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Protection against infectious bronchitis virus by spike ectodomain subunit vaccine

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ABSTRACT

The avian coronavirus infectious bronchitis virus (IBV) S1 subunit of the spike (S) glycoprotein mediates viral attachment to host cells and the S2 subunit is responsible for membrane fusion. Using IBV Arkansas-type (Ark) S protein histochemistry, we show that extension of S1 with the S2 ectodomain improves binding to chicken tissues. Although the S1 subunit is the major inducer of neutralizing antibodies, vaccination with S1 protein has been shown to confer inadequate protection against challenge. The demonstrated contribution of S2 ectodomain to binding to chicken tissues suggests that vaccination with the ectodomain might improve protection compared to vaccination with S1 alone. Therefore, we immunized chickens with recombinant trimeric soluble IBV Ark-type S1 or S-ectodomain protein produced from codon-optimized constructs in mammalian cells. Chickens were primed at 12 days of age with water-in-oil emulsified S1 or S-ectodomain proteins, and then boosted 21 days later. Challenge was performed with virulent Ark IBV 21 days after boost. Chickens immunized with recombinant S-ectodomain protein showed statistically significantly ($P < 0.05$) reduced viral loads 5 days post-challenge in both tears and tracheas compared to chickens immunized with recombinant S1 protein. Consistent with viral loads, significantly reduced ($P < 0.05$) tracheal mucosal thickness and tracheal lesion scores revealed that recombinant S-ectodomain protein provided improved protection of tracheal integrity compared to S1 protein. These results indicate that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 domain with S1 might be promising for better viral vectored and/or subunit vaccine strategies.

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1. Introduction

Infectious bronchitis virus (IBV) is a highly prevalent coronavirus of chickens that causes economic losses worldwide despite extensive vaccination. Continuous emergence of new virus serotypes results from mutation and recombination followed by selection [1]. Routinely used live-attenuated IBV vaccines, which are affected by the same evolutionary processes, not only result in vaccine-like viruses with increased virulence and persistence [2,3], but may also contribute genetic material for recombination with other vaccine or wild virus populations. We previously identified five minor vaccine virus subpopulations selected in chickens from Arkansas-Delmarva Poultry Industry (ArkDPI)-derived IBV vaccines, designated components (C) 1–5 [3,4]. The selection of these viral subpopulations within 3 days post-vaccination suggests they replicate better in chickens than the predominant virus population in the vaccine prior to inoculation [3,4].

The spike (S) protein of IBV mediates viral entry into host cells [5,6]. Its S1 subunit mediates viral attachment to host cells and induces virus-neutralizing antibodies that are important for host protective immune responses [7–9]. However, the S1 subunit shows extensive amino acid sequence variability among IBV strains, which leads to the virus's immunological escape [1,10,11]. The S2 subunit of S, responsible for membrane fusion, is more conserved among IBV strains [12]. The N-terminal portion of S2 contains immunodominant regions and a neutralizing

epitope and therefore the S2 protein has been suggested for vaccine development [12,13].

Previous studies indicated that the S1 protein alone does not induce effective protection against IBV challenge. For instance, at least four immunizations with purified S1 glycoprotein were required to induce protection against nephropathogenic N1/62 strain challenge [14]. Similarly, three immunizations with KM91 S1 protein expressed by a recombinant baculovirus produced only 50% protection against virulent nephropathogenic KM91 strain challenge [15].

The S1 subunit of IBV is sufficient for attachment [5,16–19] and the S2 portion of coronavirus spike proteins has traditionally been considered to play a role only in subsequent entry [20,21]. However, a role for the S2 ectodomain in binding to cells has been demonstrated for spike proteins of Massachusetts serotype IBVs, i.e. the highly-attenuated Beaudette strain and the virulent M41 strain [22,23]. In the current study, we evaluated binding of trimeric Ark S-ectodomain compared to trimeric S1 subunit alone to multiple relevant chicken tissues. After confirming improved binding of Ark S-ectodomain, which might be explained by the presence of the S2 ectodomain altering the conformation of S1 and thus increasing its affinity for receptors, or by S2 directly contributing to interaction with receptors or co-receptors, we tested the hypothesis that immunization with recombinant soluble trimeric S-ectodomain provides more effective protection than immunization with trimeric S1 subunit alone.

