ChAd155-RSV vaccine is immunogenic and efficacious against bovine RSV infection-induced disease in young calves

Rineke de Jong
Wageningen Bioveterinary Research https://orcid.org/0000-0002-2052-5993
Norbert Stockhofe-Zurwieden
Wageningen Bioveterinary Research
Judith Bonsing
Wageningen Bioveterinary Research
Kai-fen Wang
GSK Vaccine
Sarah Vandepaer
GSK vaccine
Badiaa Bouzya
GSK Vaccine
Jean-François Toussaint
GSK Vaccine
Ilse Dieussaert
GSK Vaccine
Haifeng Song (haifeng.x.song@gsk.com)
GSK Vaccine
Ann-Muriel Steff
GlaxoSmithKline Vaccines

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Abstract

Respiratory syncytial virus (RSV) infection causes a substantial lower respiratory-tract disease burden in infants, and is a global priority for vaccine development. We evaluated the immunogenicity, safety and efficacy of a chimpanzee adenovirus (ChAd)-based vaccine candidate, ChAd155-RSV, in a bovine RSV (bRSV) challenge model. This model closely reproduces the pathogenesis/clinical manifestations of severe pediatric RSV disease. In seronegative calves, ChAd155-RSV elicited robust neutralizing antibody responses against human RSV. Two doses protected calves from clinical symptoms/lung pathological changes, and reduced nasal/lung virus loads after both a short (4-week) and a long (16-week) interval between the last immunization and a subsequent bRSV challenge. The one-dose regimen also conferred near-complete or significant protection after the short-term or long-term intervals before challenge, respectively. Importantly, immunized calves presented no clinical signs of enhanced respiratory disease. Collectively, the data supported the development of ChAd155-RSV as an RSV vaccine candidate for infants.

Introduction

Respiratory syncytial virus (RSV) infection is a major cause of lower respiratory tract disease (LRTD) in infants. Infants aged under one year have the highest incidence of severe LRTD (i.e., bronchiolitis and pneumonia)[1], and the disease burden extends even beyond the second year of life. Worldwide, there were an estimated 33.1 million new episodes of RSV-linked acute lower respiratory infection in children aged under 5 years (in 2015), and about 10% of these cases were severe enough to warrant hospitalisation[2]. In the same age group, the estimated RSV-attributable global mortality was around 59,600 in-hospital deaths and 118,200 overall deaths per year, 99% of which occurred in developing countries[2,3].

Due to this high incidence of severe disease and a lack of cost-effective preventative measures, RSV represents a major priority for vaccine development[4]. However, progress in this area has been largely hindered by the possibility of vaccination-induced enhanced respiratory disease (ERD), which manifested after vaccination with a formalin-inactivated RSV vaccine candidate (FI-RSV, Lot 100) in the 1960's[5–7]. In one of these trials, FI-RSV vaccination of 31 infants resulted in two deaths and 80% hospitalization (of the 20 vaccinees who became infected), as compared to 5% hospitalization in the control group vaccinated with parainfluenza vaccines[5]. Although the mechanisms underlying FI-RSV-induced ERD are not completely clear, many studies agreed that a T helper (Th) 2-biased cellular response, a high ratio of non-functional antibodies (Abs) to neutralizing Abs (nAbs), and a lack of $CD8^+$ cytotoxic T-cell responses are among its major attributes[8–10]. An ideal RSV candidate should therefore induce high nAb titers, a Th1-biased $CD4^+$ T-cell response, and $CD8^+$ T-cell responses. Consequently, RSV vaccines that induce cellular expression of RSV antigens, such as viral vectored or nucleic acid-based platforms, are considered particularly suited for RSV pediatric vaccination[11]. These candidates may behave biologically as a live-virus vaccination as the antigens are produced intracellularly, thus preferentially inducing $CD8^+$ T-cell responses and skewing the adaptive immunity towards a Th1 phenotype.

Adenoviral (Ad) vectors represent an attractive vaccine platform, as they are known to induce both cellular and humoral responses against the expressed antigens[12–14]. Particularly adenoviruses of simian origin, such as chimpanzee Ad (ChAd) strains, are commonly used as vaccine vectors, due to their lower seroprevalence and thus decreased vector neutralization in humans as compared to human Ad5[15–20]. Several ChAd-vectored candidate vaccines against infectious agents including RSV and SARS-CoV2 have been demonstrated to induce favorable immunogenicity, safety and efficacy profiles in clinical trials[21–25]. A pediatric RSV vaccine candidate based on a group C ChAd 155 vector, ChAd155-RSV, encodes RSV fusion protein (F) in secreted form to induce humoral responses, and intracellularly expressed transcription anti-termination protein M2-1 and nucleocapsid protein N aimed to induce a $CD8^+$ T-cell response[22,26]. ChAd155-RSV has first been evaluated in mice, cotton rats and calves for its immunogenicity, preclinical safety and efficacy[26] (manuscripts in preparation), and has subsequently progressed to Phase I, I/II and II clinical development stages[22] (NCT02927873, NCT03636906).

Preclinical studies in RSV infection models indicated that no single validated animal model can faithfully predict the clinical outcome and efficacy of an RSV vaccine candidate in the human target population[27–29]. Bovine RSV (bRSV) infection symptoms in young calves share many aspects of severe RSV infection in human infants with respect to pathogenesis and clinical manifestations, including fever, runny nose, coughing and labored breathing[27,30,31]. The experimental calf model is thus a unique translational model in the preclinical evaluation of human RSV vaccines with regard to clinical efficacy[32], conferring advantages over mice and cotton rats model which are only semi-permissive for the human challenge virus, requiring a high challenge dose (i.e., 5-6 $\log_{10}$ PFU/$TCID_{50}$), and do not show obvious respiratory symptoms[27,33–36]. Human and bovine RSV proteins including F protein have a high-level homology with >80% amino acid identity, thus inducing cross-reactive immunity[31]. In addition, FI-bRSV-induced ERD in calves has been reported by several groups[30,37–39], allowing the identification of ERD signs already in the preclinical vaccine development phases. In the current study, the humoral immunogenicity, safety and efficacy of the ChAd155-RSV vaccine has been evaluated in a bovine RSV challenge model in young calves. We report that ChAd155-RSV vaccination elicited nAb responses, and two doses protected the calves from clinical symptoms and lung pathological changes upon bRSV challenge. The presence of pre-existing RSV-antibodies did not affect the short-term efficacy of a two-dose vaccination regimen in this model, and no clinical signs of ERD were observed post-challenge. The collective data supported further development ChAd155-based RSV vaccine as a candidate for pediatric immunization.

Results

ChAd155-RSV efficacy on bRSV-induced clinical disease

In two separate studies (Figure 1), the protective efficacy of ChAd155-RSV was assessed upon bRSV challenge, which was performed after either a short (4-week) or a long (16-week) interval after the last vaccination (further referred to as a short/long duration of immunity [DOI]).

In Study 1, placebo control calves developed typical clinical symptoms of bRSV infection when challenged after a short DOI. General illness symptoms (expressed in scores) accompanied by fever (rectal temperature >39.5°C) appeared at day 6 post challenge (dpc 6), peaked at dpc 7/8 and resolved by dpc 10/11 (Fig. 2a, d). Simultaneously, their URTD scores and respiratory rates were increased (Fig. 2g, j). In contrast, calves having received 2 doses of the vaccine were nearly completely protected from clinical symptoms, including fever. Although these animals occasionally showed (slightly) increased URTD scores and respiratory rates, these levels remained significantly lower than those for the controls ($P \leq 0.01$ and $P \leq 0.001$, respectively). As compared to the two-dose vaccine group, the single-dose vaccine group exhibited slightly higher general illness and URTD scores. However, the protective efficacy in this group was overall comparable to that in the two-dose group, and clearly higher relative to the control group.

Upon challenge after a long DOI (Figures 2b, e, h and k), clinical symptoms in the controls were similar to those in the short-DOI controls. Two vaccinations completely prevented general illness, fever and increased respiratory rates. However, mild URTD symptoms were observed on a limited number of days (Figure 2h).

