgG and RSV-nAb measured pre-vaccination (Day 1) and Day 15 post-vaccination were assessed as CoPs against RSV-mediated acute respiratory infection (ARI) and lower respiratory tract disease (LRTD). An increase in both preF-IgG and RSV-nAb at Day 15 was significantly associated with reduced occurrence of RSV-mediated ARI and LRTD during follow-up. Based on controlled vaccine-efficacy curves and Prentice criteria, both markers were identified as CoPs for RSV-mediated ARI and LRTD. These results were validated using samples from a second randomized, phase 3 efficacy trial. Comprehensive profiling of preF-specific antibody responses showed that preF IgG3 and IgG2 may also contribute to protection from RSV.

INTRODUCTION

Respiratory syncytial virus (RSV) is a highly contagious pathogen and a major cause of acute respiratory infections (ARIs) worldwide, with >60 million cases reported annually.¹ Two major antigenic subtypes of RSV exist, A and B, whose predominance varies during different epidemic seasons. While RSV infection typically causes mild upper respiratory tract disease in healthy adults, it poses a greater risk of severe lower respiratory tract disease (LRTD) in vulnerable populations, such as young children, older adults, and individuals with underlying comorbidities, and can lead to hospitalization and death in some cases.²⁻⁵ The recent United States Food and Drug Administration approval of three RSV vaccines for adults ages 60 years and older⁶ holds promise for the prevention of RSV-mediated LRTD in older adults.

The investigational Ad26.RSV.preF–RSV preF vaccine is a combination of a recombinant, replication-incompetent, adenovirus serotype 26 vector encoding a conformation-stabilized RSV pre-fusion F-protein (preF; Ad26.RSV.preF) and recombinant RSV preF, with no adjuvant. In clinical trials involving adults over 60 years of age, Ad26.RSV.preF–RSV preF showed strong humoral and cellular RSV-specific responses, with durable RSV-specific neutralizing, binding, and T-cell responses up to 1.5 years post-vaccination.⁷⁻⁹ Importantly, the vaccine was efficacious against RSV-mediated LRTD in a phase 2b study (CYPRESS, ClinicalTrials.gov identifier: NCT03982199 registered 10 Jun 2019)^{8,9} and in a phase 3 study (EVERGREEN, NCT04908683 registered 27 May 2021).¹⁰

Although to date there is no universally accepted correlate of protection (CoP) against RSV infection, a growing body of evidence from clinical and pre-clinical studies indicates that preF binding antibody (bAb) and neutralizing antibody (nAb) titers,¹¹⁻¹⁴ along with RSV-specific T-cell responses,¹⁵⁻¹⁷ play an important role in conferring protective immunity. Low levels of nAbs

may predispose older frail adults to RSV infection.¹² A human challenge study in 18 to 50 year-olds demonstrated that nAb titers against the RSV A2 strain following immunization with the Ad26.RSV.preF component alone reduced the risk of RSV infection and reduced symptom severity when infection occurred.¹⁸ Other human-infection challenge studies have implicated low levels of pre-existing mucosal immunoglobulin (Ig) A in older adults with susceptibility to infection.^{16,19} Serum IgG and nAbs correlated with RSV infection risk in 60–75 year-olds, but not in 18 to 55 year-olds.¹⁹ Mucosal and serum antibodies appear to be induced and offer protection by different mechanisms.¹¹

Other studies have provided insights into other antibody-mediated mechanisms of protection against viral infections, such as antibody effector functions. Non-nAb effector functions were shown to contribute to protective immunity against several viruses, such as human immunodeficiency virus,^{20,21} influenza,²² Ebola,²³ and RSV.²⁴ In a RSV challenge study, antibody-dependent Fc profiles, particularly IgG-driven Fc functions and IgA-mediated nAb activity, were determined to play a crucial role in protection against RSV re-infection.²⁴ The same study showed that vaccine-induced antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis, and antibody-dependent neutrophil phagocytosis responses, were all associated with protection.²⁴ Despite these advances, a single serologic threshold indicative of protection in a majority of individuals has not yet been identified.

Defining biomarkers of protective immunity against RSV-mediated disease is critical for the refinement of RSV vaccine development. A reliable biomarker that correlates with protection against infection would facilitate the approval of future RSV vaccines by providing an immunologic endpoint in lieu of large efficacy trials as a basis for licensure. Here we assessed anti-preF bAb and nAb against the RSV-A2 strain as CoPs against RSV-mediated ARI and

LRTD (a subset of patients with ARI) in two clinical studies. Additionally, we evaluated the contribution of other immune responses, such as Fc-mediated antibody activity, to the protection conferred by the vaccine-induced immune response.

RESULTS

Cohorts for the analysis of immune correlates

The primary assessment of immune correlates was performed on case-control cohorts selected from each study based on the measurement of preF bAb levels and RSV nAb titers at before (Day 1) and after (Day 15) vaccination with investigational Ad26.RSV.preF–RSV preF vaccine. Thus, only participants with Days 1 and 15 data available for preF bAb (preF IgG) levels and/or nAb titers were included in the case-control cohorts. PreF bAb levels and RSV nAb titers were secondary endpoints measured with validated assays as previously described.²⁵ All analyses were limited to participants in the per-protocol efficacy population.

The case-control cohort in CYPRESS consisted of all 56 study participants (placebo and vaccine groups) with a reverse-transcriptase polymerase chain reaction (RT-PCR)-confirmed RSV ARI recorded during the first RSV season after vaccination (cases), and 438 participants (placebo and vaccine groups) with no serological evidence of RSV ARI during the first RSV season (controls). The EVERGREEN case-control cohort comprised all 259 study participants (placebo and vaccine groups) who had RT-PCR-confirmed RSV ARI recorded up to final analysis (cases), and 1515 participants (placebo and vaccine groups) in the per-protocol efficacy set with no clinical or serological evidence of RSV detected during the study (controls) (**Table 1**).¹⁰ Clinical definitions of ARI and LRTD are provided in the Supplementary Material.

Detailed information about each cohort is provided in the Statistical Analysis Plan provided as Supplementary Material. Analyses were performed using inverse probability of selection weights to adjust for the case-cohort sampling design, missing data in RSV ARI cases, and the non-random sampling of controls.

Participant demographic characteristics

Demographic characteristics of the participants were similar in the placebo and vaccine groups of the CYPRESS and EVERGREEN studies and are described elsewhere.⁸⁻¹⁰

Day 15 serum PreF bAb levels and RSA-A2 nAb titers are lower in RSV cases versus Control

Primary immune markers for analysis were levels of serum preF bAb and nAb against RSV-A2. In the CYPRESS study, Day 15 preF bAb levels were significantly lower in RSV ARI cases (placebo and vaccine groups) compared with controls (**Fig. 1A**). Day 15 RSV-A2 nAb also tended to be lower in RSV ARI cases than controls (P value <0.002 in placebo groups, and P value = 0.137 in vaccine groups; **Fig. 1B**). Pre-vaccination preF bAb levels and nAb titers were significantly lower in RSV ARI cases than in controls in both placebo and vaccine groups (**Fig. 1C**).

Similar results were confirmed in the EVERGREEN study, with significantly lower bAb levels and nAb titers at Day 15 in RSV ARI cases compared with controls in both the placebo and vaccine groups (**Fig. 1**). Pre-vaccination (Day 1) preF bAb levels and nAb titers were significantly lower in cases compared with controls in the placebo group, but not in the vaccine group (**Fig. 1C**). Fold increases in preF bAb or RSV-A2 nAb from Day 1 to Day 15 were not

significantly different in cases compared with controls in either study (**Fig. 1C**). We observed similar findings in RSV LRTD cases (**Fig. S1**).

