Protective Efficacy of a Recombinant Newcastle Disease Virus against Infectious Bronchitis

by

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Abstract

A previous study has reported that Newcastle disease virus (NDV) recombinant LaSota strain (rLS) expressing infectious bronchitis virus (IBV) Arkansas-type (Ark) trimeric spike ectodomain (Se) (rLS/ArkSe) provides suboptimal protection against IBV challenge. This study was aimed at developing rLS expressing chicken granulocyte-macrophage colony-stimulating factor (GMCSF) and IBV Ark Se in an attempt to enhance vaccine effectiveness. We first compared protection conferred by vaccination with rLS/ArkSe and rLS/ArkSe.GMCSF. Vaccinated chickens were challenged with virulent Ark-type IBV, and protection was assessed by clinical signs, viral load, and tracheal histomorphometry. Results showed that challenged chickens immunized with rLS co-expressing GMCSF and the Se had significantly reduced tracheal viral load and tracheal lesions compared to chickens vaccinated with rLS/ArkSe. In a second experiment, we evaluated enhancement of cross-protection by a Massachusetts (Mass) serotype attenuated vaccine after rLS/ArkSe.GMCSF priming or boosting. Vaccinated chickens were challenged with Ark-type IBV, and protection was evaluated. Results showed that priming or boosting with the recombinant virus significantly increased cross-protection conferred by Mass vaccine against Ark virulent challenge. Greater reductions of viral loads in both trachea and lachrymal fluids were observed in chickens primed with rLS/ArkSe.GMCSF and boosted with Mass. Consistently, Ark Se antibody levels measured with recombinant Ark Se-protein-coated ELISA plates 14 days after boost were significantly higher in these chickens. Unexpectedly, the inverse vaccination scheme, i.e., priming with Mass and boosting with the recombinant vaccine, proved somewhat less effective. We concluded that a prime and boost strategy using rLS/ArkSe.GMCSF and the Mass attenuated vaccine, ubiquitously used world-wide, provides enhanced cross-protection. Thus, rLS/GMCSF

co-expressing the Se of regionally relevant IBV variants could be used in combination with live Mass vaccines to protect against these regionally circulating variant strains.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ark	Arkansas-type IBV
BPL	Beta-propiolactone
CAM	Chorioallantoic membrane
cDNA	Complementary DNA
CEK	Chicken embryo kidney
CK	Chicken kidney cells
DMSs	Double membrane spherules
DMVs	Double membrane vesicles
DPC	Days post-challenge
DPV	Days post-vaccination
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EID_{50}	50% Embryo infective dose
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HVR	Hypervariable region
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
ILT	Infectious laryngotracheitis
IM	Intramuscular

M	Membrane protein
Mass	Massachusetts-type IBV
MDAs	Maternally derived antibodies
N	Nucleocapsid protein
NDV	Newcastle disease virus
nts	Nucleotides
OEV	Oil emulsified vaccine
OIE	Office of International Epizootics
ORF	Open reading frame
rAdV	Recombinant adenovirus
RBD	Receptor binding domain
rLS	Recombinant Newcastle disease virus LaSota-type
rLS.ArkSe	rLS expressing Se of Ark-type IBV
rLS/ArkSe.GMCSF	rLS co-expressing Ark Se and GM-CSF
S	Spike protein
S1	S1 subunit of spike protein
S2	S2 subunit of spike protein
Se	Spike ectodomain
UTR	Untranslated regions

I. Introduction

Being a highly contagious disease affecting the respiratory, reproductive, and renal tract, infectious bronchitis (IB) is responsible for substantial economic losses to the global poultry industry. For the last 8 decades, vaccination with embryo-attenuated infectious bronchitis virus (IBV) has been an extensively employed strategy to prevent the disease (175). However, attenuated vaccines are associated with grave consequences such as reversion to virulence (162, 399), vaccine viral persistence (178), rolling reactions (247), selection of vaccine subpopulations in chickens (128, 248, 262, 360), and recombination with wild-type strains (179, 185, 210, 215). Ultimately, these phenomena lead to the emergence of vaccine-like variants, which complicate the control of IB (7, 144, 179). In contrast, inactivated vaccines are safer but elicit insufficient immune responses (163, 250, 377) and require priming with a live vaccine and parenteral administration (135). Similarly, recombinant DNA vaccines (14, 191, 304, 336, 342, 382) have been demonstrated to provide limited protection. Although some subunit vaccines have promised better protection (113) than others (214, 383, 321), a necessity to inject these vaccines restricts their applicability in commercial settings. Therefore, a novel approach involves construction and evaluation of homologous (18, 50, 115, 155, 198, 372) or heterologous viral vectors (67, 187, 222, 315, 351, 370, 396-398) expressing antigenic sequences of IBV. While these vectors have conferred protection of variable magnitudes, their drawbacks have been discussed (sections 4.3.3 and 4.3.4.). Interestingly, recombinant Newcastle disease virus (rNDV) has been experimentally used as a promising vaccine vector against various other avian (169, 200, 260) and mammalian diseases (207, 331). However, LaSota-type (rLS) rNDVs expressing IBV antigens have induced suboptimal protection thus far, especially upon single vaccination with the recombinant constructs (1, 317, 318, 334, 335, 401). Considering an enhanced immunogenic potential of spike ectodomain (Se) in

comparison with S1 subunit (113) (page 30), a recent attempt demonstrated partial protection provided by vaccination with rLS expressing Se of an Arkansas-type (Ark) type IBV (rLS/ArkSe) (394). To enhance the efficacy of avian vaccines, researchers have tested a variety of cytokines as recombinant genetic inserts or co-administered adjuvants (67, 315, 330, 370). Specifically, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been demonstrated to augment efficacy of vaccines against various human (5, 41, 183, 271, 284), non-avian animal (10, 109, 408, 409), and avian diseases (137, 368, 369), including IB (333, 396). Taking into account a diverse functionality of GM-CSF in both innate and adaptive immune responses and its established potential as a vaccine adjuvant, we collaborated with Dr. Qingzhong Yu at USDA's Southeast Poultry Research Laboratory to produce and evaluate rLS co-expressing Ark-type Se and GM-CSF (rLS/ArkSe.GMCSF).

II. Literature Review

1. Infectious bronchitis

IB is a highly transmissible respiratory disease primarily affecting chickens and causing significant economic losses to the global poultry industry. Even though initial descriptions of IB were published about 100 years ago (17, 194), its control is still an unresolved problem. The etiologic agent of IB, infectious bronchitis virus (IBV), is a rapidly evolving avian coronavirus belonging to the genus *Gammacoronavirus*. IBV is transmitted horizontally by aerosol and direct contact with diseased birds or fomites. As the disease is highly contagious, morbidity is usually around 100%. However, the mortality rates depend on the IBV strain, bacterial co-infections, and host factors. The virus initially replicates and damages epithelial mucosas of the upper respiratory tract. Following a short viremia, the virus reaches the urogenital tract and other tissues. Depending on viral strain, the reproductive, renal, or enteric systems may be more severely affected (175).

1.1. History

A comprehensive literature review provides ample evidence that IB was reported before the 1930s (194, 195, 295), contrary to the pervasive and mistakenly reiterated belief that IB was first observed and documented in 1930s (175). Salmon, who first described a clinical picture similar to IB in 1899, termed the disease “bronchitis” and described labored breathing as one of the clinical signs. However, he considered non-infectious environmental factors such as dampness, dust, cold air, temperature changes, and irritating vapors as the causative agents (295). The contagious nature of bronchitis was reported by Kaupp in 1917, who described respiratory signs, laying complications, and intra-tracheal treatment for the disease (194). In the 1920s, various names such as tracheitis, trachea-laryngitis, Canadian flu, gasping disease, and infectious bronchitis were used to designate the same disease (17, 44, 47, 148). In 1930, the first comprehensive account of IB was

published by Kernohan, who reported transmission of the disease through intra-tracheal inoculation of the exudate from the infected birds and post-infection immunity (199). In 1931, Schalk and Hawn reported that the symptoms and lesions of the emerging disease were clinically comparable to infectious laryngotracheitis (ILT) (298). Bushnell and Brandly compared clinical symptoms and pathological lesions with other diseases in 1933, and concluded that the causative agent was probably the same filterable microbe i.e. virus that caused ILT (45). However, Beach and Schalm performed virus neutralization and cross-immunity experiments in 1936, revealing that the disease was dissimilar to infectious coryza, and the pathogen of interest was a virus differing from that of ILT (15). In 1937, Beaudette and Hudson reported the ability of IBV to propagate in the allantoic cavity of embryonated eggs (16). These findings were confirmed by Delaplane and Stuart in 1939 (101) and 1941 (102), who further demonstrated a decline in the lethality of embryo-propagated viruses with successive passaging. Consequently, the reduction of pathogenicity through this process established the foundation of immunization against IBV (102). In the 1940s, van Roekel et al. reported the first vaccination of layers in Massachusetts, using inocula obtained from laboratory birds infected with IBV (358). Subsequently, Hofstad published a detailed account of IB in 1945, describing microscopic lesions in both young and old chicks (158), rejecting the potential of experimental and field cases to serve as recovered carriers of IB (159), and invalidating the authenticity of hemagglutination test as a diagnostic tool for IBV (160). In 1948, Jungherr and Terrel discovered maternally derived antibodies (MDAs) against IBV in embryo yolks and young chicks (188). However, in 1950, Hofstad and Kenzy found that despite high serum antibody content, chicks of age 4, 6, 7, and 10 days were susceptible to IBV (157). In the same year, Loomis et al. comprehensively described the gross and microscopic alterations in IBV-infected embryos through the first seven passages (229). A major breakthrough was made in

1956 by Jungherr et al., who reported antigenic differences and lack of cross-protection between a IBV isolates obtained in Connecticut and Massachusetts (189). In 1973, Hitchner suggested standardization of virus neutralization test for screening and classification IBV field strains (153). The introduction of an enzyme-linked immunosorbent assay (ELISA) by Marquardt et al. in 1981, was a major advancement in IBV diagnostics, as it was more sensitive, allowed earlier detection, and required smaller volumes of sera as compared to virus neutralization test (243). Raj and Jones discussed the long-term persistence in 1997, local immunity in the oviduct, duodenum and caecal tonsils, and the increase in CD8 cells following IBV infection (288).

1.2. Transmission

As a highly transmissible virus, IBV spreads quickly in the flocks leading to the morbidity of around 100% (100). The transmission of IBV occurs primarily through the aerosol route (91, 217). Additionally, it is transmissible through direct contact with infected materials (199) and mechanically through fomites and the movement of personnel across the farms (283). Birds recovered from IB may continue shedding the virus, thereby transmitting the disease to a susceptible population (205). Experimental infection of antibody-free chickens revealed intermittent tracheal and cloacal shedding up to 63 days and isolation of the virus from various organs up to 163 days post-inoculation (261). Other routes likely include venereal transmission (127).

1.3. Pathogenesis

The pathogenic outcomes of an infection with IBV depend on the serotype, host factors, and environmental conditions (89, 231). IBV initially replicates in the upper respiratory tract and is subsequently disseminated to other tissues via viremia (249). Tropism of IBV for ciliated epithelial

cells has been reported through immunofluorescent staining of impression smears (130). Electron microscopy of tracheal sections revealed viral invasion of epithelial cells by viropexis (354).