In Study 2, performed separately, the efficacy of a 1-dose vaccination for protection against bRSV challenge 16-weeks after immunization was evaluated (Figure 2c, f, i and l). Unexpectedly, the challenge outcome in the age-matched placebo control group in this study was more severe than in Study 1. In Study 1, all placebo control calves developed moderately to severely increased breathing rates from dpc 6 onwards, and recovered by dpc 10 or 11. In Study 2, the onset of respiratory distress was observed earlier, at dpc 5, and most of the placebo control calves developed severely increased breathing rates (Figure 2l). This resulted in persistent respiratory distress or non-reversible respiratory failure in five (out of nine) placebo control calves. On dpc 7 and 8, four calves from the placebo group were pre-terminated as they complied to humane endpoint criteria while another calf had died unexpectedly at dpc 8. Despite the more severe clinical outcome of bRSV challenge in Study 2, the 1-dose vaccination group was nearly completely protected after long-term DOI against general illness and fever ($P \leq 0.001$), while the breathing rate was slightly reduced ($P \leq 0.05$) and the URTD symptoms were comparable to those in the placebo group (Figure 2c, f, i and l). Nevertheless, none of the 1-dose ChAd155-RSV vaccinated calves was pre-terminated, and all of them had nearly recovered by the end of the study (dpc 12).

Effect of ChAd155-RSV on bRSV-induced lung pathology

At necropsy (dpc 12/13), lungs were examined macroscopically to determine the consolidated lung area (CLA) as percentage of the total lung area (Figure 3a-c). Additionally, three tissue samples per lung were examined microscopically for histopathological changes, and sections were scored for evidence of bronchitis, peribronchitis/perivasculitis, interstitial pneumonia and alveolitis. Scores are presented as total histopathological sum scores (Figure 3d-f) as well as by individual pathological symptom (Supplemental Figure 1).

In Study 1, macroscopic CLAs upon short DOI and challenge were observed in all control calves (≤7%), but were only minimally or not observed in vaccinated calves of the single- or two-dose groups (Figure

3a). All control calves showed histopathological changes (scores 5 to 34, on a scale of 48), while vaccinated calves displayed minimal alterations (scores ≤5) except one animal from the single-dose group with a score of 10 (Figure 3d). In parallel, all seven control calves with long DOI showed CLAs of up to 6%, whereas consolidation was absent (n=7) or minimal (n=1) in calves which received two vaccine doses (Figure 3b). All control calves showed histopathological changes (scores: 1–36), while all except one (with score 11) in the two-dose vaccine group were completely protected against histopathological alterations (Figure 3e).

In line with the clinical observations, lung pathology was more severe after the long DOI in Study 2. Indeed, compared to Study 1, control calves had larger CLAs and more histopathological changes (0.4-53% and scores 28-39; Figures 3c and f, respectively). In addition, greater CLAs were observed in the five animals that were either pre-terminated (n=4) or found dead (n=1) at dpc 7/8 (Figure 3c). The single-dose vaccine group exhibited at most minimal (0.8%) macroscopic consolidation, though microscopic evaluation still revealed histopathological changes (score 2-32).

ChAd155-RSV efficacy on bRSV replication in nasal and lung fluids

Following bRSV challenge, nasopharyngeal brush samples were obtained daily, and BAL samples on dpc 5, 7 and 9 (Figure 4). The viral loads determined by bRSV qPCR on all samples (Supplemental Figure 2) were consistent with the viral infectivity data generated from selected time-points (described below).

In Study 1, virus loads in BALs from control calves of both the short and long DOI groups peaked on dpc 7 (mean titer ~2.5 $\log_{10}$ $TCID_{50}$/mL), but were cleared by dpc 9 (Figure 4a, c). One or two vaccinations resulted in significantly lower viral loads at dpc 5 and 7 in both short-DOI groups ($P \leq 0.001$) and in the two-dose long-DOI group ($P \leq 0.01$ at dpc 5 and $P \leq 0.001$ at dpc 7). Kinetics in the nasopharynx of short- or long-DOI controls were similar as for BALs, with a gradual increase from dpc 3 onwards, peaks on dpc 6 or 7 (mean titers: 3–4 $\log_{10}$ $TCID_{50}$/mL), and no re-isolated infectious virus on dpc 9 (Figure 4b, d). Two vaccine doses significantly reduced viral loads as compared to controls, in both the short- and the long-DOI groups (on dpc 3, 5, 6 and 7, and on dpc 6 and 7, respectively). In the single-dose short-DOI vaccine group, viral clearance was also observed earlier than in controls, i.e. on dpc 6 and 7 ($P \leq 0.001$, Figure 4b).

In Study 2, the kinetics of viral replication were slightly advanced with a peak at day 5 and decreased thereafter (Figure 4e, f). This was likely due to the more virulent challenge. Nonetheless, a single vaccination resulted in earlier viral clearance compared to placebo, as observed in the lungs at dpc 7, and in the nasopharyngeal tract at dpc 6 or 7 (all $P \leq 0.001$).

ChAd155-RSV efficacy in the presence of pre-existing bRSV Abs

As most infants have maternal RSV Abs transferred via the placenta, we investigated whether such pre-existing responses would impact the vaccine's immunogenicity and efficacy. To that effect, newborn

calves in Study 2 received either bRSV Ab-positive colostrum (resulting in a group mean nAb titer of 1:200 at pre-vaccination) or bRSV Ab-negative colostrum, followed by two vaccine doses, 4 weeks apart, and challenge after a short DOI (see Figure 1). A control group with colostrum-derived pre-existing bRSV Ab received two PBS injections and a challenge following the same schedule. Upon challenge, this control group displayed the typical bRSV-induced clinical symptoms, including general illness, fever, increased respiratory rates and signs of URTD (Figure 5a-d). Additionally, increases in CLAs, overall lung pathological scores and increased viral loads were observed in their BAL and/or nasal samples (Figure 5e-h). Of note, this placebo group behaved similarly to the one-dose long term DOI placebo group from Study 2 with more severe clinical symptoms compared to that in Study 1, and leading to premature termination in 3 out of 9 animals. In contrast to the controls, calves in the two-dose vaccination groups with or without pre-existing bRSV Ab exhibited either a complete absence of symptoms, or significantly reduced symptoms of general illness, fever and respiratory rates, although they still showed URTD symptoms (Figure 5a-d). In addition, none of these vaccinated animals were pre-terminated. Although occasionally, clinical scores were slightly higher in the group with *versus* without pre-existing Abs, these differences were not statistically significant. Moreover, two vaccinations resulted in significant reductions in the CLAs –which were absent in all (9/9) animals with pre-existing Abs and in nearly all (8/9) animals without pre-existing Abs– and in reduced pathology sum scores (both $p \leq 0.001$; Figure 5e, f). The vaccinations also resulted in reduced virus titers and faster viral clearance from the lung and nasopharynx (Figure 5g, h). Overall, the presence of pre-existing Abs had minimal impact on the vaccine efficacy in calves which received two vaccine doses and were challenged 4 weeks post-dose 2. Furthermore, the fact that all these animals (irrespective of the presence of pre-existing bRSV Ab) were protected from bRSV disease, as observed in Study 1 with the same two-dose short-DOI regimen (see Figure 2), indicated that the vaccine performed similarly well after a more virulent challenge.

ChAd155-RSV humoral immunogenicity

Kinetics of vaccine-induced nAb responses were analyzed by standard neutralization assay using hRSV A Long. In Study 1 at 4 weeks post first vaccination, this vaccine dose had elicited low-level responses across the single-dose short-DOI group and both two-dose groups (geometric mean titers [GMTs]: 35.7–57.7; Figure 6a, b). Similarly, in Study 2, low titers were observed in the single-dose long-DOI group and the two-dose group without pre-existing Abs (GMTs: 154 and 76, respectively). However, the titer in the latter single-dose group was sustained at similar levels up to the time of challenge, i.e. 16 weeks after immunization.

In both studies, the second vaccination clearly increased the nAb titers (Figure 6a, b, d). At two weeks post-dose 2, this was observed in the short- and long-DOI vaccine groups in Study 1 (GMTs: 296 and 531, respectively) and in short-DOI vaccine group without preexisting Abs in Study 2 (GMT: 1323). In the latter group, the titer gradually decreased over time, but remained detectable at 16 weeks post-dose 2 (GMT 101; Figure 6b).