In addition to measuring bAb levels and nAb titers against the RSV-A2 strain, we also measured preF bAb against the RSV-B subtype in EVERGREEN and nAb against the RSV-B subtype in CYPRESS. bAb and nAb against RSV-A and RSV-B strongly correlated with each other (Day 15 correlations of 0.98 and 0.93, respectively; **Fig. S2**). With this high correlation, the effects of bAb and nAb against RSV-A and RSV-B cannot be statistically distinguished from each other. Consequently, subsequent analyses focused on bAb and nAb against the RSV-A antigen, as it matches the vaccine immunogen.

Day 15 serum preF bAb levels and RSV-A2 nAb titers are inversely correlated with RSV infection risk

There was an inverse association between RSV ARI and LRTD risk and increasing preF bAb levels and RSV-A2 nAb titers at Day 15 in the CYPRESS study, which was also observed in the EVERGREEN study (**Fig. 2, Fig. S3**). There was evidence of a continuum across the range of bAb levels and nAb titers observed in unvaccinated and vaccinated participants, suggesting that the association was independent of vaccination.

Both markers were significant predictors of RSV ARI and LRTD in the vaccine and placebo groups, as confirmed by logistic regression modelling (CYPRESS) and Cox regression modeling adjusted for baseline risk scores (EVERGREEN; **Fig. S3C**). Day 15 values were more predictive

of infection risk than those at Day 1 and were more predictive than the fold increase from Day 1 to Day 15 (**Fig. S3C**).

We explored the independent contribution of parameters that were correlated with RSV risk. Including the effects of both Day 15 preF bAb levels and RSV-A2 nAb titers in the EVERGREEN Cox regression model of RSV ARI or LRTD did not significantly improve the model fit compared with the models of individual parameters with only Day 15 preF bAb levels (**Table S2.a**) adjusted for baseline risk scores. Further, a strong correlation was observed between preF bAb levels and RSV-A2 nAb titers (weighted Pearson correlation coefficient, 0.91 in CYPRESS and 0.93 in EVERGREEN; **Fig. S4**). These results suggest that both Day 15 preF bAb levels and Day 15 RSV-A2 nAb titers are correlates of risk for RSV ARI and LRTD but are not independent predictors.

We next assessed whether adding preF bAb levels and RSV-A2 nAb titers at Day 1, the fold rise from Day 1 to Day 15, or the value measured at Days 3 to 5 of an ARI event would improve the fit of the EVERGREEN Cox regression model that already contained the Day 15 immune marker value as a predictor. Immune markers at Days 3 to 5 of an ARI event were measured in a subset of the vaccinated participants of the immune correlate analysis for whom samples were available (**Table S1**). The model fit did not significantly improve preF bAb levels nor RSV-A2 nAb titers when adding immune marker responses at Day 1, the fold rise from Days 1 to 15, or at Days 3 to 5 of an ARI (**Table S2.b**). Therefore, only Day 15 preF bAb levels and Day 15 RSV-A2 nAb titers were further assessed as potential CoPs.

We used the maximal chi-square method to identify a potential threshold protective value.

However, no protective threshold for the Day 15 preF bAb levels or RSV-A2 nAb titers was

identified in either study. The data were more consistent with a steady decrease in RSV ARI/LRTD risk with increasing Day 15 immune marker responses (**Table S3, Table S4**).

Vaccine efficacy increases with Day 15 bAb levels or nAb titers

We estimated the vaccine efficacy over different post-vaccination bAb levels and nAb titers using the principal surrogate framework.²⁶ Vaccine efficacy was defined as 1– the relative risk of RSV in vaccine recipients compared with “comparable” placebo recipients. In this calculation, “comparable” refers to placebo recipients who would have attained the same biomarker level if they had been vaccinated, based on baseline immunogenicity predictors.²⁷ **Fig. 3** and **Fig. S5** show the estimated vaccine efficacy against RSV ARI and LRTD across a range of preF bAb and Day 15 RSV-A2 nAb antibody levels. For both markers, estimated vaccine efficacy against RSV ARI and LRTD rose with increasing antibody levels. In CYPRESS, this increase was greatest for bAb: at the 10th and 90th percentiles of the bAb distribution at Day 15 after vaccination, the estimated vaccine efficacy was 50.8% and 89.8%, respectively (**Fig. 3C**). The increase with increasing nAb titers was less pronounced: at the 10th and 90th percentiles of the nAb distribution at Day 15 after vaccination, the estimated vaccine efficacy was 60.7% and 81.5%, respectively. In EVERGREEN, at the 10th and 90th percentiles of the bAb distribution at Day 15 after vaccination, the estimated vaccine efficacy was 38.1% and 75.3%, respectively. At the 10th and 90th percentiles of the nAb distribution at Day 15 after vaccination, the estimated vaccine efficacy was 40.5% and 73.0%, respectively.

We then evaluated the value of Day 15 preF bAb levels or Day 15 RSV-A2 nAb titers as CoPs by assessing if these markers conform to the Prentice criteria.²⁸ Both biomarkers met Prentice criteria for RSV ARI and LRTD in CYPRESS and in EVERGREEN (**Table 2**). Therefore, based on the vaccine efficacy curves and Prentice criteria, both preF bAb levels and Day 15 RSV-A2

nAb titers measured at Day 15 post-vaccination can be considered CoPs for RSV ARI and LRTD.

preF IgG3 levels at Day 15 contribute to protection against RSV ARIs in addition to RSV nAb Titers

Fold increases in preF bAb or nAb from Day 1 to Day 15 were not significantly different in RSV ARI cases compared with controls in either study. Therefore, we searched for other humoral features that might differentiate cases from controls. We sought to understand whether other antibody functionalities in addition to preF bAb levels and RSV-A2 nAb titers could contribute to the CoP model. In a subset of EVERGREEN cases and controls (**Fig. 4A**), we profiled preF-specific antibody responses in more detail by measuring exploratory immune markers, including preF-specific antibody isotype and subclass titers, Fc receptor binding profiles, and ADCD. By Day 15, all measured immune markers showed a significant increase after vaccination with Ad26.RSV.preF–RSV preF (**Fig. 4B, Table S5**). The responses for all immune markers measured in vaccine recipients, except IgA2, were significantly lower in RSV ARI cases compared with controls at Day 15 (**Fig. 4B, Table S5**). The responses of immune markers at Days 3 to 5 during an ARI in vaccinated participants were also lower in RSV ARI cases compared with controls (**Fig. 4B, Table S5**). Most markers for Fc receptor binding and IgG were highly and positively correlated with preF bAb levels or RSV-A2 nAb titers, and with each other post-vaccination (**Fig. 4C**, lower left panel). For other markers, low to moderate positive correlations were observed post-vaccination.

To understand whether these immune markers could contribute additional protection against RSV, on top of the confirmed RSV-A2 nAb surrogate, we evaluated the effect of each Day 15 marker on the probability of an RSV ARI. When adjusting for RSV-A2 nAb levels, IgG3

demonstrated the most significant effect in vaccinated participants, with the highest log-odds ratio (adjusted P value = 0.028; **Fig. 4D**). IgG2 showed a lower yet significant effect (adjusted P value = 0.045). None of the markers showed a significant effect in the placebo group, suggesting the effect may likely be specific to RSV vaccination. Therefore, in addition to the RSV-A2 nAb, which can be considered a CoP, preF IgG3 or IgG2 may additionally contribute to protection against RSV ARIs following RSV vaccination.

DISCUSSION

Defining the immune CoP required to protect against RSV-mediated disease is fundamental to guide the development of RSV vaccines. Numerous studies have demonstrated that bAb levels or nAb titers play an important role in conferring protective immunity against RSV infection.^{11,12,18,29} Our study demonstrated that increasing levels of either the preF bAb levels and/or the RSV-A2 nAb serum titers measured at Day 15 after Ad26.RSV.preF–RSV preF vaccination were significantly associated with a reduced risk of RSV ARI/LRTD. PreF bAb levels and RSV-A2 nAb titers were strongly correlated, indicating that these markers are not independent predictors of RSV ARI or LRTD occurrence. Based on the Prentice criteria and controlled vaccine efficacy curves, our findings indicated that both of these biomarkers can individually serve as CoPs for RSV-mediated ARI and LRTD. In addition, these biomarkers were also correlates of risk since the association with decreasing RSV ARI/LRTD incidence was also observed at baseline (Day 1 and Day 15) in placebo recipients (**Fig. 1 and Fig. S3**).