1.4. Gross pathology

Nasal, tracheal, and bronchial exudate with mild tracheitis and sinusitis have been observed in early stages following experimental infections. The nature of the exudate was found to be serous or catarrhal with yellowish color and variable consistency. Moreover, congested lungs with turbid air sacs were found on necropsy (15, 158). In chickens infected with nephropathogenic strains, swollen and pale kidneys have been observed. Renal tubules and ureters were found distended with urate deposits (378, 379). Regressive ovary and oviduct were determined as primary gross lesions in controlled experiments involving weight and length measurements. Shriveled, wrinkled, and "fig-shaped" ova were found, with discontinuous yolk membranes allowing the accumulation of "cheesy" yellow material into the peritoneal cavities (88, 89, 306). Exposure to IBV during the first 18 days of age can induce permanent anomalies of the reproductive organs, including non-patent and cystic oviduct (39). IBV-infected embryos show stunting, dwarfism and curling with clubbed appearance of feathers. Moreover, retarded lung development with pneumonia and serous exudation can be observed 8 days post-infection (229).

1.5. Histopathology

Early histopathological studies discovered deciliation and desquamation of the tracheal epithelium in conjunction with diffuse leucocytic infiltration in the mucosa and submucosa 3 days post-infection. Microscopic evaluation of air sacs showed a marked increase in lymphoid and mononuclear cells. In addition, an influx of eosinophilic granulocytes and mononuclear cells was observed in the turbinates (158, 286). Following viremia, IBV replicates in renal and reproductive organs. The severity of the damage in these tissues depends on the IBV strain. Nephropathogenic

strains cause acute and chronic progressive nephritis/nephrosis. Renal cortex and medulla with foci of mononuclear infiltration and extensive viral replication have been observed. Chickens chronically infected with nephropathogenic strains have been diagnosed with fibrotic and atrophied kidneys (70, 90, 378). Infected oviducts show inflammatory cells with focal or diffused distributions. Loss of cilia and cellular infiltration with copious lymphocytic foci has been reported in lamina propria and inter-tubular stroma of affected oviducts. Proliferation of fibroblasts and absence of tubular glands have also been observed in the oviduct (306).

1.6. Clinical signs

Infected birds present respiratory signs, including gasping, coughing, sneezing, nasal discharge, increased lachrymation, and tracheal rales (199). In addition, the birds appear dull and depressed, with reduced feed intake and reduced weight gain (280). In laying birds, decline in egg production with deteriorated interior and exterior egg quality is observed. Moreover, eggshells can be wrinkled, misshaped, non-uniform in size, bleach-colored, and of variable thickness (306, 359). The interior egg quality is also affected, as indicated by watery albumen (46, 87).

1.7. Economic significance

From 2011 to 2019, about 65000 outbreaks and 359 million worldwide cases of IB have been reported to OIE (266). An IB outbreak results in significantly declined egg production, reduced feed conversion efficiency, mortality, higher medication costs, increased culling from the flock, and increased condemnation rates at slaughter (39, 306). Depending on IBV strain, in egg production may decline from pre-infection levels of 70% to 2.4% within two weeks. The losses are recurring as the recovered flock does not regain its optimal production level, which could be explained by permanent post-infection changes in the oviduct (298, 359).

2. Infectious bronchitis virus

2.1. Taxonomy

IBV has been classified in the order *Nidovirales* and family *Coronaviridae* which comprises subfamilies *Orthocoronavirinae* and *Letovirinae*. *Orthocoronavirinae* consists of four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* (134, 171). A coronavirus derives its name from the characteristic electron micrographic appearance of negatively stained particles, the outer surface of which bears a resemblance to a crown (4). Birds have been determined as the ideal hosts for the species belonging to genera *Gammacoronavirus* and *Detlacoronaviruses.* IBV is a G*ammacoronavirus* that primarily affects chickens. Some reports indicate that peafowls, pheasants, and parrots might also be susceptible (136, 228, 380).

2.2. Virus structure

IBV is an enveloped virus with a diameter ranging from 80 to 120 nm. The virions appear to be pleomorphic in electron radiographs, although generally spherical with about 20 nm long club-shaped projections (4, 24). IBV has a mean density of around 1.18 on the sucrose gradient (29, 53). The structural proteins include spike (S), envelope (E), membrane (M), and nucleocapsid (N) (53). The S protein has a molecular mass of 354 kDa (56) and forms club-shaped spikes. The M protein is integral to the membrane and partially exposed at the surface of the virion. It consists of a 23 k-Da polypeptide about and can have a molecular mass up to 36 kDa depending on the extent of glycosylation (54, 328). The inner core of IBV consists of N protein, a 50 k-Da protein associated with the RNA genome (53, 237).

2.3. Genome

IBV possesses a positive sense, non-segmented, single-stranded RNA genome. The sequence of IBV comprises approximately 27.6 kilobases (339). The 5’ end has a 5’ untranslated region (5’-

UTR) contains 528 nts (7). Interestingly, the genome contains several ORFs, involving a discontinuous extension of transcription and generation of several subgenomic RNAs (297, 403). The first 20 kilobases of the genome consist of genes 1a and 1b that encode polyproteins (33, 34). The terminal sequence of about 8 kilobases encodes four structural proteins: S, E, M, and N (7, 34). Researchers have demonstrated a critical role of the replicase gene 1a in the pathogenesis of IBV by using recombinant IBVs with mutated 1a or 1b ORFs. The mutations in gene 1b appeared not to affect replication of IBV (400). The role of four accessory genes 3a, 3b, 5a, and 5b is still indeterminate. Despite the diffused and punctate localization of 3a proteins to smooth endoplasmic reticulum (ER) as observed by confocal microscopy (277), it has been reported that proteins encoded by 3a and 3b sequences are not critical for replication (154). Researchers have demonstrated that gene 5 and ORF 5a are not critical for *in vitro* viral replication (50, 388). In addition, *in ovo* and *in vivo* pathogenicity evaluation have shown that mutations in 5a and S can attenuate the virulent IBV (404). The 3' end of the genome has a more than 500 nts long UTR followed by polyadenylate sequences of varying lengths (7, 300).

2.4. IBV attachment and entry

The so-called receptor-binding domain (RBD) on the S1 subunit of the spike protein mediates the binding of IBV to the host cell receptors and is responsible for cellular tropism (51, 281, 374). An α-2,3 sialic acid receptor has been determined as an attachment factor for the virus (301, 308, 376). Moreover, a competitive exclusion experiment has revealed that cell surface heparan sulfate could be responsible for an extended host range of Beaudette-type IBV (238). Before viral entry into the host cells, proteolytic maturation of surface glycoproteins and conformational alteration have been observed among enveloped viruses (201, 364). Even exogenous proteases have been shown to enhance cellular tropism and infectivity. For example, in a very recent study, higher viral

titers were obtained in an experiment using exogenous trypsin during the propagation of IBV in the cell culture (329). Unlike some other coronaviruses, the S protein of IBV is cleaved into S1 and S2 subunits in the virus producing cells, and this cleavage is catalyzed by host cell serine proteases (60, 61). Furin is a calcium-dependent serine protease that cleaves S at the consensus motif RRFRR537/S in addition to cleaving the S2 subunit at RRRR690/S in the virus-infected cells (58). Contrary to other class I fusion proteins, the membrane-anchored subunit of IBV S protein is not exposed by processing just upstream of the fusion peptide. Interestingly, the furin-dependent processing of IBV S protein happens further away from the predicted fusion peptides (31, 32). The cleavage recognition site of the S protein mainly comprises highly conserved amino acid sequences and does not explain host range or serotype divergence (176). The concerted interaction of S1 and S2 with the host cell surface is responsible for the viral attachment, although no receptor binding domain has been observed on the S2 subunit (57, 282). Moreover, glycosylation of the N-terminal RBD on the S protein is indispensable for the attachment of IBV to the target cells and modulation of infectivity thereof (35, 273, 406). Following attachment, fusion activation of the host cell membranes is enabled by the S2 subunit (27). Although some earlier studies have described the optimum cell to cell fusion at pH 6.7 (220), more recent studies have demonstrated that the viral entry depends on lower pH and very little or no fusion above pH 6.0 (73). While the initial studies suggested large syncytia formation around neutral pH as a representative model for fusion and entry (219, 220, 381), electron microscopic evidence showed viropexis as the key mechanism for IBV entry into the tracheal epithelium, chorioallantoic membrane (CAM), and chicken kidney cells (CK) (274, 354). Viropexis, by definition, is a mechanism by which a virus is ingested into the cytoplasmic vacuoles of the host cells and has been extensively described for influenza viruses (98, 274). Therefore, the initiation of entry into

the cells is depends on an endocytic pathway as elucidated by an experiment involving endocytosis-inhibiting drugs monensin and chlorpromazine (74). As the endocytic environment is acidic (299), these findings corroborate with data suggesting low-pH-dependent fusion and entry (73). A study using baculovirus described the clathrin-dependent signal transduction by using an endocytosis inhibitor monodansylcadaverine (265). More recently, a detailed study involving chemical inhibitors, RNA interference, and dominant negative mutants has investigated the requirement of clathrin-coated vesicles for IBV entry into the target cells. The pathway involves the attachment of IBV to the lipid rafts, its movement along the depolymerized actin filaments, the vesicular scission through GTPase dynamin 1, transportation through early and late endosomes, and ultimate fusion of the viral envelope with the late endosomal membrane leading to release of the genome into the cytoplasm (365).

2.5. Viral replication

Following the cytoplasmic release of the positive-sense, single-stranded RNA genome, IBV ORF 1ab is recognized by the host ribosomal machinery. The first two-thirds of the genome encodes two large polyproteins: 441-kDa pp-1a and 741-kDa pp-1a/1b. The fusion polyprotein pp-1a/1b is translated through a unique ribosomal frameshifting mechanism (34, 38). Subsequently, the cleavage of the pp-1a and pp-1a/1b produces several non-structural proteins (NSPs). This processing is catalyzed by ORF 1a-encoded papain-like and 3C-like viral proteinases (225, 411). Unlike some other coronaviruses that encode 16 NSPs (NSP1-16) as proteolytic cleavage products, IBV lacks NSP1(410). IBV's 15 NSPs play multiple roles in the replication and assembly of the virus (107, 263, 337). The assembly of these proteins leads to the formation of replication-transcription complexes.

The positive-sense RNA genome is used by the RNA-dependent-RNA-polymerase as a template to produce a full-length negative sense replica of the IBV genome, which is further utilized to produce genomic and subgenomic RNA copies (305, 403). These subgenomic mRNA (sg-mRNA) species constitute a 3' co-terminal or so-called 'nested' set of varying lengths, are 3' polyadenylated, and share a common 5' leader sequence. The translation of sg-mRNAs leads to the production of structural polypeptides of the virus (40, 325-327). A recent transcriptomic analysis of genomic and sg-mRNAs performed through deep sequencing and ribosomal profiling of IBV infected CK cells established an sg-mRNA encoding N protein as the principal species, while the amount of sg-mRNA encoding M protein was strain-dependent (103). An sg-mRNA has been discovered with the potential ORF located between the M gene and gene 5. The translation of an 11 k-Da accessory protein from this previously unidentified sg-mRNA is controlled by a transcriptional regulatory sequence (TRS) (19). More recently, a similar sg-mRNA has been identified, the transcription of which occurs through a non-canonical TRS located between the N gene and 3' UTR (197).