2. Materials and methods

2.1. Genes and expression vectors

The amino acid sequence of S proteins representing an IBV ArkDPI vaccine subpopulation previously designated C2 (GenBank accession ABY66333) was chosen to produce recombinant proteins. C2 was strongly selected in chickens after vaccination with an ArkDPI-derived attenuated vaccine [3,4]. Its S1 is almost identical to that of the unattenuated parent ArkDPI isolate [24] and represents the consensus sequence of vaccine subpopulations rapidly positively selected in chickens after vaccination with ArkDPI-derived attenuated vaccines [2–4,25,26]. To generate recombinant S1 protein, a human codon-optimized sequence encoding C2 S1 [amino acids (AA) 19-538] was synthesized (GeneArt, Regensburg, Germany) and cloned into the pCD5 vector. To generate recombinant S-ectodomain, a human-codon optimized sequence encoding the C2 S2 ectodomain (S AA 544-1097) was cloned into the pCD5 vector already containing the S1 domain as described [22]. At the S1/S2 border, the furin cleavage site sequence RRSRR was replaced by GGGVP to avoid cleavage of the full length S-ectodomain [22]. These S1 and S-ectodomain-coding sequences were flanked by sequences encoding an N-terminal CD5 signal sequence and sequences encoding C-terminal artificial GCN4 trimerization motif and Strep-tag II for purification and detection of proteins, as described [16].

2.2. Recombinant S protein production and purification

Soluble trimeric recombinant S1 and S-ectodomain proteins were produced in human embryonic kidney (HEK) 293T cells as described [16,22,27]. In brief, the expression vectors encoding S1 or S-ectodomain were transfected into HEK293T cells and recombinant proteins purified from tissue culture supernatants 6 days post-transfection using Strep-Tactin® Sepharose columns according to the manufacturer's instructions (IBA GmbH, Göttingen, Germany). The concentration of purified proteins was determined by Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA). The purified proteins were confirmed and concentrations normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels (Bio-Rad, Hercules, CA).

2.3. Binding to tissues by protein histochemistry

The binding efficiency of S1 and S-ectodomain proteins to tissue sections prepared from healthy specific pathogen free (SPF) 40-day old white leghorn chickens was assessed by protein histochemistry as described [22,27] with minor modifications: antigen retrieval was conducted at 80 °C for 30 min, Tris buffers were substituted for phosphate buffers, slides were blocked with universal negative serum (Biocare, Pacheco, CA) instead of 10% goat serum, and the addition of most reagents and washing steps were performed by an intelliPATH FLX automated slide stainer (Biocare, Pacheco, CA). S proteins and 3-amino-9-ethyl-carbazole (AEC+; Dako, Carpinteria, CA) were added manually. Briefly, S proteins (100 μg/ml for S1 and 50 μg/ml for S-ectodomain) pre-complexed with Strep-Tactin-HRP (IBA GmbH, Göttingen, Germany) were incubated with deparaffinized and rehydrated tissue sections overnight at 4 °C. Bound S protein was visualized with AEC+ chromogenic substrate. The tissues were counterstained with hematoxylin and mounted with Lerner AquaMount (Covance, Princeton, NJ). Images were captured from an Olympus BX41 microscope with an Olympus DP71 12 mp camera.

2.4. Protection trial

2.4.1. Chickens

White leghorn chickens hatched from SPF eggs (Charles River, North Franklin, CT) were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

2.4.2. Experimental design

Four groups of chickens (each n = 16–17) were used. Chickens were primed at 12 days of age (DOA) by subcutaneous injection in the neck region of 0.2 ml containing 10 μg of S1 (group A) or 20 μg of S-ectodomain protein (group B) emulsified in Montanide™ ISA 71 VG adjuvant (Seppic, Paris, France). Twice the amount of S-ectodomain protein was used because recombinant S-ectodomain is 1.96-times the molecular weight of recombinant S1. Thus, approximately equimolar amounts of protein were administered. Chickens in groups A and B were subsequently boosted with the same adjuvanted protein 21 days later. Control group C (non-vaccinated) was primed and boosted with PBS and the adjuvant, and group D was the unvaccinated/unchallenged control group. Chickens in groups A, B and C were challenged 21 days after boost by ocular and nasal instillation of 10^5 50% embryo infective doses (EID$_{50}$) of a virulent IBV Ark-type strain (GenBank accession JN861120) previously characterized [28]. Protection was evaluated 5 days post-challenge (DPC) by viral load in tears and tracheas, tracheal histomorphometry, and tracheal histopathology lesion scoring. In addition, antibodies in sera specific for IBV or S protein were determined by ELISA before prime (11 DOA), three weeks after prime (32 DOA), two weeks after boost (45 DOA) and 5 days post-challenge.

2.4.3. Viral load by qRT-PCR

Relative IBV RNA levels in tears and tracheas were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Viral RNA was extracted from individual tear samples using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA), and

from homogenized tracheas with TriReagent® RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH) following the manufacturers' protocols. Relative viral RNA concentrations in tear and tracheal samples were determined by TaqMan® qRT-PCR as described [29]. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-test.