Importantly, even though titers were lower in participants who became infected compared with those in participants who remained uninfected during the follow-up period, no definitive seroprotective threshold could be established for either bAbs or nAbs, which is consistent with other studies.^{11,29} It is evident that absolute titers for both preF bAb and nAb were correlated with

protection, but there was no correlation with fold increases post-vaccination. This could be due to the variability in baseline titers in this pre-exposed population. This suggests that RSV vaccine effects should be evaluated not just by fold increases but also by absolute titers induced in pre-exposed populations, on the basis that ‘more is better’.

Neutralization is one of the many functionalities by which antibodies can interfere with viral infection.³⁰ Consistent with previous studies in humans,³¹ we showed that Ad26.RSV.preF–RSV preF vaccination elicited a strong and functional humoral immune response, inducing preF-specific IgA1, IgA2, IgG, IgG1 to G4, IgM, Fc receptor binding, and ADCD.

In addition to RSV neutralization, we previously demonstrated in a human challenge study, that other markers such as RSV-specific F phagocytosis, natural killer cell activity, IgA titers, and Fc glycan modifications were also associated with protection after vaccination with Ad26.RSV.preF.²⁴ In unvaccinated participants who naturally resisted RSV challenge, we observed that preF-specific IgG3 and neutralization were strongly enriched.²⁴ Consistent with these data, the EVERGREEN results showed that out of the functionalities measured, only IgG3 and to a lesser extent IgG2 levels post-vaccination contributed to protection against RSV ARI and LRTD in addition to RSV neutralization. It has been suggested that IgG3 exhibits the strongest effector functions amongst all IgG subclasses and is associated with protection from viral infections.³²⁻³⁶ Additional research is required to more specifically understand the contribution of IgG3 in RSV protective immunity.

Taken together, our data demonstrated that preF bAb levels and RSV-A2 nAb titers can individually serve as CoPs for RSV-mediated ARI and LRTD in older adults. The observation of a continuum in vaccine efficacy independent of whether antibodies were naturally acquired or induced by vaccination suggests that the association between these biomarkers and protection is

independent of vaccination. This could have implications for assessment of risk in unvaccinated individuals and could indicate that our findings could be relevant to immunity induced by other RSV vaccines manufactured on other platforms. While current knowledge does not support the use of CoPs as a basis for the registration of RSV vaccines, these endpoints can be used as a surrogate to optimize vaccines in terms of regimen, dosing, and so on, during early developmental stages.

Strengths of our study include the use of serum samples from two separate studies conducted in different parts of the world. Limitations are the relatively low number of RSV events observed in the vaccine group due to the high efficacy of Ad26.RSV.preF–RSV preF vaccination in preventing RSV LRTD. This may limit the power of the analyses, particularly in detecting a protective threshold. The estimated vaccine efficacies against ARI and LRTD differed between the two studies even though the preF binding titers post vaccination were similar for RSV A and RSV B proteins tested (**Fig.3** and **Fig. S5**). This is likely to be due to differences in the time to first occurrence of RSV in each study (RSV cases in the CYPRESS analysis occurred within 200 days from vaccination whereas RSV cases in the EVERGREEN analysis occurred up to 550 days post-vaccination), and that EVERGREEN was conducted during an RSV B dominant season and CYPRESS during an RSV A dominant season. In spite of the differences in vaccine efficacy, the relationships between post-vaccination bAb and nAb levels and vaccine efficacy were consistent across the two studies. The longer delay between vaccination and RSV infection in EVERGREEN may be a confounding variable and could in part explain the apparent difference in vaccine efficacy observed between the studies.

The univariate analyses presented here did not take into account multicollinearity across the measured features, restricting our understanding of the relative importance of the different

variables. A multivariate analysis that accounts for the overall correlation structure of the features is warranted. Finally, samples for testing cell-mediated immunity could not be obtained from all participants in these large trials. In view of the incompleteness of the data, we were not able to include cell-mediated responses to this CoP analysis. However, this does not negate the fact that cellular responses could be contributing to the protective immunity observed. As observed in the phase 1 study data, the T cell responses induced by this immunogen combination lasted for more than 1 year following one dose in the same target population.³⁷

Our data confirms and complements an analysis of immune CoPs following vaccination with mRNA-1345 RSV vaccine, which also concluded that preF bAb levels and nAb titers were CoPs for RSV-mediated ARI and LRTD.³⁸ Consistent with our study, close correlation of bAb and nAb results were observed for both RSV-A and RSV-B, there was an interaction between baseline titer and response to vaccination. Neither study identified a threshold for protection, and both largely concurred on the limited sensitivity and utility of fold-increases as CoPs.

Importantly, Ma et al, 2024³⁸ evaluated immunogenicity at Day 29 versus Day 15 used in our analysis. It is feasible that differential affinity maturation of nAbs may lead to a tighter association with efficacy using a later time point. Ma et al, ruled out postfusion bAb responses as a CoP post-mRNA-1345. Our study expanded the analysis to include functional antibody responses as biomarkers, identifying a potential role for IgG3 in protection against RSV disease. Both studies used similar statistical approaches with some differences in case definitions.

In conclusion, although we were unable to identify a single threshold value indicative of protection against RSV infection, our findings mark a significant advancement in establishing an accepted common surrogate immunologic endpoint for RSV vaccines. The consistency of our findings with another CoP analysis following vaccination with mRNA-1345 RSV vaccine

support the generalizability of the findings across different RSV vaccine platforms. These findings could potentially also be applicable to other viruses in the *Pneumoviridae* family.

METHODS

Trial Designs

A full description of both the CYPRESS and EVERGREEN study designs is found in the clinical trial protocols provided as Supplementary Material.

CYPRESS was a double-blind, placebo-controlled, phase 2b, proof-of-concept trial that was conducted at 40 centers in the US.^{8,9} Adults 65 years of age or older who were in good or stable health were eligible for participation. Enrollment began on August 5, 2019, and injections occurred up to November 13, 2019. A total of 5782 participants were randomized (1:1 ratio) to receive one intramuscular injection of Ad26.RSV.preF–RSV preF vaccine (1 mL; 1×10^{11} viral particles of Ad26.RSV.preF plus 150 µg of RSV preF) or saline placebo at Day 1. Randomization included stratification according to increased risk of severe RSV (yes/no) and age group (65-74 years of age, 75-84 years of age, or ≥ 85 years of age).

EVERGREEN was a multicenter, randomized, double-blind, placebo-controlled, phase 3, confirmatory efficacy study in participants 60 years of age and older that was conducted in Australia, Brazil, Canada, Chile, China, Estonia, Finland, New Zealand, Poland, South Africa, Taiwan, Thailand, the United Kingdom, and the US. Enrollment began on July 26, 2021, with the last injection on January 31, 2023. A total of 25,076 participants were enrolled and randomized to receive the vaccine or placebo at Day 1 (2:1 ratio). Randomization was stratified by increased

risk of severe RSV (yes/no) and age group (60-64 years of age, 65-74 years of age, 75-84 years of age, or ≥ 85 years of age).¹⁰

RSV case definitions and primary immune correlates study cohort

In both clinical studies, serum samples collected at Days 1 (baseline) and 15 and antibody measurements were evaluated as primary correlates against RSV ARI and LRTD (details on case definitions are provided in the Supplementary Material). In addition, antibody measurements at the time of ARI (Days 3-5 of an ARI episode) were explored as a secondary analysis. The correlates analyses used the combination of immunologic markers that were correlates of risk and can be used as CoPs.