Transcriptional regulation is dictated by N protein due to its localization to host nucleolus through fibrillarin and interaction with nucleolin. One consequence of this interaction could be the prolongation of interphase, thereby maximizing the viral mRNA translation (66). A fairly recent study involving mass spectrometry and fluorescence has revealed a complicated interactome of N protein with host cellular proteins that could potentially modulate IBV replication and translation (116).

Generally, the replication of positive-sense RNA viruses induces rearrangement of host cellular membranes to perform essential functions. To assess the precise location of replication organelles (RO), studies using electron tomography predicted zippered ER with double membrane

spherules (DMSs) to be the active sites of viral RNA synthesis (239). While a more recent investigation has confirmed an abundance of these DMSs, it has demonstrated an involvement double-membrane vesicles (DMVs) in viral RNA synthesis instead of ER and DMSs (320). These spherules protect newly synthesized viral RNA and permit the exchange of materials with the cytoplasm (240). Recently, accumulation of a double-stranded RNA 3-4 hours post-infection has been established as a marker of IBV replication in CK cells (241). Despite the general question of whether these dsRNA molecules function as intermediates or not, they are still believed to be indicative of active viral replication (139, 239, 241).

Experimental translation of IBV proteins has been investigated as early as the 1970s using rabbit reticulocyte and wheat germ expression systems (150). The 5' ORF of each mRNA is translated through ribosomal scanning, which happens in a cap-dependent fashion. Experiments involving murine hepatitis virus (MHV, a *Betacoronavirus*) have concluded that the 5' leader sequence augments the translation of viral mRNA as a compensatory mechanism during a host cell translational shut-off (332). The cap-dependent translation of mRNA1 yields two polyproteins 1a and 1a/1b, which are co- or post-translationally cleaved into several proteins through papain-like and 3C-like proteinases (225, 263). Other sg-mRNAs yield structural proteins S, E, M, and N and accessory proteins 3a, 3b, 5a, and 5b of unknown functionality (259). The translation of tricistronic mRNA3 initiates with the cap-independent recognition of 3 overlapping ORFs, thereby providing 3a, 3b and 3c proteins (226).

Recently, a study has revealed that IBV manipulates apoptotic pathways of the host cells, thereby enhancing cell survival and viral replication (392).

2.6. Viral assembly and release

An exocytic secretory pathway transports coronaviral structural proteins to an intermediate compartment between ER and Golgi complex (209), variably referred to as budding compartment (345), salvage compartment (371), cis-Golgi network (110), or ER-GIC (145). By virtue of its diverse enzymatic profile, the so-called 'budding compartment' offers an enriched environment for the newly synthesized glycoproteins and permits the sequential post-translational processing of these proteins (206).

A study involving immunofluorescence and cell fusion analysis demonstrated that IBV S protein is transported to the plasma membrane following expression. The presence of putative transport signals in the S-ectodomain and putative retention signals in the cytoplasmic tail was also proposed (361). However, M protein (previously labelled as E1 glycoprotein) of IBV accumulates in the cis-Golgi cisternae, as determined by immunoelectron microscopic analysis of cloned complementary DNA (cDNA) expression (234). Moreover, the withholding of M protein inside Golgi has been suggested to be regulated by an active or passive retention signal on the N-terminal transmembrane domain.

Additionally, the budding of virions from plasma membranes can be a consequence of the intracellular concentration of M protein (235). Earlier studies involving confocal and immunoelectron microscopy revealed that smaller amounts of IBV M and E proteins were released upon co-expression leading to reduced efficiency of virus-like particle formation (85). Further studies using co-immunoprecipitation assays (224) and *in vivo* chemical crosslinking assays (86) reported the physical interaction of IBV E and M proteins through specific amino acid motifs on the cytoplasmic tails. As the amount of E protein found in mature virions (227) and virus-like particles (33) was found to be minuscule, higher amounts of E protein at the subcellular levels and

its interaction with M protein could explain its role in viral budding (85, 86). Another study involving biochemical analysis of four different coronaviruses disclosed the transportation of IBV M protein away from the budding compartment and its retention through intrinsic signaling (202).

The carboxy and amino termini of the newly synthesized IBV N protein coalesce with the 3' non-coding region of full-length genomic RNA to form a helical ribonucleoprotein structure. However, the middle region of N protein was shown to interact with 5' sequences of IBV RNA (407). A hypothetical model was elucidated to explain nucleocapsid multimerization and assembly of IBV nucleocapsid at the subcellular level using a bacterial expression system. According to this model, binding of N- and C-terminal domains of N protein to the genomic RNA could trigger the clustering of N proteins. Furthermore, the condensation of IBV RNA around oligomerized N-terminal of N protein would terminate into a compact ribonucleocapsid (119).

A hydrophobic domain of E protein has been demonstrated to affect the cell secretory pathways by influencing the Golgi apparatus. This alteration could promote viral infectivity by reducing the premature syncytial formation and minimizing antigenic presentation to the host immune system (294). Another study highlighted the significance but not essentiality of the transmembrane domain of E protein for the release of infectious IBV particles (236). The latest research has revealed pH alterations in the luminal microenvironment of Golgi regulated by the transmembrane domain of IBV E protein. Such a modulation prevents premature cleavage of S protein and modifies the secretory pathway, thereby promoting the release of the particles (373).

3. Spike (S) protein

The S protein constitutes an array of projections or peplomers on the surface of the IBV envelope (4). Electron microscopic analysis observed the lengths of bulbous or tear-drop-shaped IBV surface projections ranging from 12.8 to 27.3nm with a mean of about 20nm. The observed

width was somewhere between 6.2 to 13nm, averaging about 10.5nm (95). Biochemically, it is a glycoprotein consisting primarily of 90-kDa S1 and 84-kDa S2 subunits (53, 55). The demarcation of subunits results from cleavage of the spike precursor at a specific amino acid sequence preceding the N-terminal serine residue of S2 (61). An extensive glycosylation explains the difference in molecular weight of about 50 kDa observed between the glycosylated and unglycosylated S polypeptides (30). The N-terminal bulbous S1 subunit comprises around 519 amino acids (the numbers may vary among different IBVs) (61) and is responsible for viral binding to the host cells through the RBD (281). The S2 subunit consists of 625 amino acids (61), and its carboxy terminus anchors the S into the viral membrane (30). The importance of S2 for fusion with host cells has been widely established (32). The coiled-coil structure of the S2 stalk results from the interaction of α-helices formed by an array of hydrophobic residues. The presence of two heptad repeat regions and their helical interaction leads to the oligomerization of the S protein (96). Although initial studies described either a dimeric or trimeric structure of IBV (56), a modern investigation involving cryo-electron microscopic density maps and protein modeling has revealed a trimerized structure of IBV S-ectodomain (309).

3.1. Role of S protein

S protein has multifaceted functionality defined by its structural orientation and biochemical composition. The attachment of IBV to α-2,3-sialic acid receptors on the host cells is mediated by an RBD located on the S1 subunit (281). Moreover, the S2 subunit drives the fusion of IBV with the cellular membranes and entry into the host cells (309). For Beaudette-type IBV, a fusion peptide is exposed by the action of serine proteases on a furin activation site. Such a site comprises a two amino acid conserved motif located 153 amino acids downstream of the S1/S2 cleavage site (381).

The role of S protein in determining host specificity and cellular/tissue tropism has been extensively documented. As IBV diversity is determined by S protein (59, 190), the ability of certain strains to replicate in specific cell lines or otherwise an inability of the others thereof (269), is a testament that the host and tissue specificity is directed by S protein. Earlier attempts documented successful attachment and replication of IBV in heterologous cell culture systems to propagate IBV (82, 269). However, the syncytial formation was only observed in cultures derived from avian species (pheasant, quail, turkey, and duck) and viral titers were lower in both avian and non-avian cultures when compared with chicken embryo kidney (CEK) cell cultures (82). Although the adaptation, replication, and passaging of IBV in African green monkey kidney cell line Vero was reported much earlier (93, 118), a relatively recent study has registered mutations in S protein (along with other mutations) contributing to adaptation and multiplication of IBV in this atypical host system (27, 314). A study involving S protein histochemistry has revealed that the S1 attachment blueprints of various strains correlate with their host/cellular/tissue tropism and define their *in vivo* pathogenicity potentials. More specifically, as little as 5% variation in amino acid sequence among S1 proteins of various strains is sufficient to change their avidity toward the host tissues and sialylated glycans (374). Recent work has documented the relevance of amino acid sequences in the hypervariable region (HVR) 2 of S1 for recognizing sialylated glycan receptors in kidneys, thereby differentiating a non-nephropathogenic M41 strain from a nephropathogenic QX strain (35). Even within Ark-type IBV, a single mutation in the S1 sequence has been shown to either alter binding specificity or affinity of S1 towards different tissues. This S protein polymorphism ultimately contributes to the enhanced viral pathogenicity and evasion from vaccine-induced immune responses (218). Moreover, the immunogenicity associated with

antigenic epitopes of S protein is of immense significance from the perspective of cross-protection and vaccine development (62, 216, 264).

3.2. S protein-driven serotype divergence

The characterization of IBV serotype phylogeny is contingent upon the variability of the amino acid sequence of S1 (55, 56), which further depends on the hypervariability of the S1 sequence (211). The percentage of similarity in the HVR sequences is considered to be a determinant of serotypic affiliation. Generally, a dissimilarity of about 20-25% in the amino acid sequence of S1 categorizes the viruses in the different serotypes (2). However, exceptions to this rule may exist (63). This extended variability and predominance of specific serotypes is a consequence of the evolutionary routes undertaken by IBV. Genetic diversity and subsequent selection of the fittest viral populations in the host microenvironment engender different IBV types and subtypes (128, 349). The generation of genetic diversity involves random mutations (substitutions, insertions, and deletions) (248, 349) and recombination episodes (185, 208, 210, 215). IBV, like all other coronaviruses, expresses an exoribonuclease protein (NSP14). The proofreading activity associated with the N-terminal domain of NSP14 removes the mismatches introduced by RNA-dependent RNA polymerase (111, 253). Despite this proofreading activity, mutations in the IBV genome can be attributed to a rate of about 2.43×10^{-5} to 9.77×10^{-5} substitutions/site/year depending on the gene (405). Specifically, the importance of S protein as a determinant of IBV serotype diversity is explained by the presence of a hypervariable region in S1 (59, 211). Particularly, the S1 sequences undergo an enormous rate of substitutional mutations (2.93×10^{-5} substitutions/site/year) (405), as well as recombination (366), thereby contributing to the genetic diversity.

A comparison of IBV evolution with other coronaviruses can only stand valid if the genetic divergence is viewed from the chronological perspective (184), considering that IBV was the first coronavirus experimentally isolated and extensively attenuated for live vaccination (15, 358). Interestingly, the rate at which evolutionary changes accumulate within a given IBV serotype may differ (179). Among eight strains of Massachusetts (Mass) serotype collected over 30 years, a maximum of 4% difference in S1 nucleotide sequence, and 6% dissimilarity in amino acid sequence was observed (59). Within the D274 group, seven different isolates of IBV collected over the span of 8 years, displayed a maximum dissimilarity of about 3% in nucleotide sequence, and a difference of approximately 5% in amino acid sequences (58). Regardless of the chronological evolutionary track undertaken by IBV, numerous types and subtypes of regional significance are prevalent globally (174, 177, 179, 355). The prevalence of Mass, Ark, and additional serotypes has been extensively documented during the last two decades (6, 99, 105, 292, 346).