2.4.4. Tracheal histomorphometry and histopathology

Histomorphometry of the tracheal mucosa was evaluated blindly as described [30]. Briefly, formalin-fixed sections of trachea collected from challenged and control birds at 5 days post-challenge were processed, embedded in paraffin, sectioned at 4–6 μm and stained with hematoxylin and eosin for histopathological examination. The tracheal mucosal thickness and the thickness of lymphocytic infiltration were measured using ImageJ (https://imagej.nih.gov/ij/download.html), and the average of five measurements for each chicken calculated. The severity of lesions (tracheal deciliation and epithelial necrosis) was scored (1 = normal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) and the average of the two scores determined as a lesion score for each chicken. Histomorphometric data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons post-test. Lesion scores were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons post-test.

2.4.5. Antibodies measured by ELISA

2.4.5.1. IBV-specific ELISA. IBV-specific ELISA was performed as previously described [31]. Briefly, ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated with heat-inactivated IBV (ArkDPI vaccine strain; S AA sequence GenBank #ABY66334) purified as described [31]. Individual chicken sera diluted 1:100 were loaded and plates incubated at 4 °C overnight. IBV-specific IgG was detected using biotinylated monoclonal mouse–anti chicken IgG [(clone G-1) Southern Biotechnology Associates, Inc., Birmingham, AL], streptavidin-conjugated HRP (Southern Biotechnology Associates, Inc.) and tetramethylbenzidine (TMB; Invitrogen Corp., Frederick, MD) HRP substrate. Absorbance at 450 nm was measured with a Powerwave XS (BioTek Instruments, Inc., Winooski, VT).

2.4.5.2. S1 and S-ectodomain protein-specific ELISA. ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated with 100 μl of 0.25 μg/ml of either recombinant S1 protein or S-ectodomain protein at 4 °C overnight. Plates were drained and blocked with 200 μl of 1% bovine serum albumin and 0.05% Tween 20 in PBS for 1 h at room temperature. Plates were drained and individual chicken sera (diluted 1:100) were loaded and incubated 30 min at room temperature. Plates were washed and antibodies detected using reagents in a commercial IBV ELISA kit (Idexx Laboratories, Inc., Westbrook, ME) following instructions in the kit. Absorbance at 650 nm was measured with a Powerwave XS. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test.

3. Results

3.1. S Binding to tissues

The binding affinity of recombinant S-ectodomain to relevant chicken tissues was compared to that of recombinant S1 protein using protein histochemistry. As seen in Fig. 1, the S1 protein bound weakly to the epithelium of trachea, nasal mucosa, choana (not shown), cecal tonsils, and cloaca, and to secretory cells of trachea, nasal mucosa, and choana, while binding was not detected in the lung and kidney. Extension of S1 with S2 subunit ectodomain (S-ectodomain) increased binding affinity to trachea, choana, nasal mucosa, cloaca, and cecal tonsils and enabled binding to lung and kidney. It should be noted that the molar concentration of S-ectodomain used for spike histochemistry was approximately one-fourth that of S1, indicating that the binding affinity of S-ectodomain is much greater than that of S1.

3.2. Viral load

Chickens immunized with recombinant S-ectodomain protein showed statistically significant ($P < 0.05$) reductions of viral RNA both in tears and tracheas 5 days post-challenge compared to chickens immunized with recombinant S1 protein or adjuvant alone (Fig. 2). A significant ($P < 0.05$) reduction of the viral RNA in the S1-immunized group compared to mock-vaccinated chickens was detected only in tears. S1-protein immunization did not significantly reduce viral RNA levels in trachea.

3.3. Tracheal histomorphometry and histopathology

Consistent with the viral load results, the S-ectodomain-immunized chickens showed a significant reduction ($P < 0.05$) of tracheal mucosal thickness, lymphocyte infiltration, and lesion severity (tracheal deciliation and epithelial necrosis) 5 days post-challenge compared to recombinant S1 protein alone-immunized and adjuvant-only chickens (Fig. 3). In contrast, no significant differences ($P < 0.05$) were detected between recombinant S1 protein-immunized and adjuvant-only groups. Remarkably, no significant differences in any of the tested tracheal histopathology parameters were detected between chickens immunized with S-ectodomain protein and unvaccinated/unchallenged controls, indicating that immunization with recombinant S-ectodomain protein provided complete protection of tracheal integrity.