In Study 2, nAb responses induced by two vaccinations were also compared between calves with *versus* without pre-existing bRSV Abs (Figure 6d). While at 4 weeks post-dose 1, low responses were detected in vaccinated calves without pre-existing Abs, titers in calves with pre-existing Abs were similar between the placebo and vaccine groups. This suggest that the nAb responses in the latter vaccine group largely comprised pre-existing Abs derived from the Ab-positive colostrum. The titers in both vaccinated groups increased after the second dose, but were two- to three-fold lower in the calves which received Ab-positive colostrum (GMTs with *versus* without pre-existing Abs, at 2 or 4 weeks post-dose two: 436 *versus* 1323, and 382 *versus* 725, respectively). Our data therefore indicate that the pre-existing bRSV Abs may have dampened the vaccine-induced nAb response, consistent with observations made for different vaccines in human infants with maternal Abs[40,41]. However, despite the potential blunting effect of pre-existing responses on the humoral vaccine immunogenicity, the clinical efficacy upon bRSV challenge appeared to be uncompromised, as described above (see Figure 5).

Upon challenge, titers increased across all groups in both studies. Of note, among the short-DOI groups in Study 1, titers were lower in the single-dose group than in the two-dose group at pre-challenge (GMTs: 58 *versus* 316, respectively), but similar between these groups two weeks after the challenge (Figure 6a). Among the two-dose groups, post-challenge titers were comparable between the short- and long-DOI groups (Figure 6a, b). A similar trend was seen in Study 2. Indeed, the low titer in the single-dose group at pre-challenge increased by 53-fold upon challenge, whereas fold-increases post-challenge were lower in the two-dose groups without or with pre-existing Abs (4.3-fold and 1.5-fold, respectively; Figure 6c). This suggests that the nAb titer before challenge impacts the magnitude of the Ab response to viral infection, such that higher titers before infection exhibit a lower increase after infection. A likely explanation is that a more robust nAb response will better contain viral replication, and the resulting lower viral levels will then induce a lower increase in Ab titer.

Discussion

A pediatric vaccine to protect infants against RSV-linked LTRD is urgently needed to cover the large burden of disease in infants. Vector-based RSV vaccines, such as the ChAd155-RSV candidate vaccine based on a chimpanzee-derived Ad-vector, elicit immune profiles that have not been associated with ERD, and may be a suitable solution for pediatric use. We have evaluated the immunogenicity, safety (in terms of ERD) and vaccine efficacy of the Chad155 RSV pediatric vaccine in a calf model, and studied different settings and regimens to mimic, as closely as possible, the human infant vaccination scenarios. We demonstrate that ChAd155-RSV was immunogenic in naïve young calves, inducing low to modest RSV nAb responses following the first dose which were further increased following the second dose. The two-dose vaccination regimen protected the calves from bRSV-induced clinical disease and lung pathology, and significantly reduced viral loads in the nasopharynx and lung after a DOI of either 4 or 16 weeks. The protection conferred by a single vaccination resembled the protection following the two-dose regimen when applying the short DOI, and was near-complete against general illness, fever and CLA after the long DOI. However, in the latter case, the effects of the single dose on respiratory rates and lung

histopathology were less profound than with two doses and a long DOI. Finally, protection after two vaccinations and a short DOI was similar in calves with *versus* without pre-existing maternal RSV Abs.

Multiple studies demonstrated that replication-incompetent ChAd vectors against several viruses, including RSV and SARS-CoV2, are capable of inducing humoral and cell-mediated responses in humans with acceptable safety profiles[16,19,24,25,42]. Activation of both arms of adaptive immunity is considered critical for protection against severe RSV disease, especially in infants and older adults[11,43–46]. Previously, ChAd155-RSV was demonstrated to induce RSV nAbs and T-cell responses in seropositive adult humans[22]. Here we show that two doses of ChAd155-RSV induced robust nAb responses that reached the estimated protective titer in human infants[47] (i.e., 256) and just exceeded the threshold of 6 log2 associated with a reduced risk of hospitalization in infants[48]. The immune responses observed here in calves, which were sustained during the 4-week study period, almost completely protected the animals from bRSV clinical disease and lung pathology, as did the two-dose regimen after the 16-week period. The latter is remarkable, considering that at challenge these nAb responses had decreased to a level (geometric mean of 101) well below the above mentioned protection threshold[47]. In animals receiving a single vaccine dose, the general illness, fever and lung consolidation readouts, and the virus concentrations in the lung and nasopharynx, were all comparable after long- and short-DOI. However, among these single-dose groups, respiratory rates and lung histopathological scores with a long DOI (Study 2), while still being lower than the controls, were less reduced than with the short DOI (Study 1). In the absence of a two-dose long-DOI group in the same study, it remains inconclusive whether this slightly compromised protection was due to the more severe challenge condition, or to a lower efficacy of this single-dose regime. Nevertheless, the overall results suggest that a single-dose regime also merits consideration in clinical development (NCT03636906).

Though not measured here, several lines of evidence suggest the presence of vaccine-induced immunity beyond the detected nAb responses. Indeed, in line with the intended pediatric use of ChAd155-RSV, calves received their first injection at a young age (3 to 12 months old). At this age, the bovine immune system is considered not fully mature, resulting in weak nAb responses due to the induction of low-frequency and short-living plasma-cell responses[49–51]. In human infants, differentiation of B cells into long-lived plasma cells after antigenic exposure is known to be reduced due to lack of B-cell survival signals in the bone marrow[52–54]. While a similar effect is expected to have occurred in the vaccinated calves, they nevertheless exhibited the typical kinetics of a B-cell response to vaccination, in which nAb titers elicited by the first dose were boosted by the second vaccination, and further boosted by the challenge. This suggests that the vaccination had successfully induced plasma-cell and memory B-cell responses targeting the live human challenge strain (RSV A Long). Moreover, the fact that all calves in the single-dose groups were strongly protected with reduced viral titers, even though their nAb titers were low, indicated that other arms of immunity, such as T cells, may also have contributed to this level of protection. Indeed, the M2-1 and N proteins are included in the vaccine construct to elicit T-cell immunity, and preliminary analysis in a different calf study showed that ChAd155-RSV induced both $CD4^+$ and

$CD8^+$ T-cell responses to RSV proteins following a single injection (unpublished data). In addition, T-cell responses were detected in the vaccinated adult humans[22].

A large proportion of the vaccine target population of young infants will have maternal-derived RSV Abs due to natural infection of the mother, and possibly increased levels due to future maternal RSV vaccination[4,55]. Moreover, many infants may receive one of the forthcoming prophylactic anti-RSV mAbs[56,57]. Importantly, although our results suggest that the presence of maternal Ab may have had some negative impact on the levels of the nAb response, as observed after the second dose, this did not appear to have affected the protection against the bRSV challenge conferred to these animals. The collective data thus suggest that the presence of pre-existing RSV Abs might have at most minimal effects on the efficacy of ChAd155-RSV in human infants. Nevertheless, future research could help determining which RSV F epitopes are the main targets of the humoral immune response, and whether the observed negative effect of pre-existing Ab has any impact on vaccine immunogenicity in humans.

Considering the (~3.2 times) higher dose of challenge virus in Study 2 versus Study 1, the clinical data post-challenge indicated a clear dose-response effect in the onset and severity of the symptoms. The higher CLA scores of controls in Study 2 may also be related to the more severe challenge condition. However, this difference between studies may have been exacerbated by the fact that the mean CLA score at necropsy was calculated on data from all animals, including those that were terminated 5 or 6 days earlier, when they most likely have been at their disease peak. Indeed, the overall higher CLA scores in the pre-terminated versus surviving animals is consistent with data showing a trend of an association between disease severity scores and CLA scores[30]. Similarly, histopathology scores were also higher in Study 2, though for these scores no difference was found between pre-terminated and recovered animals. This may be because the recovery from such microscopic symptoms takes generally longer. Nonetheless, the two-dose (short-DOI) regimens in groups without pre-existing responses displayed similar efficacies across the two studies. This indicated that the efficacy data from Study 2 can be used to support the overall conclusion on ChAd155-RSV vaccine.