3.3. Immunogenic significance of S-ectodomain

The portion of the S protein exposed on the surface of the virion, including S1 and most of S2, but excluding S2's transmembrane and cytoplasmic domains has been termed the S-ectodomain (Se) (51, 282). The implication of S1 for generating neutralizing antibodies has been established through multiple pieces of evidence showing conformation-dependent neutralization epitopes (59, 216, 255, 264). Although S2 does not furnish any additional binding site, it has been shown to enhance the avidity of S1 (282). This increase in avidity is consistent with the considerable neutralization (more than 100-fold) by monoclonal antibodies directed against an epitope cluster on S2 (203). Indeed, histochemical analysis of respiratory tissues, kidneys, cecal tonsils, and cloaca has revealed enhanced binding affinity of Se compared to S1 (113). Although earlier studies speculated that S2 contains a single linear immunodominant neutralizing epitope

(172, 203, 216), a relatively recent study involving protein modeling and epitope mapping has revealed 3 novel epitopes on or proximal to fusion peptide of S2 (9).

A study utilizing IBV expressing the chimeric Se produced using a reverse genetics system has previously demonstrated the involvement of IBV Se in determining *in vitro* cellular tropism and infectivity. Following infection of different target cell-types, the recombinant Beaudette-type IBV, with genomic sequences of Se replaced by those of donor strain M41-CK, displayed replication dynamics of the M41-CK (51). Subsequently, an *in vivo* study revealed that the expression of virulent M41-CK-type Se from an apathogenic Beaudette-type IBV does not alter its pathogenic potential (155). Although Se is one of the multitude of factors that can influence pathogenicity (11), such an approach can be valuable from the vaccine development perspective. It avoids the concern of reversion to virulence that could occur due to expression of virulent-type S from apathogenic IBV and allows the concurrent expression of immunogenic epitopes. Moreover, the induction of significant protection as evaluated by ciliostasis confirms the *in vivo* immunogenic potential of the Se (155). Rationally, the recent efforts using a recombinant viral vector containing Se sequence insert (394) and recombinant Se as a recombinant protein in subunit vaccine (113) have shown promise.

4. Control of IB

Considering the substantial economic impact of IB on the global poultry industry, control of the diseases bears enormous significance. From a broader perspective, optimal management practices, appropriate air quality, calculated bird density, and strict adherence to the all-in/all-out principle are critical for control of any disease, and IBV is not an exception (17, 175). Biosecurity is another crucial management tool conventionally employed for disease control. A recent study compared the levels of integration between two commercial settings and concluded that

biosecurity could serve as an efficient impediment to IBV transmission (126). Conversely, poor managemental practices can lead to a rise in the levels of residual ammonia, consequently inducing pathological changes such as pulmonary edema, congestion, and hemorrhages (8). This management-originated pathogenesis, together with other bacterial diseases in birds, has been demonstrated to augment the severity of IB (319, 386).

Moreover, chickens exposed to stress factors related to management and viral infections can predispose them to immunosuppression (156). Researchers have previously correlated the clinical and immunological outcomes of IB with the detrimental effects of immunosuppressive diseases such as chicken infectious anemia and infectious bursal disease (350, 356). Conclusively, appropriate flock management practices and control of other bacterial and immunosuppressive diseases are crucial for the control of IB. In recent years, efforts have been made to discover natural antiviral properties of phytopharmaceuticals to treat IB (180, 256, 385). A novel study demonstrated an *in-silico* design and subsequent evaluation of peptides involving the heptad repeat regions of IBV S. The antiviral activity of these peptides was established using chicken embryo infectivity and pathogenesis assays (367). Nevertheless, vaccination remains the most extensively practiced among all the intervention strategies being researched and undertaken by the industry (289).

4.1. Live attenuated Vaccines

Before the advent of IBV vaccines, various drugs, air sterilization procedures, and management practices were tested but remained ineffective (17, 151, 195, 199, 217). Soon after IB was recognized as a disease different from ILT, experimental efforts were launched to immunize the birds using bird-propagated IBV (16, 101, 358, 359). Ultimately, successful cultivation of the virus in embryonated eggs (16, 92) precipitated the concept of IBV attenuation

via serial passaging and subsequent immunization of the birds (37, 102). Soon after this revolutionary discovery, propagation of various IBV strains in various cell/tissue/organ cultures was also documented (69, 78, 93, 118). Moreover, IBV can adapt to the cell cultures as soon as 2-5 passages, and after only 7 cell culture passages, around 25 amino acid substitutions have been observed (120, 313). Nonetheless, embryonated eggs have been preferred for viral attenuation due to the logistics and economics of propagation. Subsequently, the applicability of attenuated vaccines in commercial settings was reinforced with the concept of mass spray and micronized dust vaccination (242, 279). Although the concept of embryo attenuation was quite innovative back in the mid-20^{th} century, the detrimental effects of live attenuated vaccines remained unanticipated. While attenuated vaccines have been demonstrated to effectively protect the birds by reducing the R_0 (a disease transmissibility measure) from >19 to 0.69 (100), recent studies have elucidated their role in viral evolution and emergence of vaccine-derived strains (128, 179, 248, 349, 360). Earlier attempts using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) followed by phylogenetic analysis revealed an S1 amino acid sequence dissimilarity of about 0.6% between attenuated and pathogenic IBV 4/91 (49). Subsequent studies indicated disparities in S1 sequence within the vaccines developed by different manufacturers against one IBV serotype, among different serial numbers, and in subpopulations within one vial of vaccine (248, 360). Ensuing live vaccination, spontaneous mutations have been documented, leading to distinct viral subpopulations and subsequent selection (64, 248, 347). Moreover, major or minor vaccine virus populations can supply genetic material for recombination of vaccine strains with field strains, thereby fostering the emergence of IBV variants. For example, genomic sequencing has suggested that the emergence of Arkansas Delmarva poultry industry (Ark-DPI) strain originated from mutations and recombination between Connecticut (Conn) vaccine strain

and field strains (7). Moreover, the control of IB through vaccination is hampered by serotype diversity (179). Vaccination against one IBV serotype provides little or no cross-protection when challenged with another serotype (213, 348). Therefore, diverse attenuated vaccines have been developed to protect chickens against numerous divergent types. Unfortunately, these vaccines also end up in regions without substantial evidence supporting the prevalence of the serotype that would justify the use of that vaccine i.e., introducing previously exotic serotypes into certain regions (121, 125, 252). Although these haphazardly introduced genotypes may disappear when vaccination is discontinued (125), novel IBV variants could emerge through recombination of genetic material furnished by the previously foreign vaccine strains (348). Furthermore, live attenuated vaccines are subject to reversion of virulence. For example, genomic analysis has provided putative evidence that H120, an extensively used and well-advocated IBV vaccine (28, 81), undergoes recombination and reversion to virulence (399). Moreover, enhanced transmission and persistence of IBV has been established following vaccination with H120, compared to vaccination with IBV M41 (246), providing another example of several drawbacks associated with attenuated vaccines.

4.2. Inactivated vaccines

Although initial attempts of physical or chemical inactivation of IBV using phenol, formalin, and ultraviolet irradiation remained unsuccessful, the first available report of effective inactivation described the use of beta-propiolactone (BPL). While the inactivated IBV induced serum neutralizing antibodies, the protection determined by clinical signs following challenge was insufficient (72). The principle of using BPL-inactivated IBV was further extended to develop the first commercially available inactivated IBV vaccine (Iblin®) using a British Mass-type strain. While egg production was established as a protection determinant instead of disease resistance

(22, 23), the reliability of such an approach was challenged through substantial experimentation (163, 250, 377). For example, an experiment revealed partial protection of the reproductive organs and no respiratory protection at all when challenged with M41 even after 2 or 3 vaccinations (250). Furthermore, an experiment evaluating the immunogenicity of this inactivated vaccine indicated suboptimal serum neutralizing antibody levels (neutralization index<2.0) against the Mass and Beaudette strains isolated in the United States (377). Another detailed study involving analysis of respiratory signs, histopathological lesions, viral isolation, and serological evaluation revealed a lack of immunity following vaccination with the Iblin® vaccine (163). Additionally, an independent study concluded that the presence of neutralizing antibodies following an IBV immunization attempt could not be correlated with disease resistance (285). Considering all these studies, it can be inferred that the very first commercially available inactivated vaccine (Iblin®) lost its scientific credibility (163, 250, 377). During the same era, a BPL-inactivated vaccine using IBV strain 33 was prepared and was claimed to reduce viral isolation post-challenge when administered through a combination of aerosol and subcutaneous routes. However, insignificant reduction in clinical signs and short duration of immunity indicated poor protection levels (84). Another attempt involving aerosol administration of BPL-inactivated viruses resulted in insignificant antibody levels and absence of resistance to infection upon challenge with IBV M41 (83). Subsequent studies indicated poor immune responses elicited by inactivated monovalent and bivalent oil emulsion or BPL-inactivated preparations when administered as primary IBV vaccines (80, 135). Therefore, a combined strategy, using attenuated vaccines as primers and inactivated vaccines as boosters, was suggested (36, 122, 135, 343). While it was argued that slower but persistent humoral immunity thus acquired was better suited for layers and breeders, a weaker cell-mediated immune response was observed with inactivated vaccine despite higher

hemagglutination inhibition (HI) titers (343). These findings greatly reduce the scope of inactivated vaccines, as studies have widely established the significance of T-lymphocytes and their memory responses in controlling IBV infection (77, 276, 303). While a recent attempt has demonstrated higher granzyme homolog A mRNA level expression following challenge of chickens vaccinated with inactivated vaccine compared to those of the unvaccinated group following challenge, both tracheal and renal CD8+ marker mRNA levels were not statistically significantly different from those of the unvaccinated group post-challenge (296). Although the constituent pathogens of inactivated vaccines do not interfere with each other even in trivalent forms (268), a time- and strain-dependent interference between live attenuated and oil emulsified vaccines has been demonstrated. Specifically, priming with the more aggressive attenuated strain (H52) resulted in suboptimal immunogenesis by the inactivated booster vaccine (36). Interestingly, serological analysis revealed low IgG quantities upon single intramuscular vaccination with oil emulsified vaccine (OEV) and negligible IgM levels in birds primed with a live vaccine and boosted OEV (244). Moreover, evidence of antigenic persistence and microscopic lesions has been demonstrated following an administration of an inactivated vaccine (212). Although more recent efforts have incorporated various immunostimulatory adjuvants such as chitosan (230) and pustulan (214), cell-mediated immune responses seem to be either overstated or irrelevant. For example, following vaccination with chitosan-encapsulated nephropathogenic IBV (IBV-CS), the renal interferon-γ (IFN- γ) mRNA levels were not significantly different in any of the vaccinated or non-vaccinated groups on 1, 5 and 11-days post-infection (dpi). Moreover, tracheal IFN- γ levels were not significantly increased following single vaccination with inactivated IBV-CS. Additionally, humoral responses were controversial as IgA levels 11dpi for inactivated IBV-CS group were similar to non-vaccinated (NV) group and IgG levels of NV group were higher than

IBV-CS group (230). Thus, inactivated vaccines confer inadequate immunity, and the prerequisite of live priming makes this vaccination alternative less appealing. In addition, parental administration of inactivated vaccines induces stress and occasional local tissue damage/reactions. From a commercial perspective, inactivated vaccines heighten the cost of production due to higher amounts of antigen required, the necessity of skilled vaccinating workforce and sterile equipment (75).