3.4. Antibodies

Chickens immunized with S-ectodomain protein showed significant ($P < 0.05$) increases in IBV-specific antibodies in sera compared to those immunized with S1 protein alone and the non-vaccinated controls before challenge at 32 and 45 DOA, as well as 5 DPC (Fig. 4A). However, no significant differences were detected between S1 protein-immunized chickens and non-vaccinated controls. Consistent with IBV-specific antibodies, S-ectodomain protein-specific ELISA also revealed significant differences between the S-ectodomain protein-immunized group and the S1 protein-immunized group at all times post-immunization (Fig. 4B). S1 protein-specific ELISA did not indicate any significant differences between the chickens immunized with S-ectodomain protein compared to chickens immunized with S1 protein alone (not shown). Collectively, these results indicate the presence of antibodies directed against S2 and/or S-ectodomain-specific conformational epitope(s) in chickens immunized with S-ectodomain protein.

4. Discussion

The evolutionary success of IBV and the problems associated with use of live-attenuated vaccines indicate an urgent need to develop novel vaccines. Alternative approaches such as subunit vaccines or viral-vectored vaccines expressing specific proteins would eliminate emergence of vaccine subpopulations and facilitate the rapid development of effective vaccines against new serotypes. We have demonstrated that trimeric S-ectodomain provides more effective protection than trimeric S1 protein.
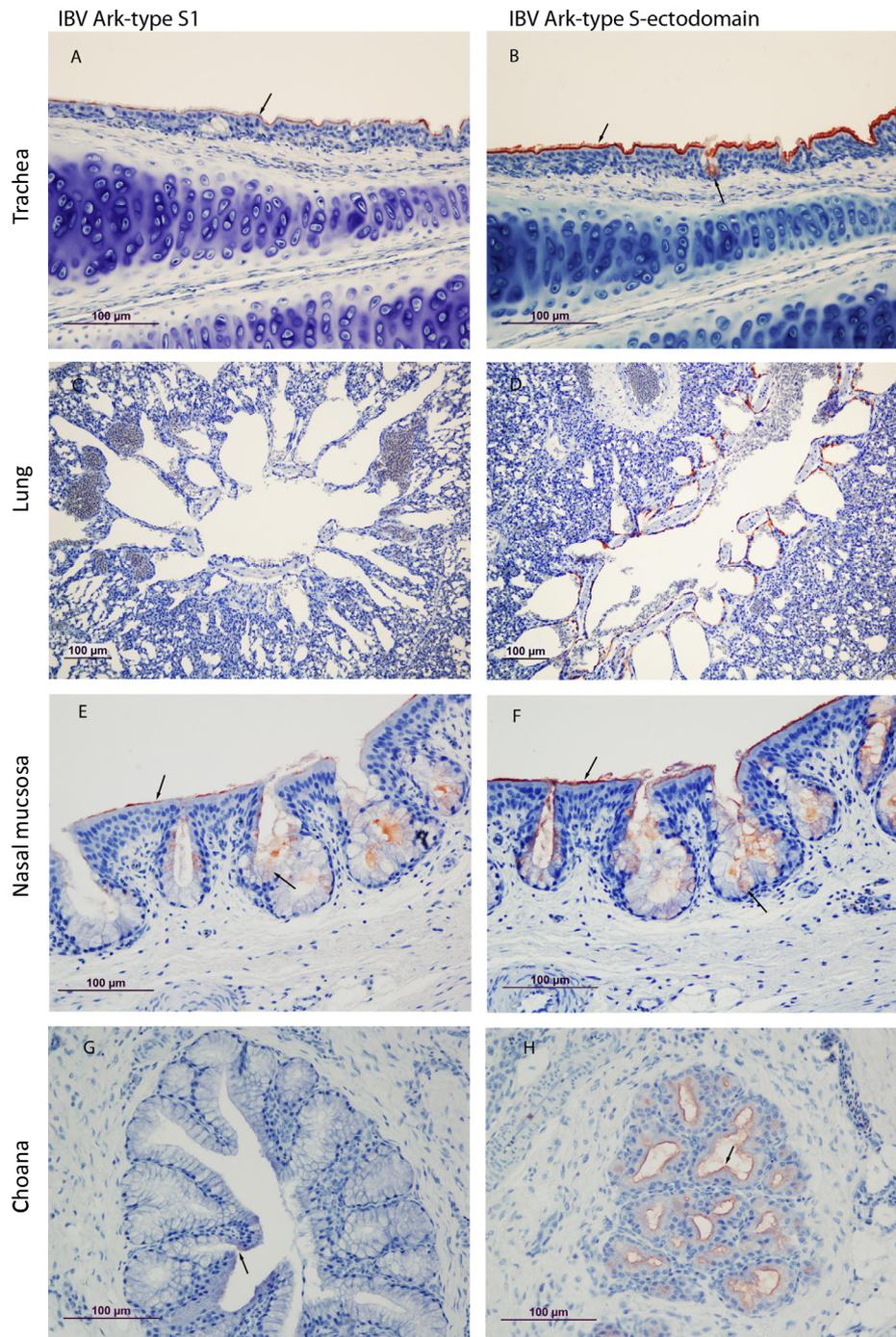
Fig. 1. Protein histochemistry demonstrating recombinant IBV Ark-type S1 and S-ectodomain binding in various chicken tissues. AEC+ chromogenic substrate was used to identify bound spike protein as indicated by red staining (arrows). (A) S1 ectodomain binds to the apical surface of the tracheal epithelium, while S-ectodomain (B) binds to the cilia, tracheal epithelium, and the mucin-containing goblet cell secretory vesicles. (C) S1 protein