Correlates analyses were performed based on all RSV ARI and LRTD cases if they occurred ≥ 15 days after vaccination through to the study date of final analysis (EVERGREEN) or during the first RSV season after vaccination (CYPRESS, up to April 2020). RSV endpoints in the correlates analyses were selected to be aligned with the primary (LRTD) or secondary (ARI) endpoints used in the clinical studies. RSV ARI and LRTD were similarly defined in both clinical studies.

Correlates analyses were performed in the per-protocol efficacy population (i.e., all participants who underwent randomization and received the assigned injection, excluding participants with protocol deviations that would be expected to affect efficacy, those with the onset of RSV-mediated ARIs within 14 days after the injection, and those who discontinued participation within 14 days after the injection). A case-cohort sampling design and inverse probability weighting was used to control for non-random sample selection in the control set, as described in the Statistical Analysis section.

The primary immune correlates case-control cohort in CYPRESS consisted of all RSV ARI cases during the first RSV season after vaccination, a control set that consisted of participants with no evidence of RSV ARI in an immunogenicity subset of the study (100 vaccinated and 100 placebo participants), and a random selection of approximately 150 vaccinated and 70 placebo participants. Only participants with Days 1 and 15 data available for preF bAb (preF IgG) levels and/or nAb (VNA A2) titers were included. In total, 438 participants (56 RSV cases and 382 non-RSV controls; 191 in the placebo group and 247 in the vaccinated group) were in the immune correlates analysis population (**Table 1**).

The primary immune correlates case-control cohort in EVERGREEN consisted of all RSV ARI cases up to final analysis and a control set that consisted of 1515 participants in the per-protocol efficacy set without RSV detected during the study and with Days 1 and 15 data available for both preF bAb (preF IgG) levels and nAb (VNA A2) titers at the time of selection. A total of 1774 participants were included in the immune correlates analysis population, with 116 and 143 RSV ARI cases in the vaccine and placebo arms, respectively, and 1188 and 327 non-RSV controls in the vaccine and placebo arms (**Table 1**).

Exploratory Correlates Study Cohort

Exploratory functional antibody responses were measured in a subset of RSV ARI cases and controls from the primary immune correlates case-control cohort in the EVERGREEN study (**Fig. 4A**).

Serum preF IgG Antibody Enzyme-linked Immunosorbent Assay

RSV-A and RSV-B preF binding was assessed with a standard enzyme-linked immunosorbent assay.²⁵ preF-specific antibodies were detected with anti-human IgG antibodies. Gen5 software

(version 2.0 secure) was used to calculate the anti-RSV preF IgG concentrations by referring the luminescence readout of samples to the 4-parameter logistic fit of the standard curve.

RSV VNAs

VNAs used an RSV-A2 firefly luciferase virus based on a recombinant RSV-A2 backbone displaying the Buenos Aires strain G-protein sequence. RSV-specific nAb were assessed in a VNA using A549 CCL-185 cells, firefly luciferase-expressing RSV-A2 virus, and serially diluted serum samples as previously described.²⁵ Briefly, a fixed number of RSV viral particles were mixed with serially diluted serum samples; 1 hour later, A549 cells were added to the mixture. RSV infection was measured after approximately 20 hours using a luciferase gene-expression system. Luciferase activity levels in serially diluted samples were plotted in a 4-parameter logistic regression curve, and the geometric mean half-maximal inhibitory concentration was reported as the nAb titer.

Exploratory Functional Antibody Assays

Antigen-specific antibody isotype and subclass titers and Fc gamma receptor (FcγR) binding profiles were evaluated using a custom multiplex Luminex assay (Luminex Corp). Briefly, RSV preF A-antigen was covalently coupled to Magplex Luminex beads (Luminex Corp) via a 2-step carbodiimide reaction. Coupled beads were blocked with 5% bovine serum albumin (BSA)–phosphate buffered saline (PBS) overnight; subsequently, beads were diluted to a concentration of 100 microspheres/μL and incubated with serum samples that were diluted 1:16,000 (IgA1, IgA2, IgG2, IgG3, IgG4, IgM) or 1:20,000 (IgG, IgG1, FcγR binding) in PBS. Following a wash with 0.1% BSA in PBS-Tween, the bound antibodies were subsequently stained with phycoerythrin (PE)-conjugated secondary antibodies that were diluted 1:200 for IgG (clone, JDC-10), IgG1 (clone, 4E3), IgG2 (clone, HP6002), IgG3 (clone, HP6050), IgG4 (clone,

HP6025), IgA1 (clone, B3506B4), IgA2 (clone, A9604D2), or IgM (clone, SA-DA4). For Fc γ R binding, the bound antibodies were stained with PE-labeled tetramerized recombinant Fc receptors (Fc γ RIIa 131H/R, Fc γ RIIb, Fc γ RIIIa 158V/F, and Fc γ RIIIb). Fluorescence was analyzed using a Stratadigm S1000EON. The data are reported as the median fluorescence intensity of PE.

ADCD assessed the recruitment of complement component C3b on the surface of antigen-coupled beads. Briefly, biotinylated RSV preF A-antigen was coupled to FluoSphere NeutrAvidin beads (Thermo Fisher). The coupled beads were blocked with 5% BSA-PBS overnight, then washed and resuspended in 0.1% BSA-PBS. Ten μ L of antigen-coupled beads were incubated with 10 μ L serum that was diluted 1:1600 in PBS for 2 hours at 37°C and then washed with PBS. Commercially available guinea pig complement (Sigma, G9774) that was diluted 1:60 in gelatin veronal buffer (Sigma, G6514) was then added as a source of complement. Following incubation, free complement was washed and a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody specific for guinea pig C3 (MP Biomedicals, 0855385) was added at a 1:200 dilution. Complement deposition was measured using Stratadigm S1000EON. The data are reported as the geometric mean fluorescent intensity of FITC.

Ethics and consent

All experiments were performed in accordance with relevant guidelines and regulations. All participants whose serum samples were assayed in this work provided informed consent.

Statistical Methods

Analyses were performed according to the immune correlates statistical analysis plans provided in the Supplementary Material. To adjust for the case-cohort sampling design, missing data in

RSV ARI cases, and non-random sampling of controls, inverse probability of sampling weights was used based on case-control status, geographic region, age category, risk category, and treatment allocated.

In general, more complex methods and more stringent significance criteria were used in EVERGREEN compared with CYPRESS, as the former has a >4 times larger sample size than the latter. For example, in EVERGREEN, the analyses were adjusted for risk scores for RSV that were constructed using Superlearner ensemble modeling based on a predefined list of potential risk factors for RSV. Modeling for CYPRESS used logistic regression modeling of RSV ARI during the first RSV season after vaccination. In addition, for EVERGREEN, Poisson and Cox regression models were used to model the rate of RSV ARI episodes and time to first RSV ARI episode, respectively, after vaccination.

In the planned analyses for CYPRESS, 1-sided P values were presented and labeled significant if <0.05 (1-sided). In contrast, for the planned analyses for EVERGREEN, 2-sided P values were presented and labeled significant if <0.05 (2-sided). In this manuscript, we present 2-sided P values for both studies and label results significant if the 2-sided P value is <0.10 for CYPRESS and <0.05 for EVERGREEN.

Immune variables were log-transformed before statistical analysis and included values at Day 15 post-vaccination and the fold rise from pre-vaccination baseline to Day 15. Immune variables were compared between RSV ARI cases and controls using the Wilcoxon rank sum test. In addition, the incidence of RSV during the RSV season following vaccination in vaccinated and placebo participants was shown by immune variable level and modeled using weighted univariable and multivariable logistic (CYPRESS) and Cox (EVERGREEN) regression.

Potential protective thresholds were derived using the maximal chi-square method.³⁹ To test the existence of a threshold effect, 3 logistic regression models were compared using Akaike's information criterion: (1) model without biomarker effects, (2) model with biomarker level as a linear effect, and (3) model with biomarker level as a binary effect.