4.3. Recombinant vaccines

Novel vaccine alternatives include subunit vaccines, DNA vaccines, and viral vectored vaccines.

4.3.1. Subunit vaccines

With the advancement and increasing convenience of the expression systems, subunit vaccines involving IBV peptides of immunological significance have been tested experimentally. For example, linear epitopes of B- and T-cells eliciting immune responses against IBV have been produced as fusion peptides, using an *Escherichia coli (E. coli)* expression system. Following administration of such a multi-epitope peptide vaccine, a higher viral neutralizing titer was observed. Although augmented $CD3^+CD4^+$ populations were observed, $CD8^+$ cells were as low as 10% (383). On the contrary, studies have demonstrated that although $CD4^+$ cells are produced in response to IBV infection (356), $CD8^+$ cells are more relevant in protection against IBV (303). Another study demonstrated 50% protection induced by three consecutive immunizations with recombinant S1 expressed by a recombinant baculovirus (321).

To enhance the immunogenicity of these subunit vaccines, the use of a β-1-6-glucan, namely "pustulan", has been suggested. Interestingly, an administration of pustulan-adjuvanted recombinant IBV N protein enhanced recall response of antigen-specific CD4+ cells but not that

of $CD8^+$ cells (214). Although pustulan elicited an enhanced *in vitro* MHC-II and cytokine expression, an *in vivo* assessment of its efficacy was not determined (214). Recently, a recombinant soluble trimeric Ark-type Se was produced in human embryo kidney 293T cells and was proven to protect the birds against homologous challenge (113). Although the adjuvant (Montanide^{TM} ISA 71 VG) used in the subunit vaccine has been shown to enhance both humoral and cell-mediated immunity in other experimental settings (181, 182), cellular and virus-neutralizing antibodies were not analyzed in this particular study (113). Subunit vaccines must be delivered parenterally. Thus, their use is restricted to smaller chicken populations such as layer and breeder hens. Individual injection is neither economically nor logistically feasible in large broiler operations.

4.3.2. DNA vaccines

One of the earliest attempts to develop a DNA vaccine against IBV encompassed identification and expression of cytotoxic T-lymphocyte (CTL) epitopes located in the carboxy terminal portion of the N protein. However, viral quantification following challenge revealed retention of the challenge virus in the lungs up to 2.5 $\log_{10}$ of 50% embryo infective dose (EID_{50}). Moreover, poor IgG response was observed due to specific targeting of CTL-epitopes of the N protein instead of neutralizing epitopes of S protein (304). Subsequently, another attempt involved *in ovo* and IM administration of recombinant DNA expressing Ark-type S1. However, *in ovo* vaccination demonstrated incomplete protection without a subsequent administration of a live vaccine and induced antibody levels similar to unvaccinated controls. Moreover, dual IM vaccination not only required a higher total amount of DNA vaccine (450µg), but also generated poor antibody titers. IM vaccinated groups exhibited viral clearance 10 days post-challenge (DPC) and not 5 DPC (191). However, viral titers naturally begin to decline 5 DPC (48). More recently, DNA vaccines expressing multiple epitopes have been developed (14, 336, 342, 382). For

example, a chimeric DNA expressing seven epitopes of S1, S2 and N proteins was developed. The vaccine candidate failed to elicit significant ELISA antibody levels. Perhaps, the antibody values were underestimated, as the commercial plate used for ELISA was coated with an antigen from a serotype different than that expressed by the plasmid (342). A multivalent DNA vaccine consisting of 3 plasmids each containing S1, M or N protein sequences provided 85% protection against a challenge of 100 EID_{50} (382). Similar DNA plasmids expressing S1, M and N proteins induced 80% protection when chickens were boosted with inactivated vaccine and challenged with 100 EID_{50} of IBV (138). Because DNA vaccines have shown limited efficacy, some researchers have used a plasmid expressing cytokines such as interleukin-2 (IL-2) (338) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (333). However, these studies challenged chickens with either 10^2 (333, 338, 342, 382) or 10^3 (138) EID_{50} and evaluated viral loads as late as 14 DPC (138, 342, 382). Measuring vaccine induced protection using low challenge dosages and at time points beyond 5 days casts doubts upon the actual effectiveness of these vaccine candidates (48). Some researchers have speculated that multivalent vaccines (186, 382) or those expressing several epitopes (336, 342, 383) from a plasmid could impact the efficacy of DNA vaccines by exhausting the host cell machinery, rendering more cellular stress and inducing tissue damage (302). A non-parenteral vaccine delivery approach involved oral and nasal delivery of IBV S1 and N expressing plasmids through attenuated *Salmonella.* However, the highest IgG levels generated by these plasmids 3 weeks post-boost were 3-4 times lower compared to those elicited by attenuated vaccines (186). More recently, a nano-carrier adjuvant system has been introduced for intranasal administration of IBV DNA vaccines. Although the CD8+ cell population was enhanced in the group immunized with the adjuvanted DNA vaccine, no detectable increments in IBV-specific IgA and IgY were detected even 20 days post-vaccination (DPV) (65). In a very recent study, a

consensus S protein-coding sequence was cloned into a plasmid and enhanced cellular responses were determined by IFN-γ and IL-2 assays. However, IBV-specific IgY levels were below threshold value through 14 DPV (413), which indicated poor humoral responses with single vaccination. Moreover, the cost-effective plasmid construction and large-scale production possibilities have not been explored for DNA vaccines. Therefore, it can be concluded that most of the DNA vaccines reported thus far not only confer inadequate protection regardless of the route of administration, but also implicate commercial, logistic, and animal welfare challenges, which necessitates the need for alternative vaccine candidates.

4.3.3. Homologous virus-vectored vaccines

Initial endeavors exploring the potential of IBV as a vector involved the development of a defective IBV RNA through mutagenesis (278). Since then, recombinant IBV (rIBV) has been experimentally tested as a vector for vaccination against IB (155, 198, 372) as well as Newcastle disease (384). rIBV has been determined to undergo very rapid genetic instability (arising as soon as passage 5) is associated with deletions around certain TRSs, and codon optimization can lead to a significant reduction in translation efficiency (18). While the first attempt employing rIBV indicated protection induced by a recombinant Beaudette-type IBV expressing S protein of M41 (BeauR-M41-S), similarity in viral titers, nasal discharge, and snicking 6 DPC between parent Beau-R and BeauR-M41-S vaccinated group was questionable (155). In another stu, chickens were inoculated *in ovo* with BeauR-M41-S and a turkey herpes virus expressing IL-2 was demonstrated and were challenged with 10^3 EID_{50} of IBV. However, the study neither involved histopathological analysis of embryos to determine safety of injecting two viruses *in ovo* nor the viral RNA quantitation to determine protection post-challenge (340). Briefly, contemporary studies have indicated insufficient protection conferred by rIBV against homologous (115) and heterologous

(198) challenges. Regardless of the protective immunity, rIBV as a vaccine virus could introduce genetic material for recombination with field viruses, thereby presenting complications no different than a live attenuated vaccine.

4.3.4. Heterologous virus-vectored vaccines

An initial investigation described using recombinant vaccinia virus expressing IBV S in mice that elicited low but measurable antibodies three weeks after the first vaccination. Unfortunately, the study was not replicated in chickens, the most important host of IBV (344).

Several studies (67, 315, 370, 390) have demonstrated varying levels of protection induced by recombinant fowlpox virus (rFPV) expressing the IBV genes. In the early 2000s, subcutaneous administration of rFPV expressing the 119 C-terminal amino acid residues of the N protein failed to protect against 10^3 EID_{50} IBV challenge (390). Two similar studies evaluated protection provided by rFPV expressing IBV S1 and IFN-γ against homologous (370) or heterologous (315) challenge. However, histopathological analyses showed tissue damage in all the vaccinated groups after a homologous challenge. Viral isolation from tracheal swabs indicated susceptibility in 60% of vaccinated chickens. Ironically, rFPV expressing both S1 and IFN- γ exhibited lower antibody levels compared to that expressing only S1 (370). The same recombinant construct did not induce sufficient protection against distantly related strains. Protection of 70-75% against closely related strains was attributed to CD8+ cell populations. Viral isolation 6 DPC 60% protection (315). Chickens vaccinated with rFPV expressing S1 and IL-18 were protected against IBV challenge with 100 EID_{50} (67). However, the challenge dose used was below international standards for vaccine evaluation (267). Limited efficacy of vaccination with rFPV expressing IBV antigens could be attributed to the fact that FPV does not elicit mucosal immunity in the respiratory tract, thereby allowing initial IBV replication when chickens are challenged via a respiratory route (390).

Moreover, the large size of the rFPV genome results in the expression of proteins immunologically irrelevant to IBV immunity (168) and, more importantly, an exhaustion of host cellular resources.

Other experimental viral vectors that have been used to express IBV antigens include recombinant adenoviruses (rAdVs) (187, 351, 396, 397), Marek's disease virus (rMDV) (398), duck enteritis virus (rDEV) (222), and Newcastle disease virus (rNDV) (1, 317, 335, 352, 394, 401). An initial effort using recombinant fowl adenovirus (rFAV) resulted in 80% renal and 70% tracheal protection protection against IBV. However, antigen ELISA revealed tracheal protection of 62.5% and 92.5% in chickens vaccinated at day zero and day 6 of age, respectively. Such differential protection can be attributed to intereference by MDAs, as the chickens used were MDA-positive (187). A replication-defective recombinant human adenovirus (RD-rAdV) expressing S1 induced protection when administered intramuscularly (351). Similarly, an RD-rAdV co-expressing S1 and GM-CSF injected *in ovo* (396), has been shown to provide protection. The replication deficiency precludes virus-induced pathology in the vaccinated individuals and transmission among different hosts (396). However, a greater initial dosage requirement (351), a potential of recombination with modified or wild-type adenoviruses (232, 396), and incompatibility with mass vaccination methods restrict their applicability in commercial settings. Following vaccination with rMDV expressing S1, pre-challenge antibody levels barely reached detection threshold levels (398). Further work is needed to establish these viral vectors as convincing candidates for IBV control.