Beyond its utility in RSV vaccine immunogenicity/efficacy studies, the calf model is also suitable for evaluations of vaccine safety, including the risk of ERD, a major focus in pediatric RSV vaccine development. In calves, this phenomenon has been characterized by advanced and exacerbated clinical symptoms upon bRSV challenge relative to control bRSV-challenged animals and by Th2-skewed cytokine profiles, IgE Ab production, pulmonary eosinophilia, and a high ratio of non-functional Abs to nAbs[30,37,38,58–60]. The current research work was not designed as a safety study to extensively investigate all ERD signs or characteristics, thus did not include a positive control vaccine (FI-RSV) inducing ERD. Nonetheless, we observed that all ChAd155-RSV-vaccinated, bRSV-challenged young calves were either free of clinical/pathological symptoms, or exhibited symptoms that were much less severe than in the PBS-injected and bRSV-challenged control groups, while in case of an ERD response the symptoms in vaccinated animals would have been more severe than in the controls. In conclusion,

our observations suggest that the risk that ChAd155-RSV will induce ERD in human infants is extremely low, supporting the further clinical development of this vaccine.

Materials And Methods

Ethical clearance

Prior to the studies' initiation, protocols were submitted for ethical review and approved by GSK's ethical committee (approval no: S001698 for Study 1, S003976 for Study 2). Both studies were conducted in Wageningen University and Research (WUR; Wageningen Bioveterinary Research institute, Lelystad The Netherlands) in accordance with the Dutch Law on Animal Experiments and the European legislations and guidelines (2010/63/EG and ETS 123). Study 1 was authorized by the Animal Ethics Committee of the Animal Sciences Group of WUR. Study 2 was licensed by the Dutch Central Authority for Scientific Procedures on Animals (no. AVD401002015194) and approved by WUR's Animal Welfare Body.

Animal Study Designs

Two placebo (phosphate-buffered saline; PBS)-controlled calf studies were performed according to the design displayed in Figure 1 (showing vaccine groups only). Because of the endemic status of bRSV, calves were recruited from conventional dairy farms within 2 hours after spontaneous birth and raised in isolation until the age of vaccination (6 or 10 weeks). Upon arrival in the animal facilities, calves in Study 1 were kept colostrum-deprived, and calves in Study 2 received a single feeding with either bRSV-Ab-negative or bRSV Ab-positive colostrum.

In Study 1, newborn calves (n=7 or 8 per group) tested negative for anti-bRSV Abs were injected either once or twice (4 weeks apart) with ChAd155-RSV or PBS. Challenge occurred either 4 weeks or 16 weeks after the last vaccine dose, henceforth referred to as the short or the long duration of immunity (DOI), on the same calendar day across groups.

In Study 2, two groups of 9 calves each were fed bRSV Ab-positive colostrum, and received two doses (4 weeks apart) of either ChAd155-RSV or PBS. A third group (n=9) was fed bRSV Ab-negative colostrum and received two doses of ChAd155-RSV, serving to bridge Studies 1 and 2 and as a bRSV Ab-negative control. All three groups were challenged with bRSV after the short DOI, to evaluate the impact of pre-existing RSV Abs on vaccine immunogenicity and efficacy. Two other groups of 9 calves each in Study 2 were fed Ab-negative colostrum, and received one dose of either ChAd155-RSV or PBS. These animals were challenged with bRSV after the long DOI. In Study 2, a total of eight animals in both placebo groups reached humane endpoints (as defined in 'Clinical Assessments' below) before termination of the study and were euthanized due to severe clinical signs of bRSV infection (see 'Results').

Blood samples were collected at different time-points pre- and post-vaccination and post challenge to assess vaccine immunogenicity. Upon bRSV challenge, clinical vaccine efficacy was evaluated through observations of clinical symptoms, measurements of rectal temperature; as well as collection of

nasopharyngeal brush samples and broncho-alveolar lavage (BAL) samples, to measure viral loads. At 12 or 13 days post challenge (dpc), calves were euthanized. Lungs of all animals were subjected to post-mortem examination to evaluate lung macroscopic and microscopic lesions, focusing on macroscopic consolidated lung areas (CLAs) and microscopic pathological changes. For the pre-terminated control animals in Study 2, these examinations were performed at dpc 7 or 8.

ChAd155-RSV vaccine and bRSV challenge stock preparation

The ChAd155-RSV investigational vaccine consists of a recombinant replication-defective ChAd155 vector construct, engineered to express the RSV F protein deleted of the transmembrane region, and the RSV N and M2-1 proteins, as described previously[22,26]. For the challenge, an *in vivo*-passaged bRSV (strain Odijk, subtype A, passage 6) was used, which was originally isolated from a calf in the field suffering from acute respiratory disease[61]. The *in-vivo* passage was performed in caesarian-derived specific-pathogen-free calves. BALs used for experimental challenge contained at least $10^{2.5}$ TCID50/mL bRSV, and were tested free from major bovine respiratory viruses, mycoplasmas and bacteria, by evaluating three *in-vitro* passages on sensitive cell cultures and classical bacterial and mycoplasma culture.

Vaccination and bRSV challenge

In both studies, calves were vaccinated intramuscularly in the pre-scapular area with $5x10^{10}$ viral particles of ChAd155-RSV in a 2 mL volume. Four or 16 weeks post-last immunization, calves were challenged by aerosolized inoculation of 2 mL bRSV (strain Odijk, doses: 3.2 $\log_{10}$ $TCID_{50}$ in Study 1, and 3.7 $\log_{10}$ $TCID_{50}$ in Study 2) using an airbrush (Harder & Steenbeck) as described previously[30].

Clinical assessments

Upon challenge, bRSV-related symptoms were monitored daily by the same veterinarian, who was blinded for the treatment groups. Clinical scores for general illness (including depression and loss of appetite), upper respiratory tract disease (URTD; nasal and ocular discharge and cough) and LRTD (increased breathing effort) were assessed according to the scoring system outlined in Supplemental Table 1. The LRTD score was partially based on the actual respiratory rate, which was counted by visual inspection. In addition, rectal temperatures were measured twice daily after the challenge. Humane endpoint criteria were defined as persistent severe depression (max. 24 hours), persistent severe dyspnea e.g. respiratory rate of >100 breathings/minute (max. 48 hours), or non-reversible respiratory failure such as intermittent open mouth breathing or frothing (immediately).

Sample collection

Blood samples for serology (8 mL) were collected at different time points (see Figure 6) by venipuncture of the *V. jugularis*. Just prior to challenge and on dpc 1 to 9, nasopharyngeal samples were collected using sterile nylon bristle brushes (MW126, Medical Wire and Equipment Co. Ltd). Following sampling,

brushes were directly agitated in 3 mL transport medium, consisting of EMEM (GIBCO), supplemented with 2% antibiotics and 2% fetal bovine serum (FBS). BAL samples were collected 6 days prior to challenge and on dpc 5, 7 and 9. BAL fluid was obtained after instillation of approximately 100 mL D-Phosphate Buffered Saline (GIBCO) via intubation of the ventral nasal meatus. Fluid recovered from the lung (50-80% of the instilled volume) was directly supplemented with 5% FBS.

Processing of samples for viral load

Nasopharyngeal brush and BAL samples were kept on melting ice immediately following the sampling procedure. In the laboratory, brushes were removed from the medium. Samples were centrifuged for 10 minutes at 4°C, at 1300g or 250g for nasopharyngeal and BAL samples, respectively. Supernatants were stored at -70 ± 10°C until further analysis.

Necropsy and pathological analysis

On dpc 12/13 (or day 7/8 for pre-terminated animals), calves of each treatment group were euthanized by pentobarbital overdose and exsanguination. The lungs were removed, and dorsal and ventral images were taken to calculate the extent of macroscopic lesions (Image Pro Premier 64-bit software.) The CLA is presented as percentage of the total lung area. Lung samples were collected, if applicable, on a transition between normal and consolidated tissue, stored in 10% neutral buffered formalin, and embedded in paraffin. For histological examination, 5 µm sections of the right apical and cardiac lobes and the left cardiac lobe were stained with hematoxylin and eosin. Each tissue section was scored from 0 (absent) to 4 (severe) for the following categories of histopathological changes according to the scoring table (Supplemental Table 2): endo-bronchi(oli)tis, peri-bronchitis/vasculitis, interstitial pneumonia and alveolitis. CLA calculations and histological examination were performed blinded.