Potential CoPs were assessed using the Prentice criteria²⁸ and the principal surrogate framework.²⁶ In the principal surrogate framework, using baseline immunogenicity predictors,²⁷ vaccine efficacy is predicted over a range of vaccine-induced biomarker levels (i.e., the “controlled vaccine efficacy curve”). To estimate the vaccine efficacy by biomarker level, the number of RSV LRTD events in the vaccinated group was compared with comparable placebo participants. “Comparable” in this setting means placebo participants who would have attained the same biomarker level if they would have been vaccinated. This is calculated by predicting the vaccine-induced biomarker levels in placebo participants from an imputation model that was developed in the vaccinated group.

Exploratory analyses of antibody functionalities were performed on a smaller case-cohort set in EVERGREEN consisting of: (1) all RSV ARI cases + two non-RSV controls per case in the vaccinated group and (2) a random subset of 100 RSV ARI cases + one non-RSV control per case in the placebo group. This analysis was based on z scores of the log-transformed data. The average z score in each marker of antibody functionality was calculated by time point (Day 1, Days 3-5, and Day 15 of the ARI), allocation group (vaccinated or placebo), and RSV status and presented as color in a heatmap. In a similar heatmap, Spearman correlations between z scores for the different assays without vaccination (Day 15 from the placebo group and Day 1 from the vaccine group) and at Day 15 after vaccination were shown. Lastly, standardized effects of each immune marker on the probability of RSV ARIs are shown as log-odds ratios by one standard

deviation change in log₁₀ immune marker level z scores. The estimates were obtained from logistic regression modeling in the placebo and vaccine groups separately. Results were presented with each assay as a single predictor separately (univariate analysis) and adjusted for RSV-A2 nAb titers.

Data Availability

The data sharing policy of Janssen Pharmaceutical Companies of Johnson & Johnson is available at <https://www.janssen.com/clinical-trials/transparency>. As noted on this site, requests for access to the study data can be submitted through the Yale Open Data Access (YODA) Project site at <http://yoda.yale.edu>. Data will be uploaded to the YODA project site once all final study data are available.

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Contributions

A.R.B contributed to study conceptualization, methodology, and supervision. J.M. contributed to study conceptualization, formal analysis, methodology, software development, and visualization and writing of the original draft. J.T. contributed to study analysis, methodology, and software development. V.R. contributed to study conceptualization, methodology, and project administration and writing of the original draft. M.G.M.v.R. contributed to study conceptualization and project administration and writing of the original draft. S.B. contributed to study data curation, investigation, resources, and supervision. J.H. contributed to study

conceptualization, resourcing, and supervision. T.B. contributed to study data curation, investigation, methodology, project administration, and supervision. J.D. contributed to study analysis and investigation. C.L. contributed to study data curation, investigation, and project administration. T.L. contributed to study data curation and investigation. L.M. contributed to study investigation and supervision. C.A.C. contributed to study conceptualization and project administration. E.G. contributed to data curation and formal analysis. E.D. contributed to study conceptualization and formal analysis. B.C. contributed to study conceptualization, funding acquisition, methodology, project administration, resourcing, and supervision. All authors contributed to writing – review and editing. All authors approved the final version of the manuscript and vouch for data accuracy and completeness.

Competing Interests

A.R.B., V.R., S.B., C.A.C., and B.C. are former employees of Janssen Vaccines & Prevention. J.M., J.T., and J.H. are current employees of Janssen Research & Development and hold shares and stock options in Johnson & Johnson. M.G.M.v.R. is a current employee of Janssen Research & Development. T.B. owns shares in SeromYx Systems. J.D. has no competing interests to disclose. C.L. is a former employee of SeromYx Systems and holds shares in SeromYx. T.L. and L.M. are current employees of SeromYx Systems and hold stock options in SeromYx Systems. E.G. and E.D. are former employees of Janssen Infectious Diseases.

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Table 1. Case-cohort sets for the primary immune correlates analyses in CYPRESS (phase 2b) and EVERGREEN (phase 3)

	CYPRESS		EVERGREEN	
	Placebo	Vaccine	Placebo	Vaccine
Total eligible population (PPE) ^a	2801	2791	7425	14,822
RSV ARI cases	43	13	144 ^b	116
RSV LRTD cases	30	6	92	60
Primary immune correlates analyses	191	247	470	1304
RSV ARI cases	43	13	143	116
RSV LRTD cases	30	6	91	60
No RSV controls	148	234	327	1188

ARI, acute respiratory infection; LRTD, lower respiratory tract disease; PPE, per-protocol efficacy; RSV, respiratory syncytial virus.

^aNo cases of RSV were detected in the participants in EVERGREEN recruited in China and Taiwan. Therefore, these participants were fully excluded from the immune correlates analyses.

^b One participant did not have a post-vaccination blood sample and was excluded from the correlates of protection analysis

Table 2. Assessment of Day 15 antibody markers as correlates of protection using the Prentice criteria

	CYPRESS				EVERGREEN			
	Day 15 preF bAb		Day 15 RSV-A2 nAb		Day 15 preF bAb		Day 15 RSV-A2 nAb	
	ARI	LRTD	ARI	LRTD	ARI	LRTD	ARI	LRTD
Vaccine has an effect on the clinical outcome (2-sided <i>P</i> value <0.1)								
<i>P</i> value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Unadjusted VE	70%	80%	70%	80%	64%	71%	64%	71%
Vaccine has an effect on the immune marker (2-sided <i>P</i> value <0.1)								
<i>P</i> value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Immune marker has an effect on the clinical outcome across all participants, regardless of treatment allocation (2-sided <i>P</i> value <0.1)								
<i>P</i> value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Vaccine has no effect on the clinical outcome when adjusted for immune marker (immune marker-adjusted VE is <50% of unadjusted VE)								
<i>P</i> value	0.240	0.348	0.262	0.440	0.245	0.119	0.344	0.936
Immune marker-adjusted VE	-102%	-101%	-75%	-58%	-36%	-66%	22%	-3%
Conforms to all Prentice criteria	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

ARI, acute respiratory infection; bAb, binding antibody; LRTD, lower respiratory tract disease; nAb, neutralizing antibody; preF, pre-fusion F-protein; RSV, respiratory syncytial virus; VE, vaccine efficacy.

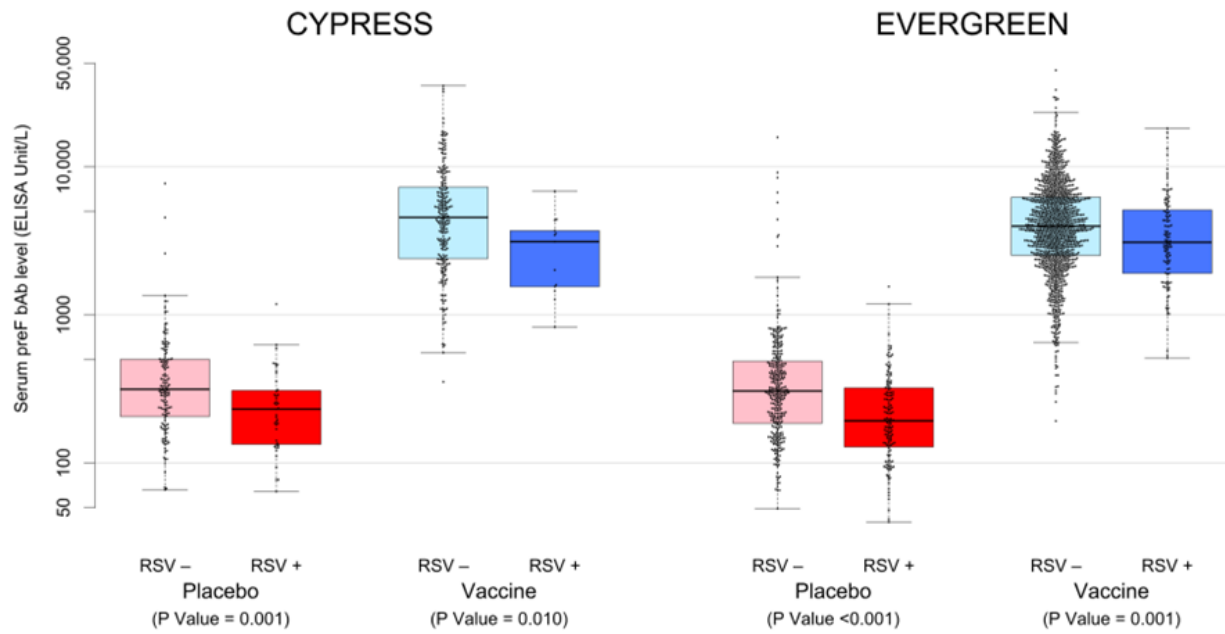
Analyses were weighted according to the selection probability of each observation and corrected for baseline risk scores, which included baseline immune marker levels.