4.3.5. Recombinant NDV

Since the first successful recovery of genetically modified NDV from cloned cDNA (275), NDV has been evaluated as a vector for vaccination against avian (166, 169, 200, 260, 352, 391, 394), mammalian (207), and human viruses including SARS-CoV-2 (331). NDV lentogenic strains

B1 and LaSota have been extensively used worldwide to prevent NDV outbreaks. Because NDV effectively replicates in epithelial cells of the upper respiratory tract, rNDV closely resembles IBV-infection and generates both humoral and mucosal immune responses when administered via mucosal routes (317, 352, 394). Previously, researchers have demonstrated construction and evaluation of recombinant LaSota (rLS) expressing IBV S1 (401), S2 (114, 352), S (1, 317, 318), and Se (394). However, single vaccination with rLS expressing S1 induced insufficient protection (401). While a combination of rLS expressing Ark S2 (rLS/S2) and live Mass-type IBV conferred protection against homologous challenge (352), only limited protection was detected against a heterologous challenge (114). A significant reduction in viral titers was demonstrated in birds immunized with rLS expressing S (rLS/S) at 4 weeks of age. However, such a decrease was not observed upon vaccination in day-old chicks followed by a challenge dose as low as 10^3 EID_{50} (317). Recently, the same researchers argued that rLS could induce clinical signs and was "not safe" for chicken embryos. Therefore, a chimeric rNDV was developed by replacing fusion and HN genes of NDV with those of a less pathogenic avian paramyxovirus serotype-2. However, neither single nor prime-boost vaccination with the chimeric rNDV reduced post-challenge viral loads (318). A thermostable NDV expressing three epitopes of S1 was evaluated for protection against IBV and NDV. The protection parameters included survival rates (335), which is of limited relevance because the IBV M41 does not cause heavy mortality in experimental settings (319). Additionally, an evaluation of viral load quantification, histopathology, cellular and humoral responses could have better explained the potency of this vaccine (267). Therefore, use of rLS expressing Se (rLS/Se) overcomes the concerns described above. rLS/Se has been shown to confer partial protection after single vaccination and moderate protection after booster-vaccination (394). rNDV has been shown to express more than one foreign gene while retaining growth kinetics and

immunogenicity as well as the low pathogenicity of the parent virus (146, 167). Accumulating evidence indicates enhancement of vaccine effectiveness using inserts expressing various cytokines (67, 333, 338, 340). We hypothesized that co-expression of granulocyte-macrophage colony stimulating factor and IBV Se from rLS/Se would enhance the vaccine efficacy.

5. Granulocyte-macrophage colony stimulating factor (GM-CSF)

The granulocyte-macrophage colony-stimulating factor (GM-CSF or CSF-2) is an immune mediating molecule that regulates the production, differentiation, and function of immune cells, including granulocytes and macrophages. GM-CSF plays a central role in generating a robust immune response by stimulating proliferation of multipotent stem cells (43, 251).

Metcalf and Burgess first purified GM-CSF in 1977 from a lung-conditioned medium and later utilized to stimulate mouse bone marrow cells *in vitro* (42). The basic properties of the protein, such as molecular weight, glycoprotein composition, and effects of the stimulation at various concentrations, were determined.

5.1. GM-CSF gene and protein structure

In humans, complete gene encoding GM-CSF comprises ~ 2.5 kilo-base pairs (kbp) which includes 3 introns (196). The mRNA encoding chicken GM-CSF consists of 435 nucleotides (GenBank accession no. AJ621253).

The chromosomal locus of the genes encoding GM-CSF depends on the species. In mice, it is encoded by genes located on chromosome 11 and is linked with those encoding IL-3 (324). While human GM-CSF gene is found on chromosome 5, precisely at 5q21-32 (170), GM-CSF is encoded in a T helper type 2 (Th2) -associated cytokine-encoding cluster found on chromosome 13 in chickens (13). Mutations in these genes lead to abnormal hematopoiesis and tumors (161, 387).

Being a member of the hemopoietin family, GM-CSF is a monomeric glycosylated protein comprising a bundle of four α-helices and two strands of anti-parallel β-sheet (293). In case of both humans (GenBank accession no. AAA98768) and chickens (GenBank accession no. ACH86023), GM-CSF consists of 144 amino acids. Chicken GM-CSF has a 19% amino acid sequence homology with human and murine counterparts (13). The glycosylation of the GM-CSF is heterogeneous. The total molecular weight ranges from 14 to 35 kDa and depends on the species and degree of glycosylation (290).

5.2. GM-CSF expression and transcriptional regulation

A conserved consensus sequence was identified in the 3' untranslated mRNAs encoding GM-CSF and other cytokines (312). After activation of T-cells, transient mRNA accumulation was observed due to increased transcriptional activity and stabilization of mRNA. The regulation of GM-CSF expression depends on a putative TATA box present in the promoter region upstream of both murine and human GM-CSF genes (254). The promoter of chicken GM-CSF does not differ much from human GM-CSF, except in NF-kB binding site (13). The transcriptional regulation by the cytokine consensus sequence (CK-1), nuclear factor kappa B (NF-kB), NF-GMa has been reported (310, 311).

5.3. GM-CSF receptors and signaling

The GM-CSF receptor (GM-CSFR) is a heterodimer constituted by a ligand-specific α-subunit and β-subunit shared with IL-3 and IL-5 receptors (97). Structural characterization of both subunits revealed type 1 transmembrane glycoprotein domains. On hematopoietic cells, GM-CSFR is expressed at minimal levels, about 100-1000 molecules per cell. The interaction of GM-CSF and its receptor involves three positions. The first is GM-CSF and GM-CSFRα, the second interaction involves GM-CSF itself and two domains of GM-CSFRβc, and the third is a stabilizing site formed

between GM-CSFRα and GM-CSFRβc (143). The signal transduction through the receptor overlaps with the interferon production regulator family. After binding of GM-CSF with the receptor, a cascade of reaction involves the activation of kinases followed by phosphorylation of signal transducers and transcriptional activators of cellular differentiation. Ultimately, this signaling cascade induces the proliferation and inflammatory responses by mitogen-activated protein kinases (MAPK) and NF-kB activation (68, 245).

5.4. Cellular production of GM-CSF

In response to specific activating signals such as antigens, microbial products, inflammatory molecules, and co-stimulatory cytokines, GM-CSF can be produced by different immune cell types (129). Immune cells such as T-cells, macrophages, fibroblasts, endothelial cells, and mast cells can be induced to accumulate GM-CSF mRNA and translate it into GM-CSF protein. Cytokine stimuli such as interleukine-1 (IL-1) and Tumor Necrosis Factor-alpha (TNF-α) act on endothelial and fibroblast cells (257, 412). Various antigens, lectins, CD28, and IL-1 act as stimuli for T-lymphocytes and lead to GM-CSF production (147, 375). Some inflammatory cytokines such as IL-10, IL-4, and IL-27 inhibit GM-CSF production (316). It can be detected at serum concentrations from 20-100pg/100ml under physiological conditions. However, after stimulation through antigens and cytokines, higher serum concentrations are observed (79). Unlike human GM-CSF, expression of chicken GM-CSF has been observed in B-lymphocytes (13).

5.5. Target cells

GM-CSF acts on various immune cells and leads to the activation and maturation of monocytes, dendritic cells, eosinophils, neutrophils, T cells, and B cells (42, 142). In addition, it has a particular affinity for dendritic cells and can generate alveolar macrophages from monocytes (152, 270).

5.6. Diversity of functions

GM-CSF has an essential position in both innate and adaptive immune responses (124). It has a significant role in the survival, proliferation, differentiation, and function of myeloid lineage cells (141). Particularly, a broad range of action through its lymphoid regulatory mechanisms enables the communication between innate and adaptive immune signals (124). Due to its demonstrated potential to affect multiple cell lineages, its role as a pro-inflammatory cytokine is central to its immunomodulatory function (112, 357). It also establishes phagocytic activity against some microbes (20, 21, 76). Some researchers suggest that GM-CSF promotes immunological tolerance through its immuno-regulatory activity (192, 204, 272). Like some other cytokines, GM-CSF has also been observed to modulate anti-tumor response along with enhanced infiltration of DCs and macrophages into the cancerous cells (12). However, the duality of function exists, and the immune response is considered to be dose and context dependent similar to other cytokines (26, 307).

5.7. Disorders associated with GM-CSF

Apart from stimulation of productive immune responses, GM-CSF can lead to disorders of varying severity. Dysregulation of GM-CSF may lead to pathogenic invasion of phagocytes and central nervous system disorders (322). In addition, it has been described to drive dysregulated hematopoietic stem cell activity. Moreover, pathogenic myeloid cell proliferation and toxic neutrophil accumulation in experimental spondyloarthritis have also been observed due to GM-CSF (291). Others have described defective expression of GM-CSF receptor (CD116) leading to inflammatory bowel disease (133). Moreover, the presence of GM-CSF autoantibodies leads to impaired antimicrobial function of neutrophils (353). The deficiency of GM-CSF has also been demonstrated to exacerbate atherosclerosis (104).

5.8. GM-CSF in cancer immunotherapy

The antitumor activity of GM-CSF relies on the presentation of tumor antigens by dendritic cells (DCs) to helper and cytotoxic T lymphocytes in the lymph nodes (233). Researchers have used irradiated tumor cells engineered to secrete GM-CSF to stimulate long-term antitumor immunity (108). A recombinant Newcastle disease virus expressing GM-CSF has also been demonstrated to enhance the efficacy of anti-tumor vaccines *in vitro* (183). DCs remain at the center of antitumor therapies mediated by GM-CSF. However, they need an ideal adjuvant to mature. Although DCs are the antigen-presenting cells in antitumor activity, they drive the production of T-regulatory cells (T-regs), which are responsible for self-tolerance. Therefore, the addition of an adjuvant is necessary to activate DCs through immunotherapy to launch a robust immune response (94). A recombinant human GM-CSF (rhGM-CSF), derived from yeast and known as sargramostim (Leukine®), has been shown to enhance the survival rates of patients with melanoma when used as an adjuvant (323).

5.9. GM-CSF as a vaccine adjuvant/recombinant insert

GM-CSF has been demonstrated to supplement the efficacy of vaccines against several human (5, 41, 183, 271, 284), non-avian animal (10, 109, 408, 409) and avian diseases (137, 368, 369), including IB (333, 396). For example, expression of GM-CSF at a supra-physiologic level after influenza A virus infection has been shown to prevent mortality in transgenic mice (140). In addition, GM-CSF and flagellin expressed by an intramuscularly and orally administered recombinant rabies virus has exhibited significantly better protection compared to parent virus control (409). Moreover, expression of either IL-3 or GM-CSF from a plasmid vector expressing swine classical fever virus E2 antigen enhanced protection against challenge (10). To prevent other

non-avian animal diseases, GM-CSF has been utilized to enhance vaccine efficacy against porcine reproductive and respiratory syndrome virus (223) and foot and mouth disease virus (109).

Among avian diseases, GM-CSF has enhanced immune responses against influenza (137, 369) and Newcastle disease (368, 389). Irrespective of the managerial inadequacies (discussed in 4.3.2 and, 4.3.4) associated with recombinant DNA (333) and rAdV (396) vectors expressing GM-CSF, the insertion of GM-CSF genes in the recombinant constructs enhanced the immune response compared to the relevant controls (333, 396).

Therefore, considering its diversified functionality in immune modulation and an established track record of vaccine potentiation, we hypothesized that co-expressing GM-CSF and Se from an rLS vectorconfers enhanced protection against IBV.

III. Enhanced Protection by Recombinant Newcastle Disease Virus Expressing Infectious Bronchitis Virus Spike-ectodomain and Chicken Granulocyte-macrophage Colony-stimulating Factor

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Abbreviations: aa = amino acid; AIV = avian influenza virus; ANOVA =analysis of variance; Ark = Arkansas-type IBV; ArkSe = Ark spike ectodomain; EID_{50} = 50% embryo infectious dose; CPE = cytopathic effect; DOA = days of age; ELISA = enzyme-linked immunosorbent assay; GM-CSF = granulocyte macrophage-colony stimulating factor; HA = hemagglutinating activity; HI = hemagglutination inhibition; IBV = infectious bronchitis virus; ICPI = intracerebral pathogenicity index; IFA = immunofluorescence assay; Mass = Massachusetts; MDT = mean death time in chicken embryos; NDV = Newcastle disease virus; nts = nucleotides; rLS = recombinant NDV LaSota virus; rLS/Ark.Se = rLS expressing IBV Ark S ectodomain; qRT-PCR = quantitative reverse transcriptase polymerase chain reaction; S = spike; Se = spike ectodomain; SPF = specific pathogen free.