Viral load assessment

All nasopharyngeal brush and BAL samples were first analyzed by bRSV qPCR to determine the course of viral replication. Subsequently, a selection of these samples collected on various time-points was also analyzed by virus titration. Infectious virus titers were determined in duplicate by end-point titration of 10-fold sample dilutions on immortalized Embryonic Bovine Trachea cells seeded in 96-wells plates. Duplicate plates were incubated for 6-7 days at 37°C and 5% CO_2. At the end of the incubation period, virus-infected cells were detected with an anti-F bRSV monoclonal Ab (mAb) followed by peroxidase staining[62,63]. For each duplicate plate, the virus titer was determined, and expressed (in $\log_{10}$ value) as the endpoint titer at which 50% of the cell monolayers was infected, as calculated according to Reed and Munch approach. The detection limit was 0.80 log10 $TCID_{50}$/mL. Samples tested negative were assigned a value of 0.30 log10 TCID50/mL.

Neutralization assay

Briefly, sera were serially diluted 2-fold in DMEM medium with 3% FBS. Sample and positive cotton rat serum dilutions were mixed with human RSV A Long (ATCC, VR-26) diluted to approximately 100 plaque forming units/well, and incubated for either 20 min at 33°C (Study 1) or 120 min at 35°C (Study 2). After

incubation, the virus-serum mixture was transferred to Vero cell-seeded plates, with virus-only wells as 100% infectivity control. Plates were incubated for 2 hours at 35°C, then the medium was removed and RSV medium containing 0.5% carboxymethylcellulose (medium viscosity) was added to all wells. The plates were incubated for 42 hours at 35°C before staining. Staining was performed with mouse anti-RSV N and anti-RSV F mAbs followed by goat anti-mouse immunoglobulin G-horse radish peroxidase. After Ab staining, True Blue substrate was added to all wells to reveal the infectious foci. Plates were scanned using a ScanLab/Axiovison reader. Reciprocal nAb titers were expressed in ED60, determined as the inverse of the serum dilution causing 60% reduction in the number of plaques as compared to the control wells (virus only, no serum).

Statistical analyses

The nAb titer and viral load data were analyzed using repeated-measurement ANOVA, with treatment, time and interaction of treatment*time as fixed effect on log10-transformed values, with the appropriate variance and co-variance assumptions. All other data were analyzed using ANOVA with treatment as fixed effect and with the appropriate variance assumption, either on the single-timepoint data (for CLAs and histopathology sum scores) or on the calculated areas under curve (AUCs; for general illness scores, rectal temperatures, respiratory rates and URTD levels). Statistical significance was set at $P \leq 0.05$.

Declarations

Authors' contribution

AMS, JFT, RDJ were involved in the conception and design of the study and/or the development of the study protocol. RDJ, JB, BB, NSZ participated to the acquisition of data. RDJ, AMS, JFT, HS, ID, KFW, SV analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

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Conflict of Interest statement

KFW, BB, JFT, ID, HS and AMS are, or were at the time of the study employees of the GSK group of companies. AMS, HS, JFT and ID report ownership of GSK shares and/or restricted GSK shares. SV was employee of Keyrus Biopharma, contracted by GlaxoSmithKline Biologicals SA in the context of this study. RdJ, NSZ, and JB are employees of the Wageningen Bioveterinary Research, Lelystad, The Netherlands, contracted by GlaxoSmithKline Biologicals SA in the context of this study. Parts of RdJ, NSZ, and JB's salaries were paid from the contract fee.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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Figures

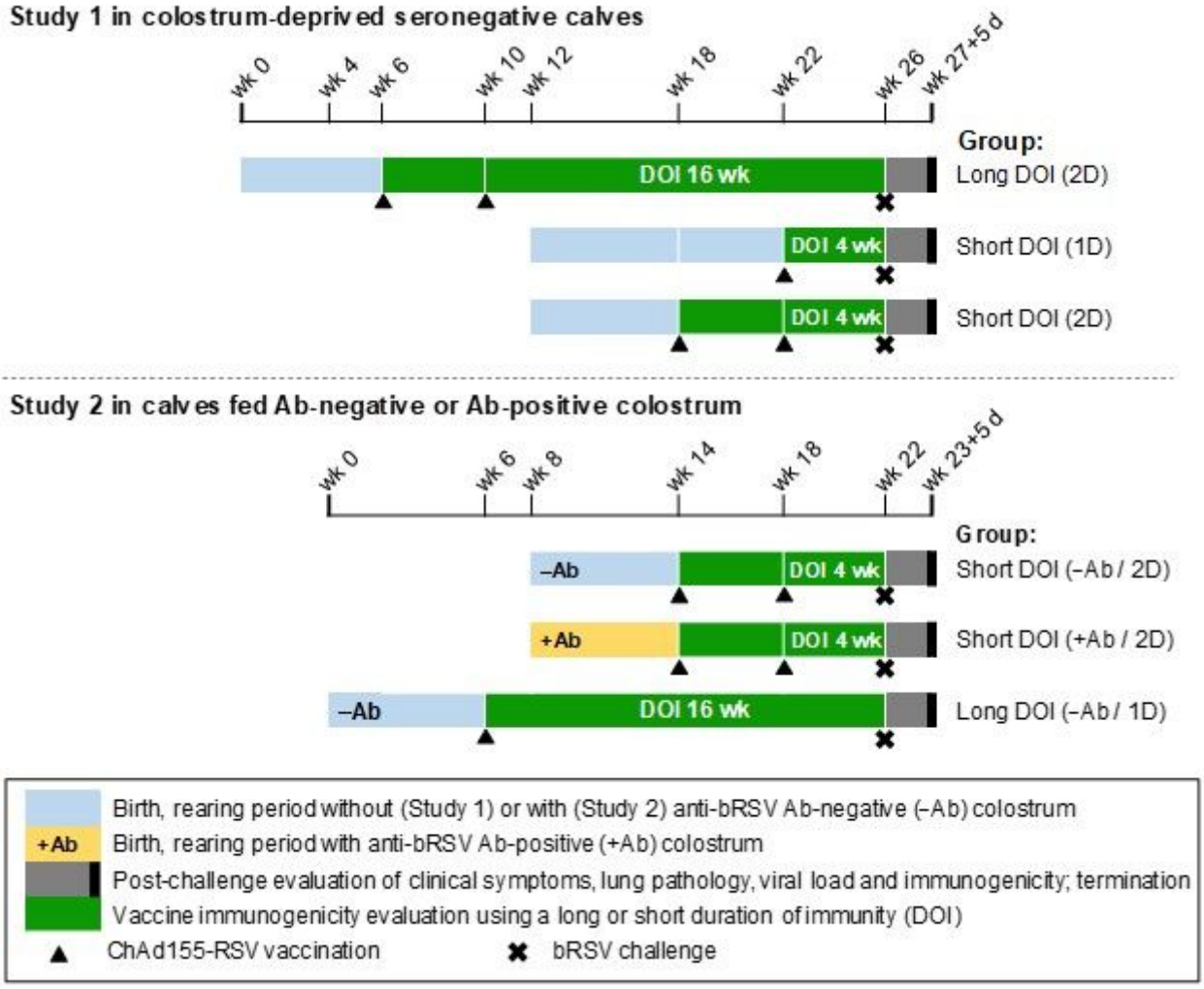

Figure 1

Study designs: Studies were conducted in bovine RSV-seronegative colostrum-deprived calves (Study 1), or in calves fed bRSV antibody (Ab)-negative (-Ab) or Ab-positive (+Ab) colostrum (Study 2). Animals (n=8 or 9/group) received 1 or 2 doses (1D or 2D, respectively) of either ChAd155-RSV vaccine or placebo (phosphate-buffered saline; controls). Corresponding vaccine and control groups challenged after either a short (4 weeks) or a long (16 weeks) duration of immunity (short DOI or long DOI, respectively) followed the same vaccination and challenge schedules. Short and long DOI controls (placebo-S and placebo-L groups, respectively) are not shown.