Figures

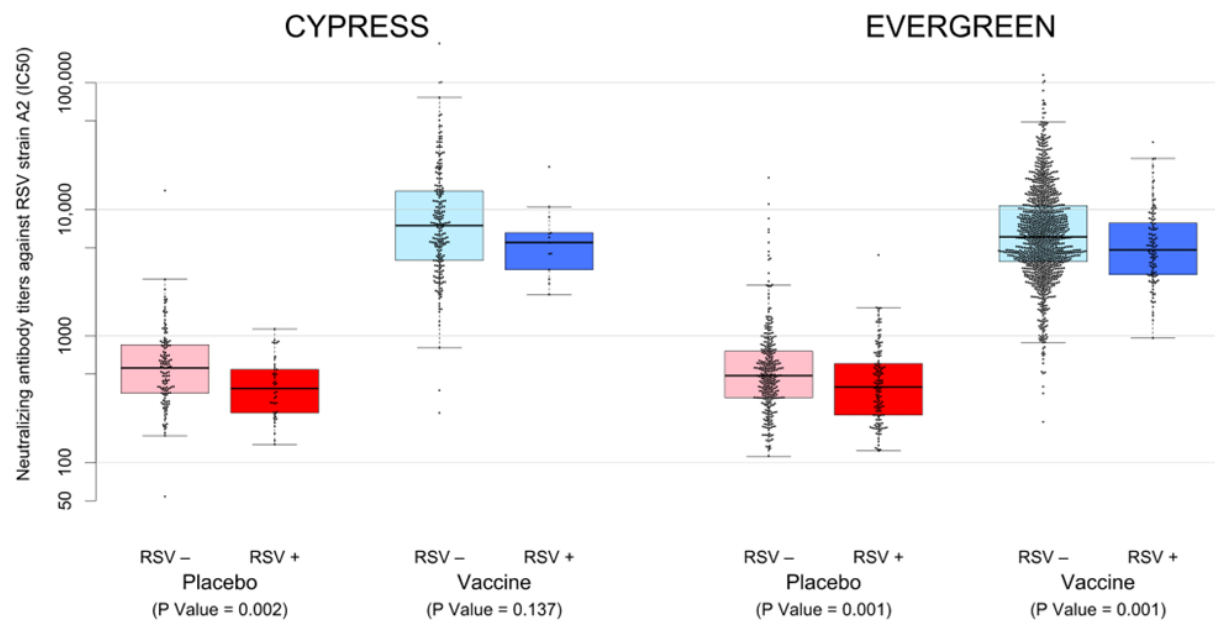
Fig. 1. bAb levels and nAb titers at Day 15 in RSV ARI cases and controls in the placebo and vaccine groups. bAb levels (**A**) and nAb titers (**B**) at Day 15 are shown for CYPRESS and EVERGREEN. Serum bAb levels were measured by preF IgG ELISA and are shown in ELISA Units/L. nAb titers against the RSV-A2 strain were measured by VNA and are expressed in IC_{50} . Two-sided P values from Wilcoxon rank sum test comparing RSV ARI cases and controls are shown. Dots present individual observations. The bolded line presents the median, the box presents the 25th and 75th percentiles, and the whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range from the box. Boxes are colored as follows: pink, RSV– in the placebo group; red, RSV+ in the placebo group; light blue, RSV– in the vaccine group; blue, RSV+ in the vaccine group. Day 1, Day 15, and fold rise at Day 15 antibody marker GMs by RSV ARI outcome status are shown in (**C**). Two-sided P values of the Wilcoxon rank sum test are shown. Cells shown in green are significant at the 10% 2-sided level (CYPRESS) or at the 5% 2-sided level (EVERGREEN).

ARI, acute respiratory infection; bAb, binding antibody; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; GM, geometric mean; IC_{50} , half-maximal inhibitory concentration; IgG, immunoglobulin G; nAb, neutralizing antibody; PBO, placebo; preF, pre-fusion F-protein; RSV, respiratory syncytial virus; VNA, virus neutralization assay.

A. bAb at Day 15



B. nAb at Day 15



C.

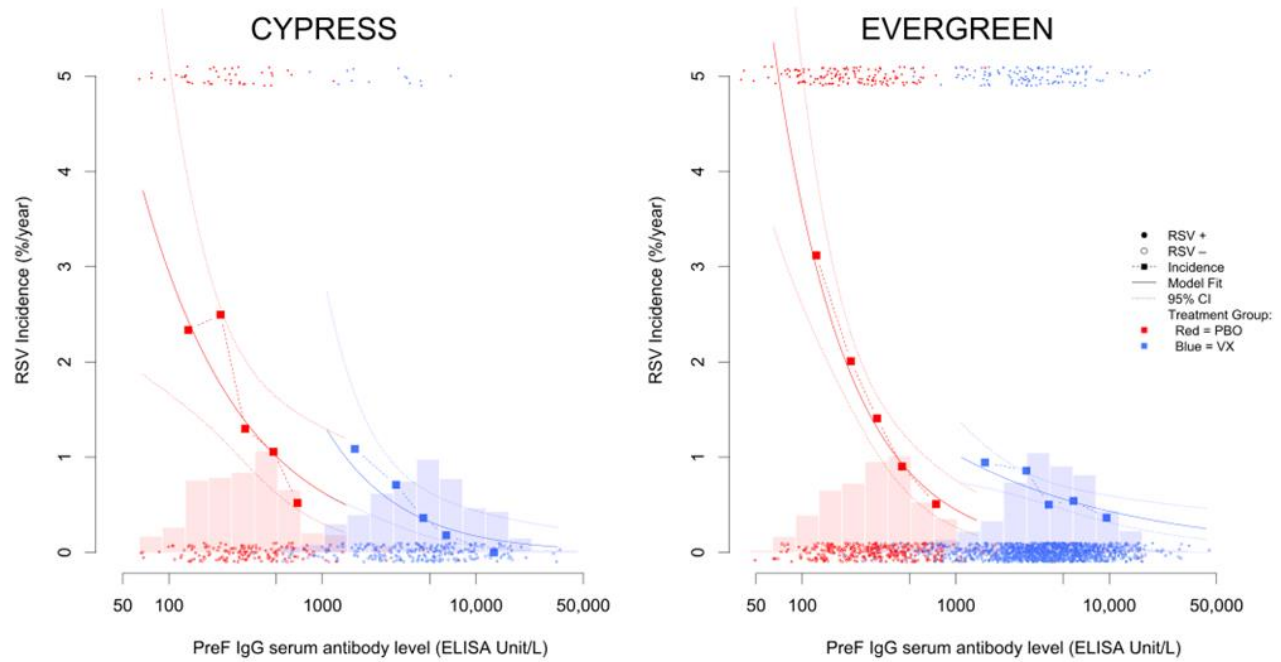
CYPRESS			Cases		Controls		Comparison	
Cohort	Marker	Day	N	GM (95% CI)	N	GM (95% CI)	Ratio of GM (cases/controls)	Wilcoxon rank sum test <i>P</i> value
Placebo	preF IgG	Day 1	43	244 (29–2059)	148	371 (32–4335)	0.66	<0.001
		Day 15	43	223 (64–775)	148	324 (69–1521)	0.69	0.001
	RSV-A2 nAb	Day 1	43	408 (129–1294)	137	566 (125–2560)	0.72	0.004
		Day 15	43	385 (130–1137)	137	560 (137–2280)	0.69	0.002
Vaccinated	preF IgG	Day 1	13	224 (48–1045)	234	318 (16–6475)	0.70	0.011
		Day 15	13	2491 (653–9507)	234	4329 (859–21,824)	0.58	0.010
		Fold rise Day15/ Day 1	13	11.1 (1.1–112.3)	234	13.6 (0.5–373.5)	0.82	0.951
	RSV-A2 nAb	Day 1	13	414 (88–1948)	228	590 (133–2616)	0.70	0.074
		Day 15	13	5338 (1345–21,190)	228	7736 (1134–52,759)	0.69	0.137
		Fold rise Day 15/ Day 1	13	12.9 (1.6–102.8)	228	13.1 (1.7–102.3)	0.98	0.969
EVERGREEN			Cases		Controls		Comparison	
Cohort	Marker	Day	N	GM (95% CI)	N	GM (95% CI)	Ratio of GM (cases/controls)	Wilcoxon Rank Sum test <i>P</i> value
Placebo	preF IgG	Day 1	143	286 (74–1101)	327	361 (81–1598)	0.79	0.004
		Day 15	143	200 (52–764)	327	319 (63–1618)	0.63	<0.001
	RSV-A2 nAb	Day 1	143	428 (110–1668)	327	528 (122–2279)	0.81	0.003
		Day 15	143	409 (107–1557)	327	522 (116–2348)	0.78	0.001
Vaccinated	preF IgG	Day 1	116	329 (89–1217)	1188	362 (82–1588)	0.91	0.384
		Day 15	116	3133 (762–12,876)	1188	3861 (904–16,483)	0.81	0.001
		Fold rise Day 15/ Day 1	116	9.5 (1.8–50.1)	1188	10.7 (1.6–72.1)	0.89	0.067
	RSV-A2 nAb	Day 1	116	496 (152–1620)	1188	531 (116–2431)	0.93	0.661
		Day 15	116	5035 (1328–19,089)	1188	6392 (1218–33,543)	0.79	0.001
		Fold rise Day 15/ Day 1	116	10.2 (1.9–54.0)	1188	12.0 (1.6–93.0)	0.84	0.056