Introduction

Worldwide infectious bronchitis virus (IBV) remains a major cause for economic losses in the poultry industry. IBV's evolutionary success relies on genetically diverse populations, which allow for quick adaptation to changes in selective pressure (349). The increasing number of Arkansas (Ark) vaccine-like viruses isolated from outbreaks of disease in chickens vaccinated with attenuated Ark, shows that live vaccine viruses augment the likelihood of recombination events and subsequently increases IBV's diversity and the virus' fitness in the environment (128, 179, 248, 360). In addition to virus populations originating directly from vaccine viruses, accumulating evidence shows that subpopulations also emerge from wild Ark viruses as result of immune selection in vaccinated chickens (132, 347, 393). Use of recombinant IBV type-specific vaccines instead of varying serotype live vaccines in the poultry industry should reduce emergence of novel IBV.

Unfortunately, expression of the IBV spike (S) S1 or S2 subunits from different viral vectors has shown varying protection levels against IBV challenge (114, 351, 352). Somewhat better results have been obtained using the whole S (1, 317) or the S ectodomain (Se), i.e. S1 extended by the S2 ectodomain (394). Expressing only the Se, which cannot be inserted into the viral envelope in a recombinant viral vector, has the advantage over expression of the complete S protein, in that it avoids the concern that the expression of the IBV S protein on the surface of the virus vector could have the potential to extend its tropism.

Insertion of foreign genes in recombinant Newcastle disease virus (NDV) LaSota (rLS) exhibits multiple advantages for vaccination of commercial broilers including safety, stability, and suitability for mass-administration. At the same time, chickens vaccinated with rLS are protected against NDV (391, 402). Insertion of the IBV S gene into the rLS genome reduces the virus'

virulence to the level of the mild NDV Hitchner B1 vaccine, thus allowing safe vaccination of young naïve chickens (352, 394). Although vaccination with IBV S expressed from rLS (rLS/IBV.S) has shown promising results, the conferred protection against IBV challenge is still suboptimal. Shirvani et al. (317) reported protection in chickens vaccinated with rLS/IBV.S but reduction in virus shedding when chickens were vaccinated at one-day of age was dependent on route of challenge. Similarly, single-dose vaccination in one-day-old SPF chickens with rLS expressing a codon optimized IBV S provided significant protection against clinical disease after IBV challenge but did not reduce tracheal virus shedding (1). We previously developed rLS expressing the IBV Ark-type S-ectodomain (rLS/ArkSe), that is, the S protein excluding the transmembrane anchor and short cytoplasmic domain of S2 that is not shown to the immune system during infection. Vaccination with a relatively high dose (10^7 EID_{50}/bird) of rLS/ArkSe reduced signs and tracheal lesions in chickens but did not decrease the viral load in tear fluids of challenged chickens. The high dose required for protection also makes this vaccine unappealing for the industry.

Others have demonstrated that DNA vaccines co-administered with plasmid cytokine adjuvants increase humoral and cellular immune responses in vaccinated animals (71, 109, 164). The cytokine granulocyte-macrophage colony-stimulating factor (GMCSF) enhances immune responses by attracting macrophages and inducing their maturation, thus resulting in increased antigen presentation (149). Accumulating evidence indicates a strong effect on differentiation and maturation of dendritic cells as well as expression of MHC and co-stimulatory molecules (123, 173, 316, 341) resulting in enhancement of antigen-specific humoral and cellular immune responses. For example, simultaneous inoculation with plasmid DNA expressing GMCSF has been reported to increase protection against classical swine fever virus (10), herpes simplex virus

(271), and foot-and-mouth disease virus (109). Prime and booster vaccination with both plasmids carrying the S1 gene of IBV and the chicken GMCSF gene have shown significant enhancement of humoral and cellular responses in chickens compared to vaccination with S1 plasmid alone (333). Recombinant human adenoviruses (rAd) expressing chicken GMCSF and the S1 gene of a nephropathogenic IBV strain have been shown to confer enhanced protection against homologous IBV challenge in chickens. Chickens vaccinated with rAd-S1 fused or co-administered with GMCSF showed reduced nephropathy and 100% protection compared to 70% protection in chickens vaccinated with rAd-S1 alone (396).

We developed rLS co-expressing the Se of an Arkansas (Ark)-type IBV and chicken GM-CSF (rLS/ArkSe.GMCSF). We initially compared the effectiveness of the construct containing GMCSF versus a previously produced construct expressing Se only (rLS/ArkSe). Because live vaccines belonging only to the Massachusetts (Mass) serotype have been licensed in most countries worldwide, in a second trial we explored enhancing cross-protection of an attenuated Mass vaccine in a prime-boost vaccination regime with rLS/ArkSe.GMCSF. If confirmed, this strategy would provide protection against regional IBV types using tailored rLS.

Materials and methods

Chickens. White leghorn chickens were hatched from specific pathogen-free (SPF) embryonated eggs (Wayward Acres, Pickens, SC) and 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 care and use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)-accredited institution.

Challenge virus. The previously characterized virulent Ark-type IBV strains with GenBank accessions # DQ458217 (350) and #JN861120 (127) were used in experiments 1 and 2 respectively. Challenged viruses were titered in SPF embryonated chicken eggs as accepted (362) with some modifications previously described (131).

Generation of rLS containing chicken GM-CSF and IBV Ark S-ectodomain (Ark.Se) genes. The previously generated pLS-I-RFP-GFP plasmid (146) was used as a backbone to clone the chicken GMCSF and IBV Se genes into the NDV LaSota genome through two-steps of cloning. First, a DNA fragment encoding the C-terminal 126 amino acids (aa) of the chicken GMCSF protein (19-144 aa, GenBank: GQ421598.1) was commercially synthesized with codon-optimization for chickens (GeneScript, Piscadaway, NJ, USA). The synthesized chicken GMCSF fragment was amplified by PCR with a pair of gene-specific primers and cloned into the pLS-I-RFP-GFP vector to replace the RFP gene using an In-Fusion® PCR Cloning Kit following the manufacturer's instructions (Clontech, Mountain View, CA, USA). Secondly, the resulting plasmid, pLS-GMCSF-GFP, was used as a vector to clone the IBV Se gene into the LaSota genome. The Ark-type IBV S-ectodomain (S1 + S2 lacking the transmembrane domain and cytoplasmic tail) gene was amplified by PCR with a pair of gene-specific primers from a previously generated plasmid pLS/ArkSe (394) and cloned into the pLS-GMCSF-GFP vector to replace the GFP gene using the In-Fusion® PCR Cloning Kit. The sequences of the primers used for the foreign gene amplification and the In-fusion PCR cloning in this study are available upon request. The final resulting plasmid, pLS-GMCSF-ArkSe, was used to rescue the recombinant virus using the reverse genetic system as described previously (117). The rescued recombinant virus, designated as rLS/GMCSF/ArkSe, was propagated in chicken embryonated eggs two more

times. The allantoic fluid harvested from infected embryos at the third egg passage was aliquoted and stored at -80 C as a stock.

Virus titration, pathogenicity assessment, and sequence analysis. The rLS/ArkSe.GMCSF and the rLS virus stocks were titrated both by standard hemagglutinating activity (HA) test in a 96-well microplate and 50% egg infective dose (EID_{50}) determination in 9-day-old SPF chicken embryos (3). Pathogenicity of the viruses was assessed by standard procedures; i.e. mean death time (MDT) in chicken embryos and intracerebral pathogenicity index (ICPI) in one-day-old SPF chickens (3). The nucleotide sequences of the rLS/ArkSe.GMCSF virus were determined by sequencing the RT-PCR products amplified from the viral genome as described previously to confirm the sequence fidelity of the rescued virus (166).

Expression of the IBV S-ectodomain protein. DF-1 cells were infected with the rLS/ArkSe.GMCSF virus and examined by immunofluorescence assay (IFA) with a polyclonal anti-Ark-type IBV chicken serum as described previously (402) with minor modifications. An NDV-specific monoclonal antibody against the HN protein (kindly provided by Dr. Ron Iorio, University of Massachusetts Medical School) was included in the IFA to detect the NDV HN protein as an NDV infection control. After incubation with primary antibodies (anti-IBV and anti-NDV HN) and secondary antibody conjugates (Alexa Fluor® 568 conjugated goat anti-mouse IgG and FITC-labeled goat anti-chicken IgG), and washing with PBS, infected cells were stained with DAPI (300nM) (Thermo Fisher Scientific, Waltham, MA) at room temperature for 5 min as an extra step of the IFA to show DF-1 cell nuclei. Cytopathic effect (CPE) and fluorescence images were monitored/photographed using an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA) at 400X magnification. Green-, red-, and blue-fluorescence images taken from the

same field of virus-infected cells were merged into a single image to examine the co-localization of the IBV Se and NDV HN proteins.

Experimental design. Two experiments were conducted to evaluate the extent of protection. In experiment 1, we compared protection conferred by single vaccination with rLS expressing Ark Se (rLS/Ark.Se) and rLS co-expressing Ark Se and GM-CSF (rLS/ArkSe.GMCSF). In experiment 2, we evaluated cross-protection induced by live Mass vaccination when administered in prime and boost regime with rLS/ArkSe.GMCSF.

Experiment 1. Protection conferred by single vaccination with rLS/ArkSe and rLS/ArkSe.GMCSF. Because accumulating evidence indicates that the immature immune system of 1-day-old chickens does not respond adequately to IBV vaccination (394, 395), to more accurately determine possible differences between the vaccine candidates, this experiment was performed in 7-day old chickens. Four groups of chickens (n=19/group) were established. Experimental groups 1 and 2 were vaccinated at 7 days of age (DOA) with 100µl per bird containing 10^6 50% embryo infectious doses (EID_{50}) of rLS/ArkSe and rLS/ArkSe.GMCSF respectively. Control groups 3 and 4 included non-vaccinated/challenged and non-vaccinated/non-challenged chickens respectively. Blood was collected 23 days after vaccination for serum NDV antibody determination by hemagglutination inhibition test as accepted (363). At 31 days of age, chickens in groups 1-3 were individually challenged with 100 µl (25 µl in each nostril and each eye) containing 10^4 EID_{50}/bird of Ark virulent virus stock (GenBank accession # DQ458217). Protection was evaluated five days after challenge by individual assessment of respiratory signs, tracheal histomorphometry, and viral load in tracheas of challenged chickens as previously described (394). In brief, nasal and/or tracheal rales were evaluated blindly by close listening to each bird and scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) and scoring data

subsequently analyzed by Kruskal-Wallis test followed by Dunn post-test. Viral load in tracheas were determined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Briefly, IBV RNA was extracted from homogenized tracheal samples with TriReagent® RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH) as per the manufacturer's recommendations. Relative viral copies were determined by quantitation of viral RNA using TaqMan© qRT-PCR as described (48). Viral RNA data were analyzed by one-way ANOVA followed by Tukey multiple comparisons post-test. Differences were considered significant with $P<0.05$. Tracheal histopathology and histomorphometry was performed as described previously (350). Briefly, tracheal sections were routinely prepared for hematoxylin and eosin stain for histopathological evaluation. ImageJ software (https://imagej.nih.gov/ij/download.html) was used to measure the tracheal mucosal thickness and lymphocytic infiltration. Tracheal histomorphometry data (arbitrary units using ImageJ) were analyzed by one-way ANOVA followed by Tukey multiple comparison post-test.