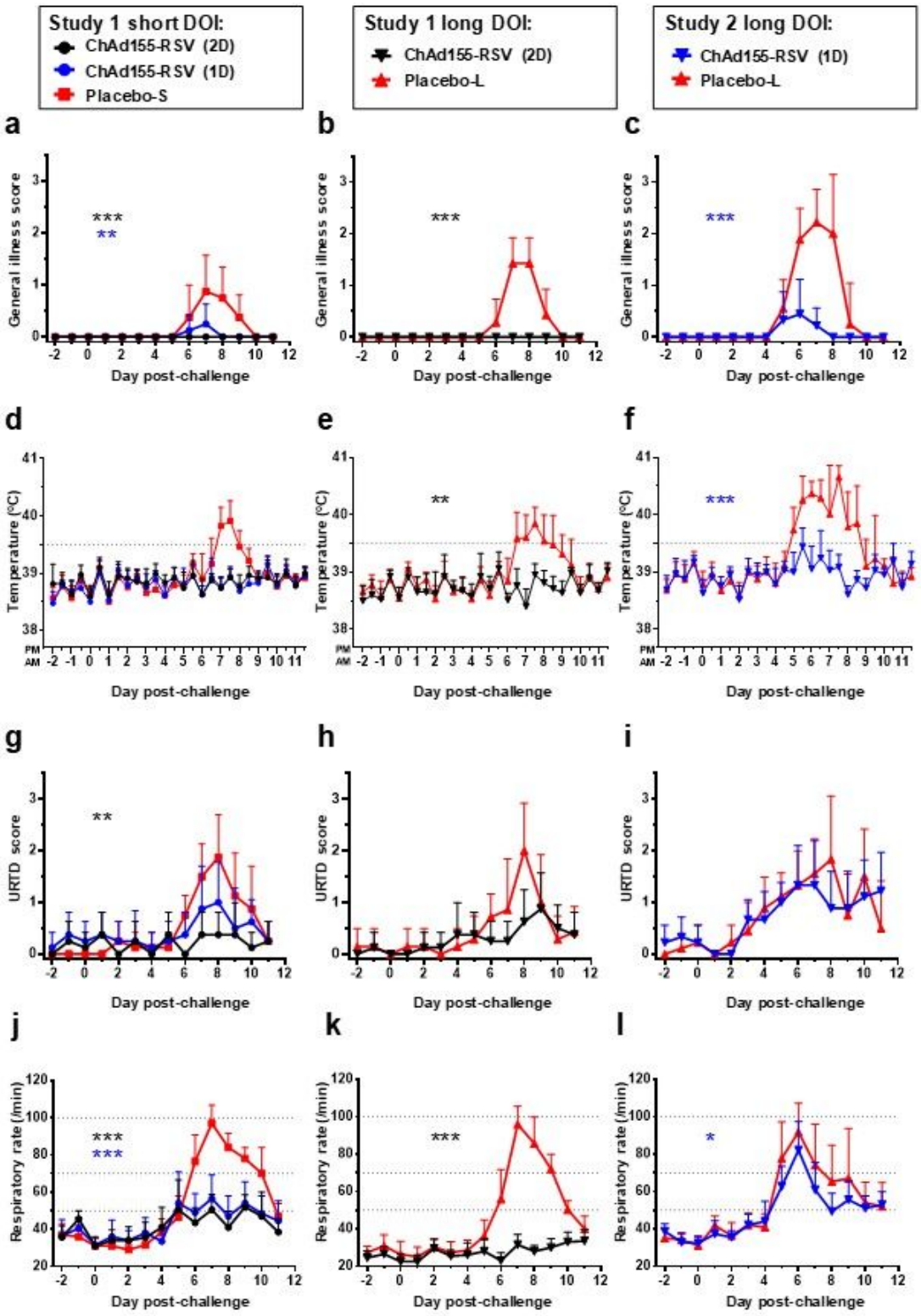

Figure 2

Clinical symptoms. Animals received either a single or two doses (1D or 2D, respectively) of ChAd155-RSV vaccine or placebo, followed by a short (4 weeks) or long (16 weeks) duration of immunity (short DOI or long DOI, respectively), and then a bovine RSV challenge. Placebo-S and placebo-L groups indicate the control groups subjected to the short DOI and long DOI regimens, respectively (note that the sample size in the placebo-L group of Study 2 decreased from day 7 post-challenge; see Fig. 3). Clinical symptoms in

the ChAd155-RSV-immunized or placebo-treated calves were evaluated before and after the challenge at the time-points indicated. Data are presented as geometric means with 95% confidence intervals (vertical bars). Scores of general illness symptoms (a-c), rectal temperatures (d-f), upper respiratory tract disease (URTD) scores (g-i) and respiratory rates (j-l) are presented. Differences between the vaccine groups and the respective control groups were estimated by calculating the area under curve (AUC), followed by an ANOVA on the AUC with treatment as fixed effect and the appropriate variance assumption. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

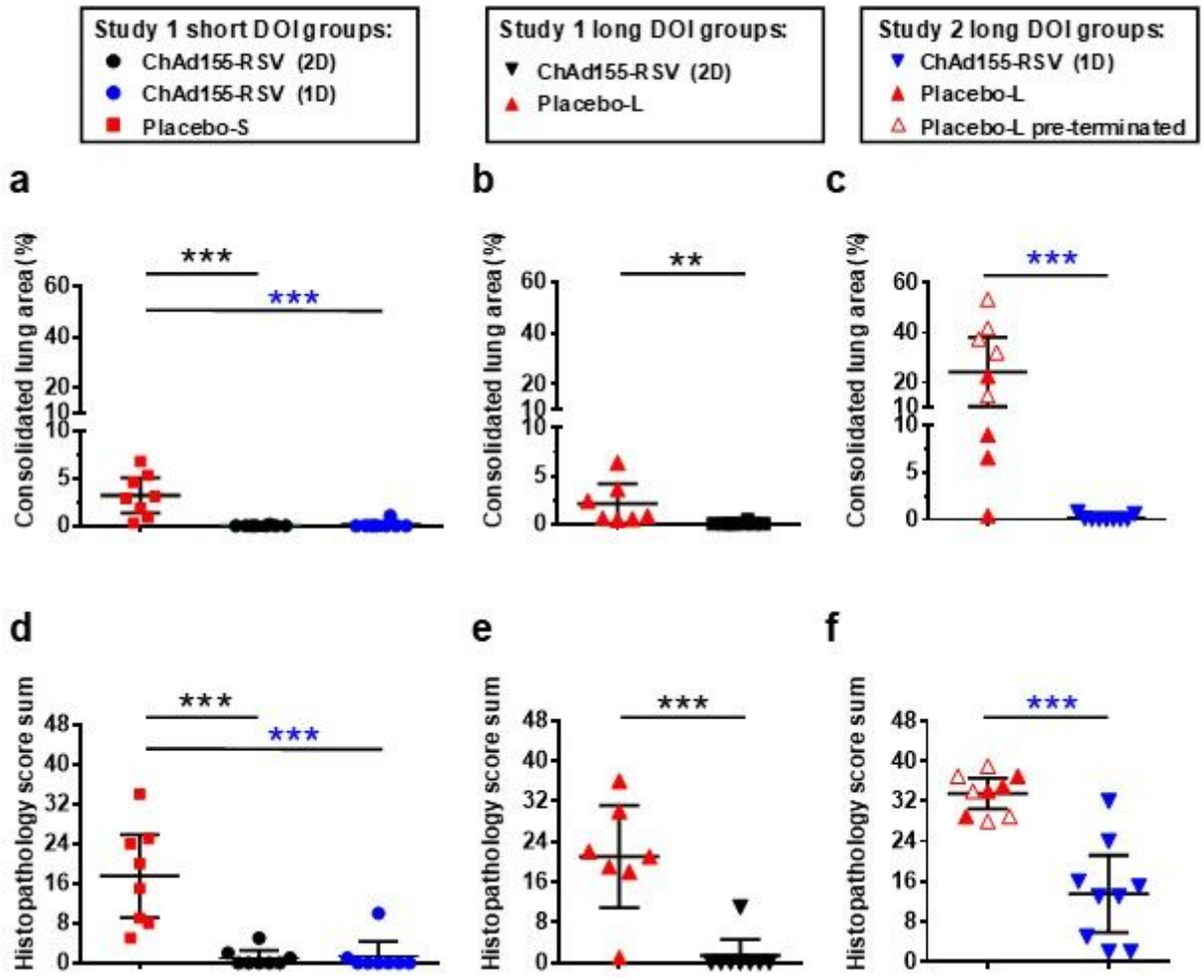

Figure 3

Lung pathology Animals received a single or two doses (1D or 2D, respectively) of ChAd155-RSV vaccine or placebo, followed by a short (4 weeks) or long (16 weeks) duration of immunity (short DOI or long DOI, respectively) and then a bovine RSV challenge. Placebo-S and placebo-L groups indicate the control groups subjected to the short DOI and long DOI regimens, respectively. Macroscopic analysis of the consolidated lung areas (CLAs; a-c) and sums of lung pathology scores determined by microscopic analysis of the lung pathological changes (d-f) are presented for the study groups indicated in the keys above the graphs. Sums of lung pathological scores were derived by addition of the scores for alveolitis, interstitial pneumonia, peribronchitis and bronchitis per individual animal. Data are presented as means ±95% confidence intervals. Each symbol represents an individual animal. Open symbols in panels c and f indicate animals that were pre-terminated or found dead. Vaccine groups were compared with the

respective control groups using one-way ANOVA for CLAs and histopathology score sums with treatment as fixed effect and the appropriate variance assumption. Significant differences are indicated by asterisks (*P≤0.05; **P≤0.01; ***P≤0.001).