binding was not identified in the lung. (D) S-ectodomain binding was recognized in the epithelium lining the pulmonary parabronchi and atria. (E) Minimal staining at the apical surface of the nasal mucosal epithelium and mucus glands with S1 protein was observed, whereas S-ectodomain (F) exhibited enhanced staining of the nasal mucosal epithelium and mucus glands. (G) Sparse punctate S1 binding occurred along the apical surface of the choanal submucosal glandular epithelium. (H) Intense S-ectodomain binding of the epithelial apical surface and secretory product in the choanal glands was recognized. (I and J) S-ectodomain binding was detected on the epithelial apex of scattered renal tubules; however, S1 binding was not observed. (K) Multifocal, weak staining at the apical surface of the cecal tonsil intestinal epithelium with S1 protein was observed, whereas S-ectodomain (L) exhibited diffuse enhanced staining of the epithelium. (M and N) There was diffuse, strong staining of the cloacal glands with S-ectodomain, and only scattered, weak staining with the S1 protein. Although recombinant S-ectodomain is twice the molecular weight of recombinant S1 protein, it was used at half the μg/ml concentration. Thus the molar concentration of S-ectodomain was one-fourth that of S1. Thus, the increased binding affinity of S-ectodomain compared to S1 is even greater than it appears. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Comparing the binding of recombinant S1 and S-ectodomain proteins of IBV Ark-type strain revealed that S-ectodomain shows increased binding affinity to chicken tissues including trachea, choana, nasal mucosa, cecal tonsils and cloaca. Interestingly, S1 protein was unable to bind to lung and kidney tissues, which are also target organs for IBV, and required the S2 ectodomain to bind. These results are consistent with reports by others, showing that while the S1 subunit of the embryo- and cell-culture-adapted
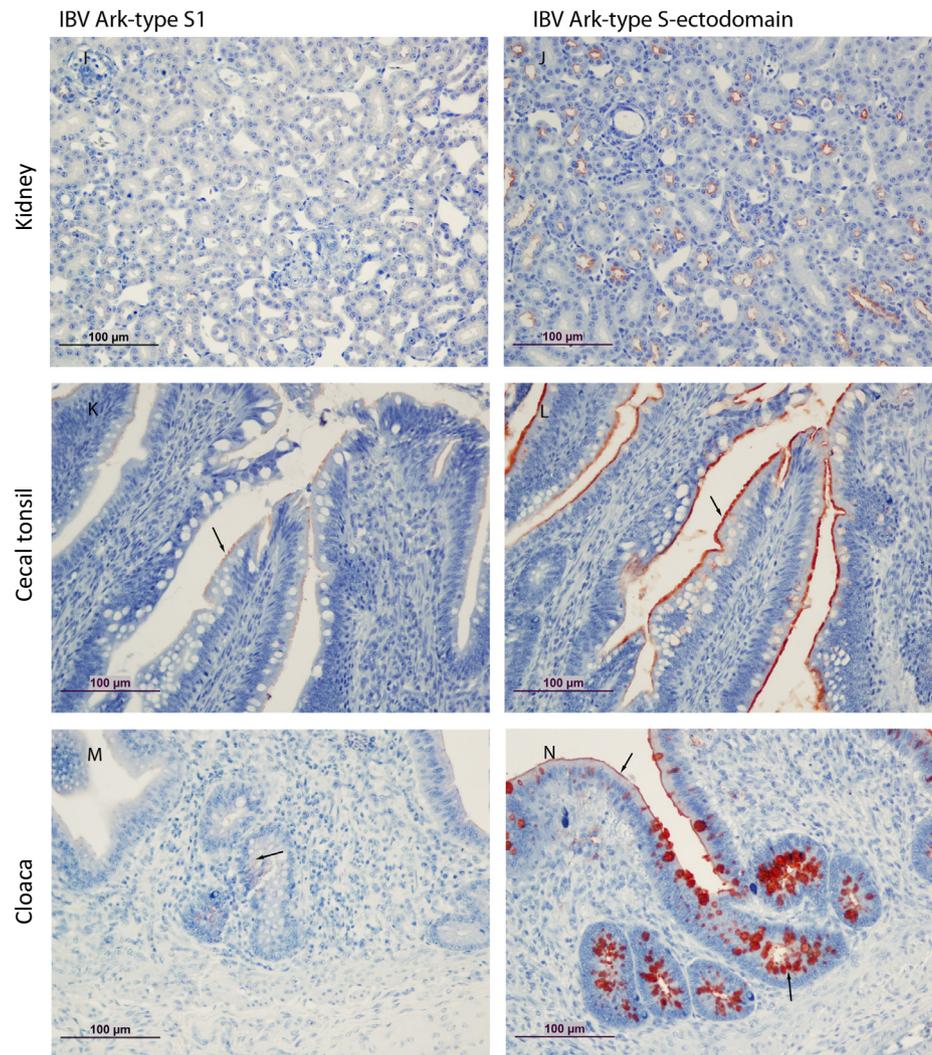
Fig. 1 (*continued*)