Fig. 2. Incidence in RSV ARI by preF bAb levels (A) and RSV-A2 nAb titers (B) at Day 15.

RSV ARI incidence by immune marker is shown for CYPRESS and EVERGREEN. Red (PBO) and blue (VX) dots present individual observations: dots at 5% Y-level are RSV+; dots at 0% Y-level are RSV-. The squares represent the RSV incidence by quintile of the immune marker. The solid red and blue lines present the fitted RSV incidence by immune marker from a logistic regression model (CYPRESS) or a Poisson regression model (EVERGREEN), with log-transformed immune marker levels as linear predictor and log follow-up time as offset. The dotted lines represent 95% CI for the RSV incidence from the same model. The histograms present the distribution of the immune marker. All analyses are performed weighted according to the selection probability of each observation.

ARI, acute respiratory infection; bAb, binding antibody; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IC₅₀, half-maximal inhibitory concentration; IgG, immunoglobulin G; nAb, neutralizing antibody; PBO, placebo; preF, pre-fusion F-protein; RSV, respiratory syncytial virus; VX, active vaccine.

A. bAb at Day 15



B. nAb at Day 15

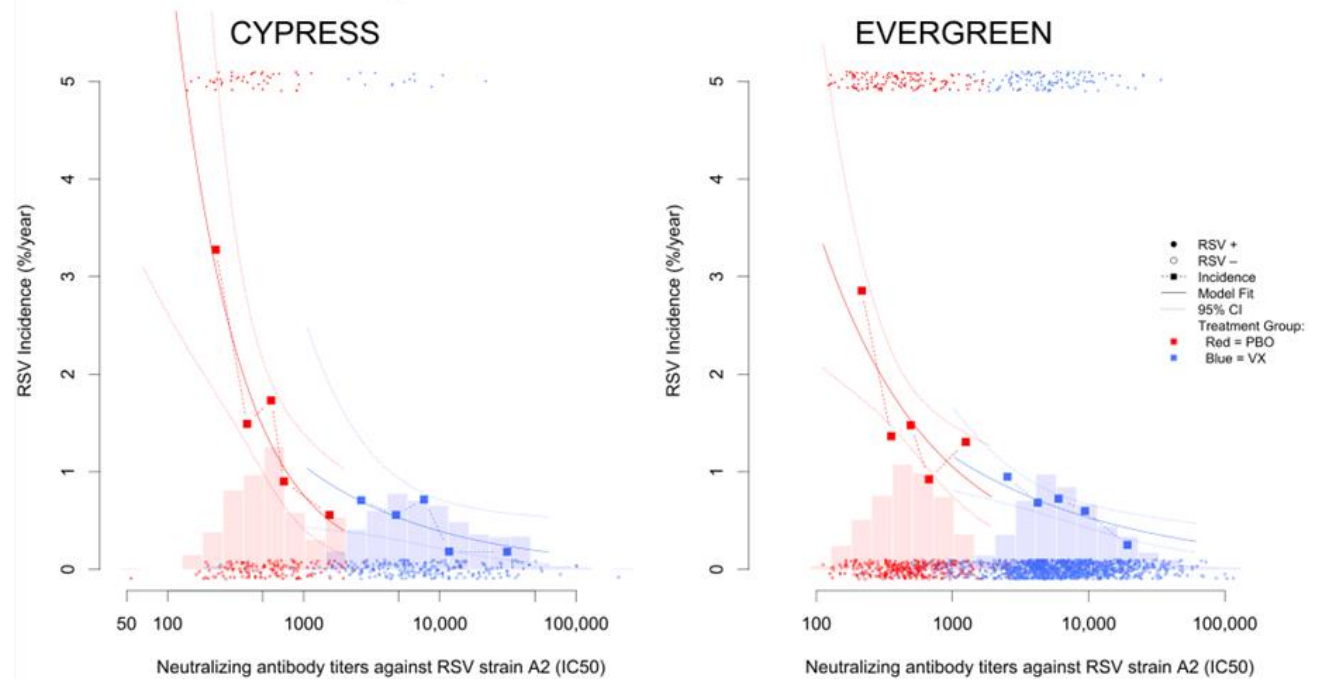
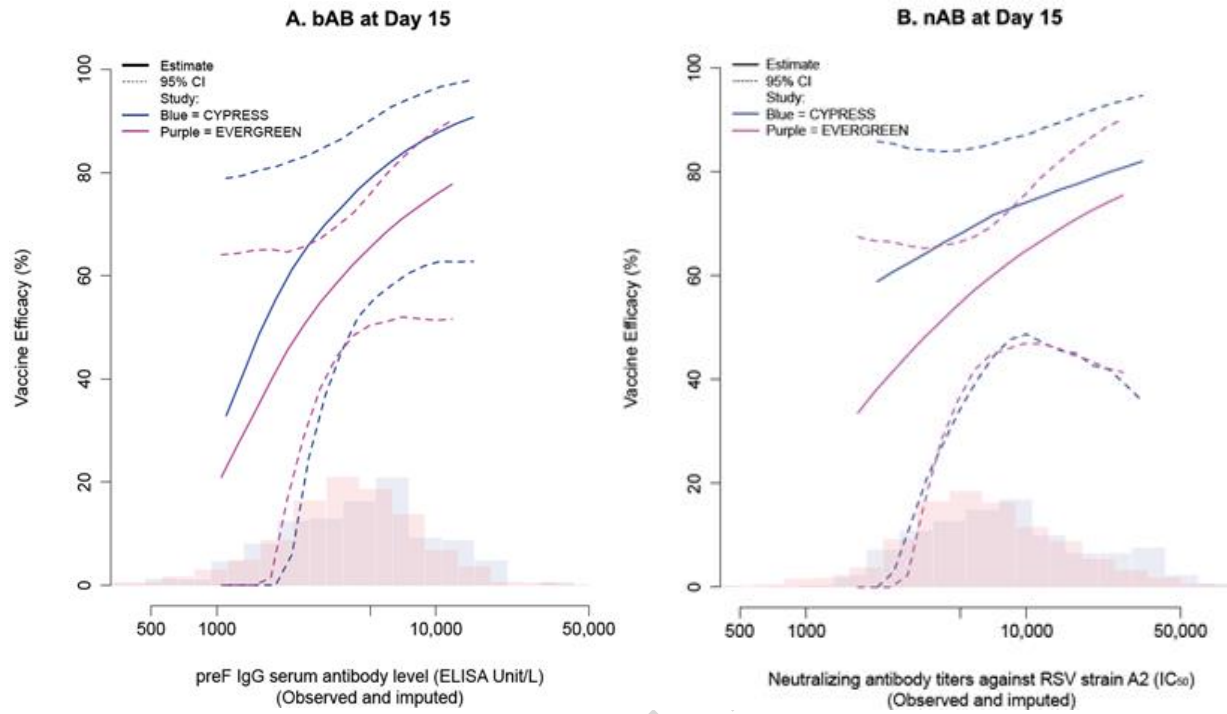