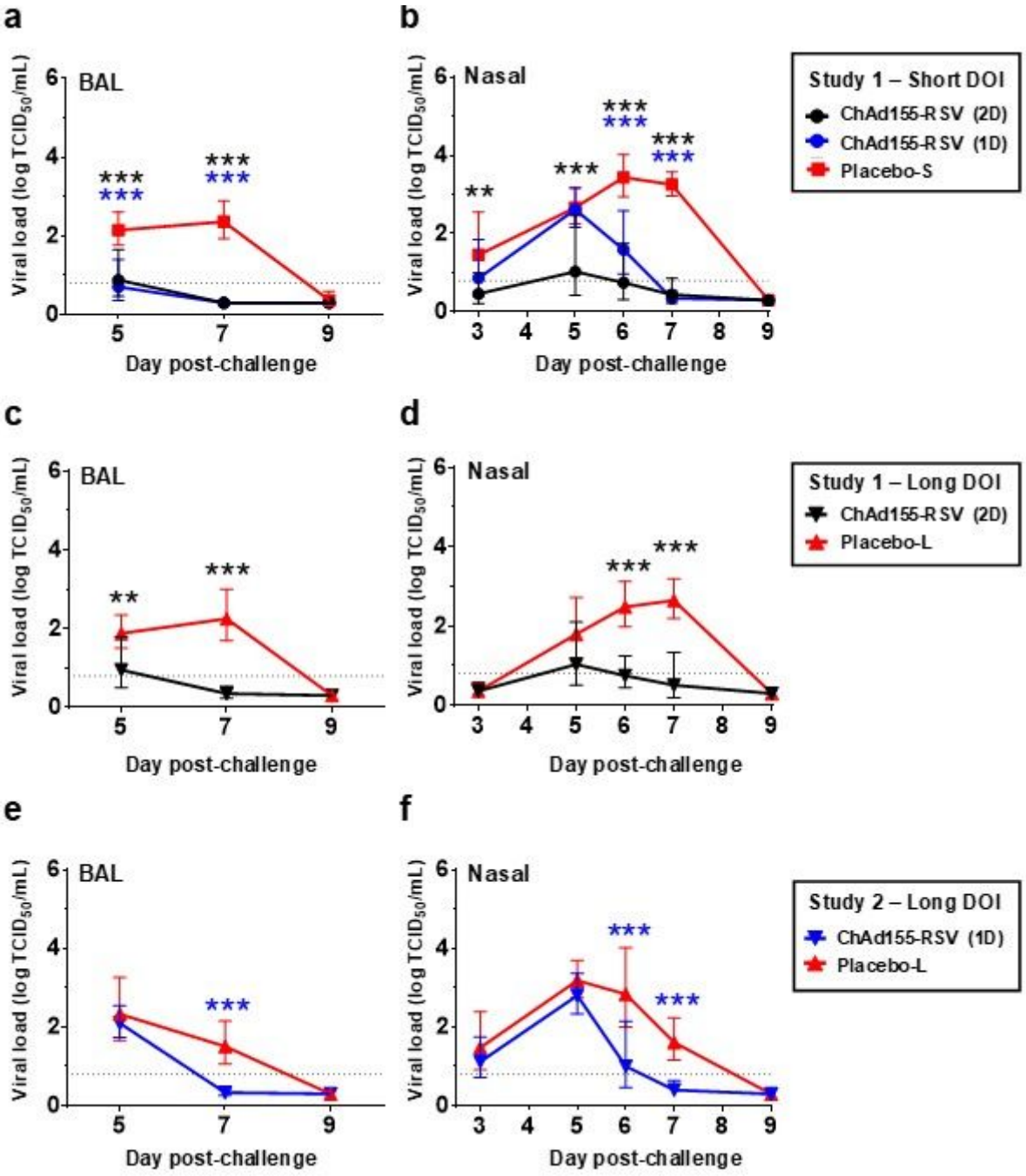

Figure 4

Viral loads. Animals received either a single or two doses (1D or 2D, respectively) of ChAd155-RSV vaccine or placebo, followed by a short (4 weeks) or long (16 weeks) duration of immunity (short DOI or long DOI, respectively), and then a bovine RSV challenge. Placebo-S and placebo-L groups indicate the control groups subjected to the short DOI and long DOI regimens, respectively (note that the sample size in the placebo-L group of Study 2 decreased from day 7 post-challenge; see Fig. 3). Virus loads of lung broncho-alveolar lavage (BAL) samples (a, c,d) and nasopharyngeal samples (b, d, f) are presented as

geometric mean concentrations with 95% confidence intervals. Dotted lines (0.8 log10 TCID50/mL) represent the lower limit of detection (LOD). Negative samples were assigned a value of 0.3 (i.e. 0.5 log below LOD). Vaccine groups were compared with the respective control groups using ANOVA mixed models for repeated measurements. Significant differences are indicated by asterisks color-matched with the vaccine group indicated in the keys above the figure (*P ≤0.05; **P ≤0.01; ***P ≤0.001).