Beaudette strain is unable to bind to chorioallantoic membrane, the Beaudette S-ectodomain binds efficiently [22]. Furthermore, the extension of the M41 S1 with the M41 S2 ectodomain domain increased binding to chicken trachea [23]. The M41 S1 shows only 77% amino acid sequence identity with the ArkDPI S1 used herein. Thus, the current results confirm these findings for another IBV serotype and additional tissues. Using chimeric S-ectodomain proteins, Promunktod et al. concluded that S2 does not contain an additional independent receptor binding site that would explain its contribution to the affinity of S for receptors [22]. Another possible explanation for improved tissue binding of S-ectodomain is that the S2 subunit is necessary for the S1 protein to adopt a conformation optimal for binding. Structures of trimeric S-ectodomains of other coronaviruses determined by cryo-electron microscopy, e.g. [32,33], suggest that the trimeric structure is important for the conformation of S1, because the S1 domains of the monomers are interwoven in the trimer. In the recombinant S1 protein used in this study, the artificial trimerization domain immediately follows the S1 domain and could thus artificially constrain the trimeric S1 in a suboptimal conformation. When the S2 ectodomain is included between S1 and the trimerization domain, the trimers might be closer to their normal conformation. However, our unpublished results indicate that a single

amino acid change in the S2 domain can reduce the binding of the S-ectodomain (S. Farjana et al., unpublished results). Thus, S2 may influence the conformation of S1 in a more specific way.

Most IBV neutralizing antibodies recognize conformational epitopes in S1 [8,34–36]. Thus, if the S2 ectodomain allows S1 to adopt a conformation optimal for attachment, antibodies generated against this conformation might more effectively neutralize virus than antibodies generated against the suboptimal conformation of S1 adopted in the absence of S2. Therefore, we considered the possibility that extension of recombinant S1 protein with the S2 ectodomain would improve the protection afforded by a subunit vaccine. Indeed, our protection trial results indicated that immunization with trimeric S-ectodomain protein significantly reduces viral loads in tears and trachea, as well as tracheal damage, compared to immunization with trimeric S1 protein. Moreover, there were no significant differences in tracheal damage between chickens immunized with S-ectodomain protein and unvaccinated/unchallenged control chickens, indicating complete protection. Conversely, no significant differences were observed between chickens immunized with S1 protein and the mock-immunized group except for the viral load in tears. This limited protection conferred by S1 protein is in agreement with results of others [14] who found that at least four immunizations with
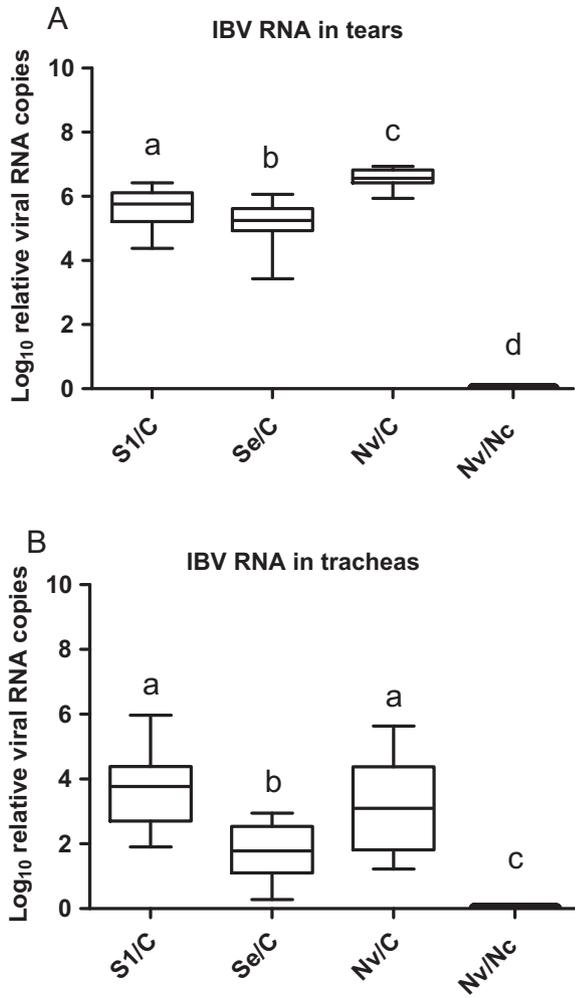
Fig. 2. Relative IBV RNA in (A) tears and (B) trachea of chickens primed at day 12 of age with adjuvanted trimeric recombinant S1, or S-ectodomain (Se), boosted 21 days later, and challenged with virulent Ark-type IBV 21 days post-boost. Nv/C = non-vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc = non-vaccinated/non-challenged. Relative IBV RNA levels determined 5 days post-challenge by qRT-PCR. Lines indicate median $\log_{10}$ relative RNA copy numbers, boxes indicate 25th to 75th percentile, and whiskers indicate minimum and maximum values. Different letters indicate significant differences ($P < 0.05$). Nv/Nc were assigned $\log_{10}$ values of 0 to be included in the graphs with log scale Y axes.