Fig. 3. Estimated VE against RSV ARI by vaccine-induced preF bAb levels and RSV-A2 nAb titers (Day 15).

VE against RSV ARI, estimated using a principal surrogate framework, is shown for preF bAb levels **(A)** and RSV-A2 nAb titers **(B)** at Day 15. Results from the CYPRESS VE estimates are shown with a blue line and those of EVERGREEN with a purple line. 95% CIs of the VE estimates are indicated with dotted lines. The median Day 15 immune marker responses (post-vaccination) are depicted to indicate the expected VE for a participant with median vaccine-induced response. The histogram presents the distribution of the immune marker post-vaccination. **(C)** Estimates of vaccine efficacy by the distribution of Day 15 binding and neutralizing antibodies in CYPRESS and EVERGREEN.

ARI, acute respiratory infection; bAb, binding antibody; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IC₅₀, half-maximal inhibitory concentration; IgG, immunoglobulin G; nAb, neutralizing antibody; preF, pre-fusion F-protein; RSV, respiratory syncytial virus; VE, vaccine efficacy.



C.

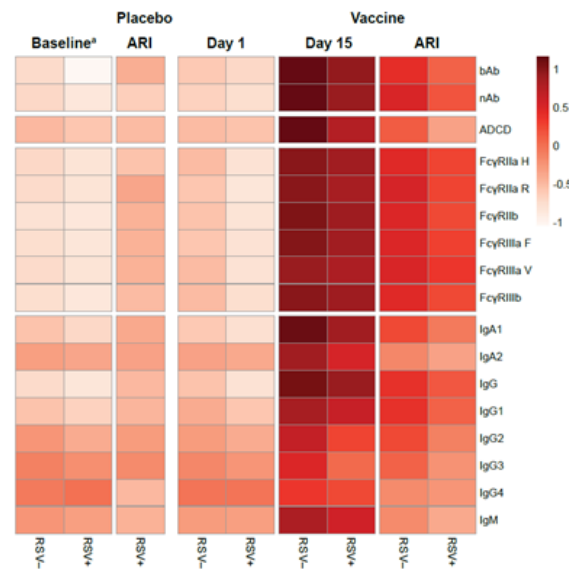
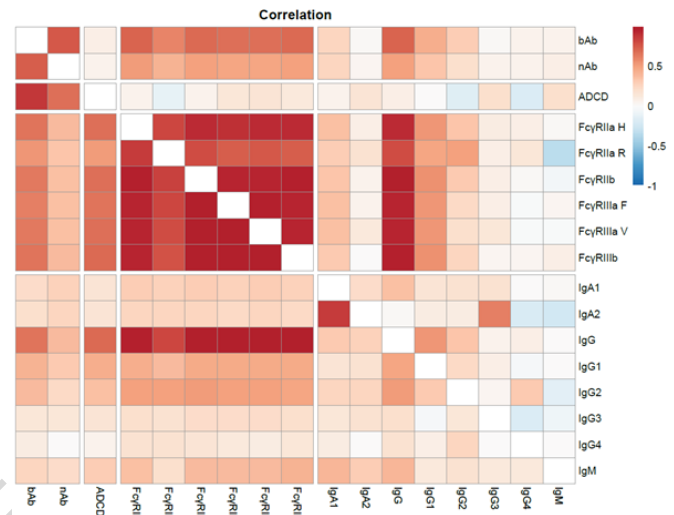
Percentile of immune marker distribution	CYPRESS Estimated VE in % (95% CI)		EVERGREEN Estimated VE in % (95% CI)	
	bAb at Day 15	nAb at Day 15	bAb at Day 15	nAb at Day 15
10%	50.8 (0.0–80.2)	60.7 (0.0–84.1)	38.1 (0.0–64.7)	40.5 (0.0–65.8)
25%	64.3 (25.6–83.3)	65.7 (26.0–83.2)	51.8 (29.2–66.1)	49.8 (28.0–65.4)
50%	77.2 (50.9–89.4)	71.9 (41.8–85.4)	61.7 (46.6–71.8)	57.9 (43.7–68.1)
75%	83.9 (56.6–94.0)	76.9 (43.6–89.8)	69.4 (49.4–80.2)	66.1 (48.9–78.7)
90%	89.8 (58.1–97.6)	81.5 (40.8–94.4)	75.3 (46.5–87.1)	73.0 (47.5–86.9)

Fig. 4. preF IgG3 levels at Day 15 contribute to protection against RSV ARIs in addition to RSV-A2 nAb titers. **(A)** Case-cohort sets for evaluation of exploratory antibody functionalities in EVERGREEN. In this cohort (1) all RSV ARI cases + two non-RSV controls per case in the vaccinated group and (2) a random subset of 100 RSV ARI cases + one non-RSV control per case in the placebo group were selected. Numbers indicate the number of selected participants with samples available for assessments at the specific time points. **(B)** Z scores for the mean level for each immune marker are given at the time points indicated in RSV ARI cases (RSV+) and non-RSV controls (RSV-). ^aBaseline for the placebo group refers to Day 15 results in this group. **(C)** Spearman correlations between the indicated assays are shown. The top-right half of the grid shows correlations between assays from participants/time points without a vaccine effect (Day 15 from the placebo group and Day 1 from the vaccine group combined). The lower-left half of the grid shows correlations between assays from participants after vaccination (Day 15 in the vaccine group). Red colors indicate positive correlations and blue colors indicate negative correlations. **(D)** Standardized effect of each immune marker on the probability of an RSV ARI is shown in log-odds ratios by 1 unit (SD) change in log₁₀ immune marker level z scores. Log-odds ratios without adjustment for RSV-A2 nAb titers are shown in the vaccine and placebo groups. *P* values are shown only for significant effects ($P \leq 0.05$).

ADCD, antibody-dependent complement deposition; ARI, acute respiratory infection; bAb, binding antibody; FcγR, Fc gamma receptor; Ig, immunoglobulin; nAb, neutralizing antibody; preF, pre-fusion F-protein; RSV, respiratory syncytial virus; SD, standard deviation.

A

	Placebo		Vaccine	
	RSV -	RSV +	RSV -	RSV +
N	100	97	229	117
Day 1	-	-	227	116
Day 15	100	97	229	117
Day 3-5 of ARI	-	97	114	107

B**C****D**