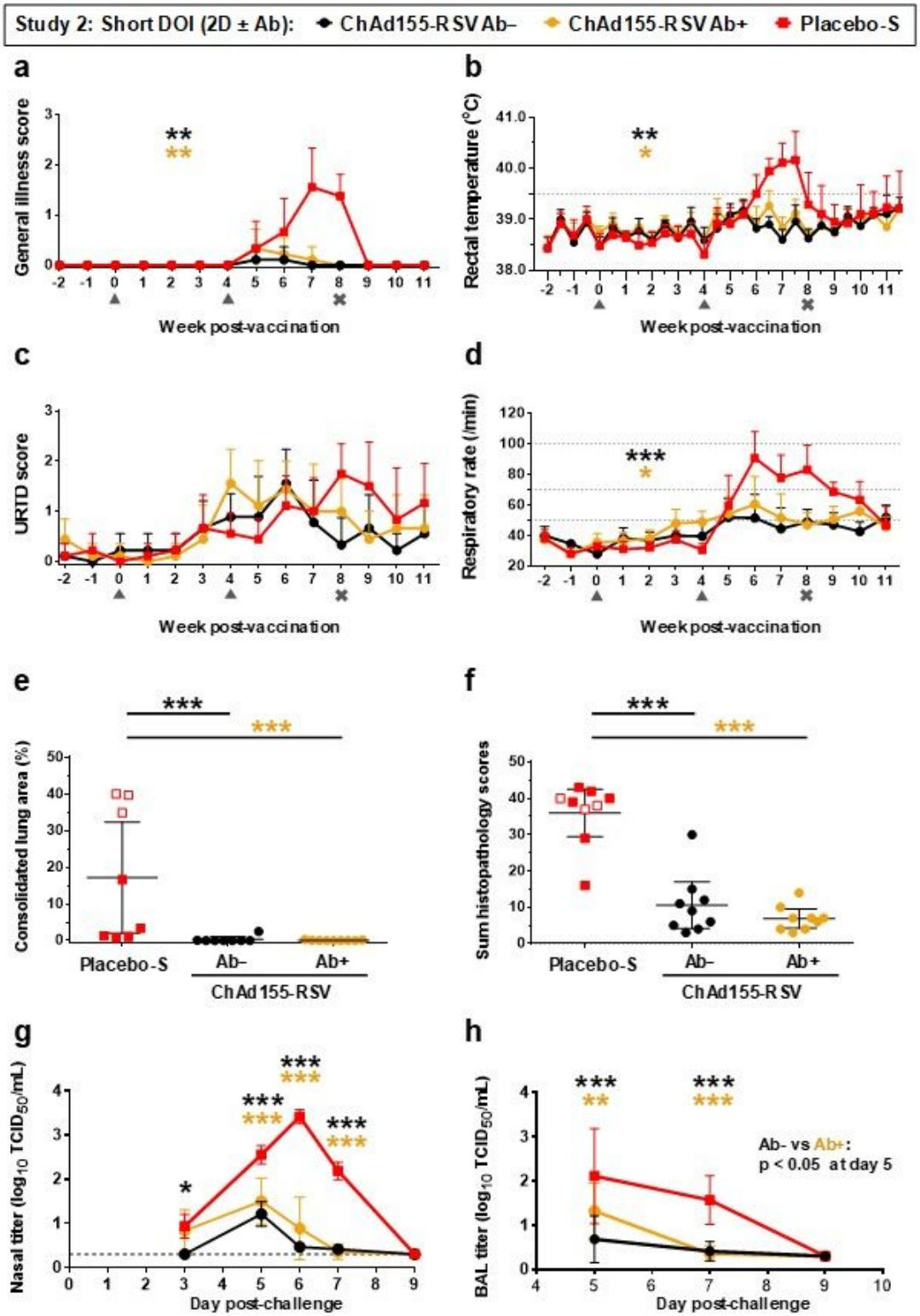

Figure 5

Impact of pre-existing bRSV Ab on ChAd155-RSV vaccine efficacy. In Study 2, calves with (Ab-) or without (Ab+) pre-existing antibodies derived from bovine RSV Ab+ or Ab- colostrum, received two doses (2D) of ChAd155-RSV or placebo, and were challenged after a short (4 weeks) duration of immunity (DOI). The placebo-S group indicates the control group. Triangles and crosses below the x-axis indicate the time-points of placebo or vaccine injections and the bovine RSV challenge, respectively. General illness scores (a), rectal temperatures (b), upper respiratory tract disease (URTD) scores (c), respiratory rates (d), consolidated lung areas (CLAs; e), lung pathological changes at termination time-point (f), and nasopharyngeal and lung virus loads (g and h, respectively) were analyzed. Differences between vaccine groups and the respective control groups were determined by ANOVA (with treatment as fixed effect and the appropriate variance assumption) performed either on the calculated areas under the curve (a-d) or on the single-timepoint data (e, f), or by ANOVA mixed models for repeated measurements (g, h). Statistically significant differences are presented as p-values or asterisks color-matched with the vaccine group indicated in the key above the figure.

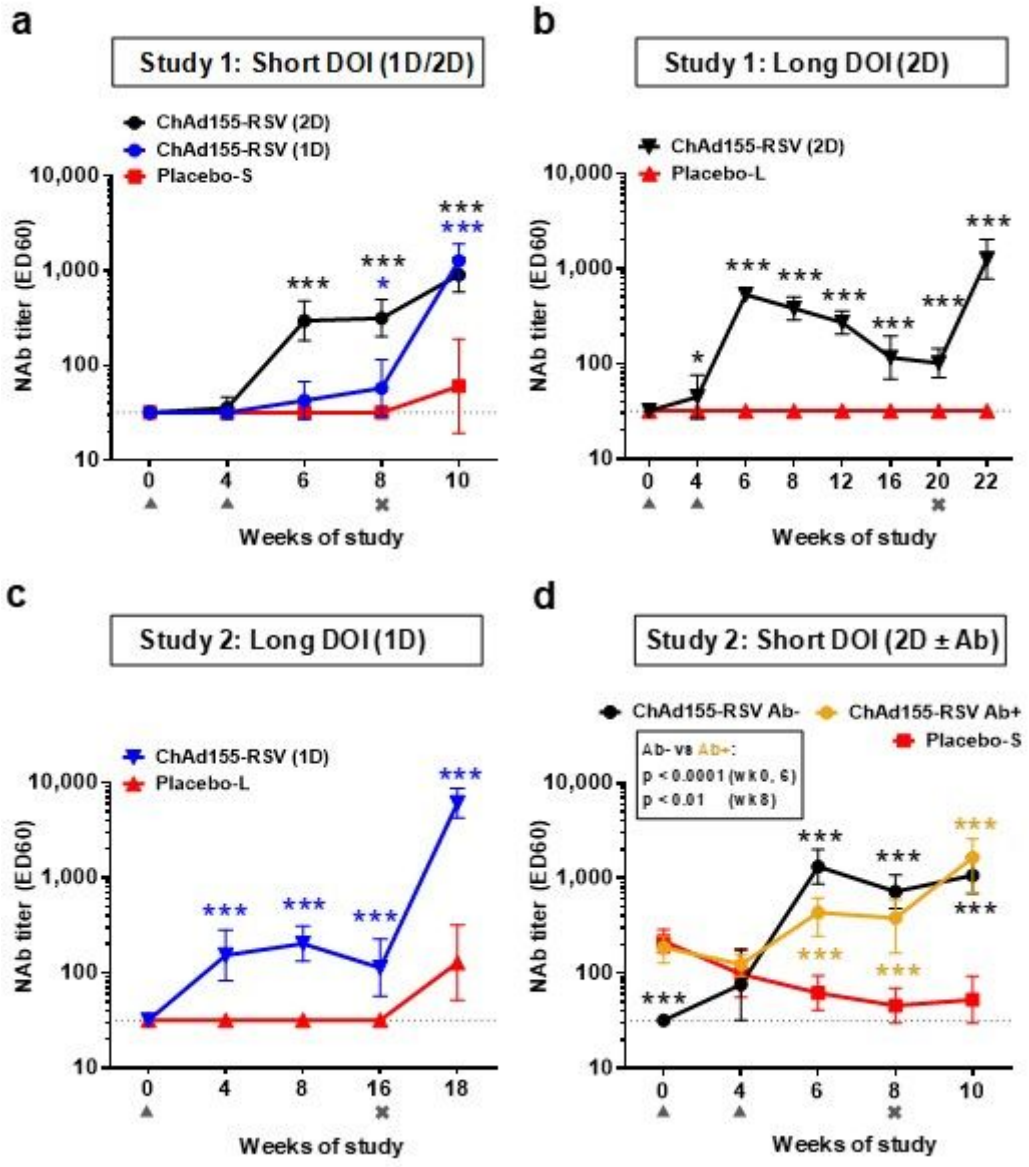

Figure 6

Human RSV A-specific nAb titers. Human RSV subtype A (hRSV A) neutralizing antibody (NAb) titers are presented. Titers are expressed as the inverse of the serum dilution causing 60% reduction in the number of plaques as compared to the virus control wells (ED60). Geometric means with 95% confidence intervals (error bars) for each group are color coded according to the keys in each graph. Animals received a single or two doses (1D or 2D, respectively) of ChAd155-RSV vaccine or placebo, followed by a short (4 weeks) or long (16 weeks) duration of immunity (short DOI or long DOI, respectively), and then a bovine RSV challenge. Placebo-S and placebo-L groups indicate the control groups subjected to the short DOI and long DOI regimens, respectively (note that the sample sizes of the placebo-L and placebo-S groups in Study 2 decreased post-challenge; see Figs. 3 and 5, respectively). Groups in (d) included calves with or without pre-existing bovine RSV Ab groups (Ab+ or Ab-, respectively). Dotted lines represent the limit of detection (LOD) i.e. 32 ED60. Negative samples were assigned the value of the LOD. Titers of vaccine and control groups were compared using ANOVA mixed models for repeated measurements. Significant differences between vaccine groups and the respective control groups are presented as asterisks color-matched with the vaccine group indicated in the keys. Triangles and crosses below the x-axis denote the time-points of placebo or vaccine injections and the bRSV challenge, respectively.

Supplementary Files

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- SupplementaryTablesandFigures.docx