the purified S1 glycoprotein of nephropathogenic N1/62 strain of IBV were necessary to induce protection, even though they used a considerably larger amount of purified S1 antigen (50 μg) for immunization.

One possible explanation for improved protection following immunization with S-ectodomain, as already mentioned, is that antibodies produced to S1 in the ectodomain conformation neutralize the challenge virus more effectively than antibodies produced to S1 protein alone. Alternatively, the conserved immunodominant linear neutralizing epitope within S2 [13] might also contribute to improved protection. Although we did not attempt to demonstrate neutralizing antibodies, our ELISA results using both purified IBV and S-ectodomain protein showed a significant increase of antibody level in chickens immunized with S-ectodomain protein compared to those immunized with S1 protein alone, indicating that antibodies to S2 epitopes were generated. Furthermore, a peptide near the amino terminal end of S2 has been shown to induce a protective cell-mediated response [37]. The adjuvant used has been reported to stimulate both antibody and cell-mediated immune responses [38–40]. The addition
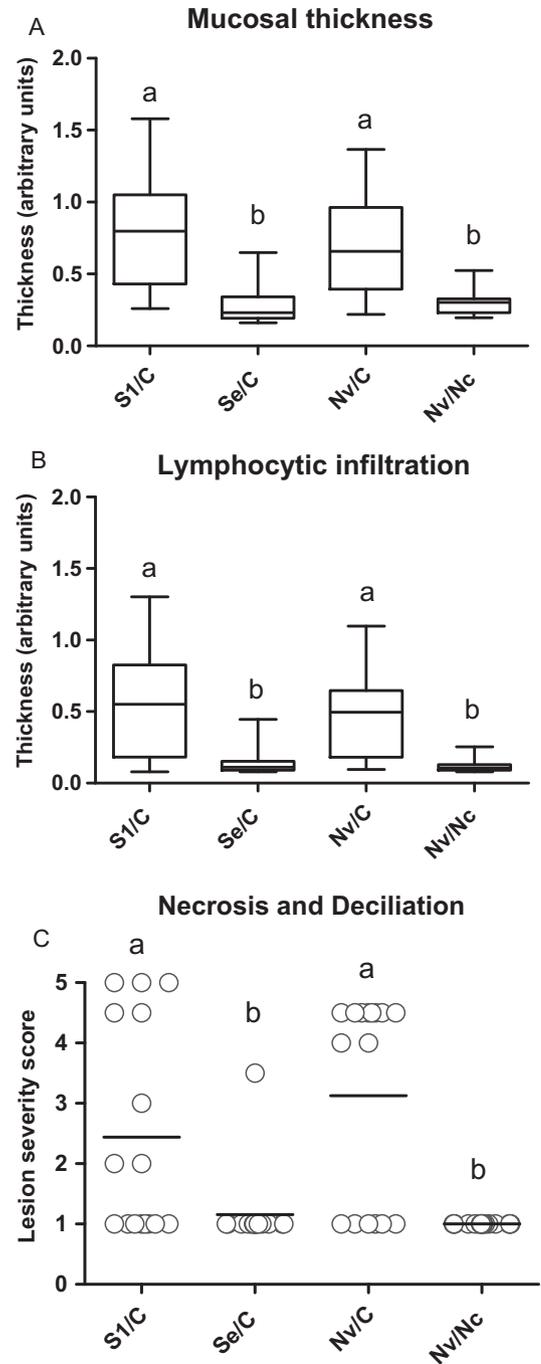
Fig. 3. Tracheal histomorphometry and histopathology 5 days after virulent IBV Ark challenge in chickens primed with adjuvanted trimeric recombinant S1, or S-ectodomain (Se), boosted 21 days later, and challenged with virulent Ark-type IBV 21 days post-boost. (A) Mucosal thickness and (B) thickness of lymphocytic infiltration by tracheal histomorphometry. (C) Severity of tracheal mucosal necrosis and deciliation scored blindly (1 = normal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) for each chicken. In box and whisker plots (A and B), lines indicate the median thickness, the boxes indicate the 25th and 75th percentiles, and the whiskers indicate minimum and maximum values. In the scatter plot (C), each point indicates the lesion score for an individual chicken and the lines indicate mean scores for each group. Nv/C = non-vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc = non-vaccinated/non-challenged. Different letters indicate significant differences ($P < 0.05$).

of the HA2 domain of the influenza hemagglutinin has also been demonstrated to increase the immunogenicity and protective capacity of IBV S1, possibly by increasing thermostability [41].
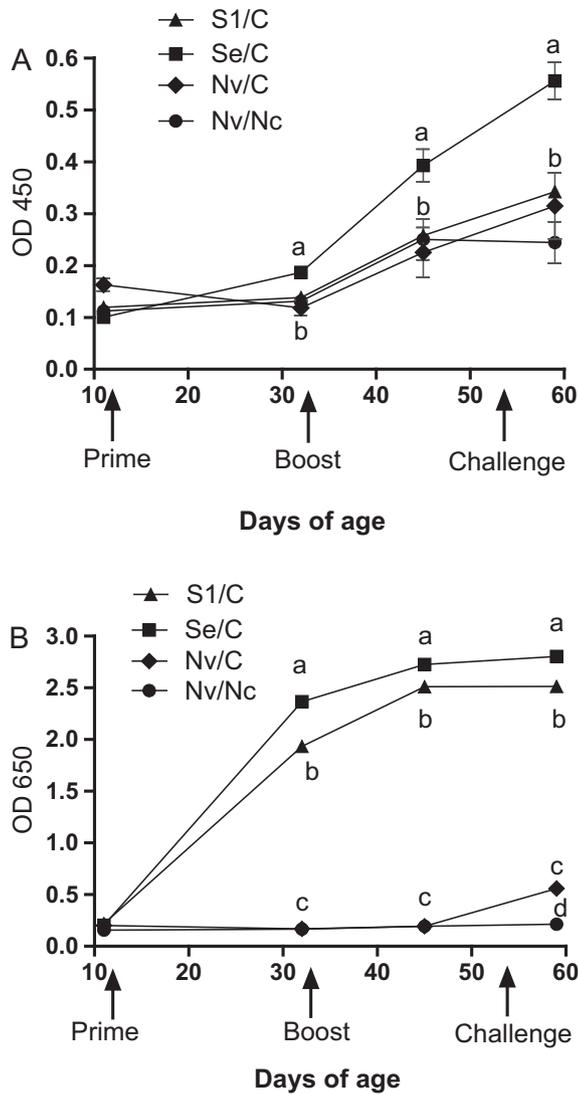
Fig. 4. IBV antibodies in chicken sera before prime (11 DOA), 3 weeks after prime (32 DOA), 2 weeks after boost (45 DOA) and 5 days post-challenge (59 DOA) determined by ELISA. IBV-specific ELISA (A), and S-ectodomain protein-specific ELISA (B) of chickens primed with recombinant S1, S-ectodomain (Se), boosted 21 days later, and challenged 21 days post-boost. Nv/C = non-vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc = non-vaccinated/non-challenged. Mean absorbance values and SEM are shown. In (B) the error bars are so small that they are obscured by the symbols. The S-ectodomain-immunized group showed significantly higher antibody levels (as measured by optical density) than the S1 protein-immunized, adjuvant only, and non-vaccinated non-challenged groups (*P* < 0.05). Statistically significant differences for each time-point post-vaccination indicated by letters.

The findings that recombinant S-ectodomain protein shows improved binding to cell receptors and elicits improved protection against challenge suggests that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 ecto-domain with S1 provides a promising option for a subunit vaccine and expands options for better viral vectored vaccines.

Acknowledgments

Conflict of interest

The authors have no conflict of interest to declare.

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