Experiment 2. Cross-protection induced by prime-boost regime with live Mass vaccine and rLS/ArkSe.GMCSF. Chickens were divided into five groups. Groups 1 and 2, consisting of 28 birds each, were vaccinated with the recommended dose of an attenuated Mass vaccine (Pfizer, New York, NY) at 1 DOA. Group 2 was boosted 16 days after prime with 10^6 EID_{50} of rLS/ArkSe.GMCSF. Group 3 was primed at 1 DOA with 10^6 EID_{50} of rLS/ArkSe.GMCSF and boosted 16 days after prime with the commercial Mass vaccine. Groups 4 and 5 were non-vaccinated/challenged (n=19) and non-vaccinated/non-challenged (n=27) controls respectively. Sera were collected 14 days post-boost from all groups. Twenty chickens of groups 1, 2, 3, and 4 were challenged as described above with 10^4 EID_{50}/bird of virulent Ark (GenBank accession #JN861120) at 33 DOA (i.e. 16 days after booster in groups 2, 3, and 4). Protection against

challenge was evaluated 5 days post-challenge by clinical signs, viral load in trachea, and tracheal histomorphometry as described above. In addition, histopathology scoring of tracheal necrosis was performed. Finally, viral load was also determined in lachrymal fluids. IBV RNA was extracted from tear samples using the QIAmp Viral RNA Mini Kit (Qiagen, Valencia, CA) and qRT-PCR performed as described above. The remaining eight chickens of these groups were separated and remained non-challenged for antibody determinations at 22 days after boost.

Ark Se serum antibody. Ark Se specific antibody levels in sera were determined at 14 and 22 days after boost using a Se specific ELISA previously described (394). In brief, ELISA plates (Nunc MaxiSorp®, San Diego, CA) were coated overnight with 100µl per well of 0.25µg/ml soluble trimeric recombinant spike ectodomain protein. Plates were blocked with 200µl/well of phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.05% Tween 20. Individual chicken pre-challenge sera were diluted 1:100 in PBS and incubated in the wells for 30 minutes at room temperature. All following steps were performed with reagents of a commercial IBV ELISA kit (Idexx Laboratories Inc., Westbrook, ME) following the manufacturer's guidelines. Absorbance values were analyzed by ANOVA followed by Tukey post-test.

Results

Biological properties of the rLS/ArkSe.GMCSF virus. As shown in Table 1, the rLS/ArkSe.GMCSF virus replicated in chicken embryos but achieved slightly lower HA and EID_{50} titers compared to those of the LaSota strain. This result suggests that the relatively sizable foreign gene insertion (totally 3,840 nts, representing approximately 25% of the LaSota genome) likely influenced the virus' replicating ability. As reported previously, the recombinant virus showed low pathogenicity with a similar ICPI (0.18) to the LaSota virus (0.15) and higher MDT (>150 hrs) than the LaSota virus (134 hrs). The nucleotide sequence analysis revealed that the

rLS/ArkSe.GMCSF virus maintained its sequence fidelity after three passages in chicken embryos. The nucleotide sequences of the chicken GMCSF gene (381 nts), the IBV Se gene (3,459 nts), and the complete genome of rLS/ArkSe.GMCSF (19,812 nts) are available upon request.

Expression of IBV Ark Se and NDV HN proteins in cells infected with the rLS/ArkSe.GMCSF virus. The red, green, and blue fluorescence shown in Figure 1 represent the NDV HN protein, IBV S-ectodomain protein, and cell nuclei, respectively. The red fluorescence was observed in LS-infected cells, but no green fluorescence was detected in the same field of infected cells, demonstrating the antibodies' specificity. When examining rLS/ArkSe.GMCSF virus-infected DF-1 cells, both green and red fluorescence were observed, indicating expression of the IBV Se and NDV HN proteins. After merging fluorescent images that were taken in the same field, green and red fluorescence co-localized to the same infected cells. This result confirmed that the IBV Se protein was co-expressed with the NDV HN protein from the rLS/ArkSe.GMCSF virus-infected cells.

Experiment 1. The rLS/ArkSe.GMCSF vaccine was safe, as no adverse side effects were detected after vaccination in 7-day-old chickens. Both chicken groups receiving rLS showed NDV HI titers significantly higher than non-vaccinated controls (Figure 2). As seen in this figure, the group vaccinated with rLS not expressing GMCSF exhibited slightly higher average NDV HI titers compared to chickens vaccinated with rLS/ArkSe.GMCSF, but the difference did not achieve statistical significance.

The parameters used herein to evaluate protection in this experiment showed improved protection by rLS co-expressing the IBV Se and the GMCSF cytokine. Figure 3A shows mostly absence of respiratory signs in chickens vaccinated with rLS/ArkSe or rLS/ArkSe.GMCSF not differing significantly from non-vaccinated/non-challenged controls. However, vaccination with

rLS/ArkSe.GMCSF resulted in significantly lower (P<0.05) viral IBV RNA in the trachea after challenge (Figure 3B) compared to chickens vaccinated with rLS/ArkSe as well as non-vaccinated controls. Tracheal histomorphometry showed a trend consistent with tracheal viral load. Chickens vaccinated with either recombinant construct showed reduced tracheal lesions compared to non-vaccinated/challenged birds. However, the reduction of mucosal thickness and lymphocytic infiltration only achieved statistical significance (P<0.05) in chickens vaccinated with rLS/ArkSe.GMCSF (Figure 4).

Experiment 2. As seen in Figure 5, all vaccinated groups showed significantly lower respiratory signs, and viral loads in both tears and tracheas (P<0.05) than non-vaccinated controls after virulent Ark challenge. Although the viral loads in tears and tracheas were reduced in all vaccinated groups, the group primed with rLS/ArkSe.GMCSF and boosted with Mass achieved maximum reduction of Ark IBV RNA. These levels were significantly lower (P<0.05) than chickens vaccinated with Mass only and chickens primed with Mass and boosted with rLS/ArkSe.GMCSF. When comparing the two latter groups, chickens primed with Mass and boosted with rLS/ArkSe.GMCSF showed lower values than Mass-only vaccinated chickens but without achieving statistical significance. The results of tracheal histomorphometry and histopathology (Figure 6) showed prime and boost with rLS/ArkSe.GMCSF and Mass in either direction protected the tracheal mucosa more effectively than Mass alone against Ark challenge. Indeed, tracheal mucosal thickness and lymphocyte infiltration in chickens vaccinated with rLS/ArkSe.GMCSF and Mass were not significantly different (P<0.05) from non-vaccinated/non-challenged controls. In contrast, chickens vaccinated with Mass only were significantly different from these controls. In addition, chickens vaccinated only with Mass did not differ significantly from non-vaccinated/challenged controls.

Se antibody levels in prime/boost chickens. Antibody levels detected 14 days after booster vaccination were highest ($P<0.05$) in the group primed with rLS/ArkSe.GMCSF and boosted with Mass compared to prime and boost in the other direction as well as Mass vaccination only (Figure 7). Consistently, 22 days post-boost antibody levels were found to be significantly higher in the chickens primed with rLS/ArkSe.GMCSF and boosted with Mass compared to those primed with Mass and boosted with rLS/ArkSe.GMCSF. However, on day 22 antibody levels for the boosted groups were not significantly different when compared with chickens vaccinated with Mass only.

Discussion

The current results demonstrate that the recombinant LaSota vaccine construct expresses the IBV Se successfully in cell culture and is stable after passages in embryonated eggs. Corroborating previous results (394), although the replication rate is reduced compared to the parental LaSota strain, the low pathogenicity, as determined by both MDT and ICPI, allows safe vaccination of young chickens. Finally, the recombinant virus replicates well in chickens as it elicits antibody responses against NDV.

Breeder and layer hens are commonly vaccinated against NDV. In the southeastern U.S., some companies use up to four live NDV vaccines in broiler breeders until 16 weeks of age. In addition, some companies may vaccinate the progenies with recombinant HVT-NDV *in ovo* (Dr. J. Cline, Wayne Farms, personal communication). The presence of maternal NDV antibodies and/or antibodies resulting from active *in ovo* immunization may interfere with rLS vaccination in commercial settings. Indeed, others have shown that presence of NDV maternal antibodies in chickens at the time of vaccination with rNDV expressing avian influenza virus (AIV) proteins can prevent development of immunity from rNDV expressing the H5 of AIV (25). In addition, presence of maternal antibody to AIV has also been shown to interfere with active vaccination

with NDV expressing the AIV hemagglutinin (258). We presently lack information on use of rLS expressing the IBV spike glycoprotein in chickens of commercial origin. The levels of antibodies in broilers certainly vary around the world depending on NDV pressure of infection and NDV vaccination programs used. Thus, varying levels of immunity against NDV may allow or impede the vaccine to break through maternal immunity and replicate successfully at variable times after hatch. However, we presume that initial replication of the recombinant virus in the upper respiratory tract will induce mucosal immunity and protect the birds at the port of entrance of IBV. This presumption is based on the fact that vaccination with live IBV vaccines in commercial chickens with maternal antibodies to IBV, does induce an immune response that protects the birds against IBV challenge. In addition, use of better adjuvants should also have an effect on the effectiveness of recombinant NDV vaccines in commercial chickens.

Several studies have shown that use of rLS expressing IBV S or subunits of S induce less than optimal protection against IBV virulent challenge (1, 317, 351, 352). Thus, improved adjuvants may be needed to achieve optimum protection. As discussed above, others have reported that both co-administration of subunit vaccines with GMCSF and DNA vaccines with GMCSF show significant improved immunogenicity. Similarly, co-expression of foreign proteins and GMCSF as well as co-administration of GMCSF with recombinant virus constructs have also shown enhanced protection (10, 71, 109, 164, 271, 333, 396). However, having to inject such vaccines individually in large broiler populations limits their applicability for the poultry industry. In the current experiments, the successful replication of the rLS after administration via a mucosal route indicates that mass administration via spray is feasible. Based on viral load and tracheal histopathology, experiment 1 showed that co-expression of GMCSF and IBV spike-ectodomain from rLS enhances protection induced by this recombinant vaccine. This result corroborates the

results by others (discussed above) who have demonstrated enhanced protection when using GMCSF to accompany other vaccine settings. However, the level of effectiveness achieved by single vaccination with the dual recombinant vaccine still does meet effectiveness requirements of commercial vaccines.

IBV's extensive genotypic and phenotypic variability is the result of genetic diversity generated by mutations made by the viral RNA dependent RNA polymerase and by recombination events. The evolutionary process continues when abundant new variants serve as the material of selection (349). Thus, it has become clear that the use of live attenuated IBV vaccines may have solved the problem in the short term but has favored recombination events worsening the problem in the long term. In addition, because often more than one serotype is acting in a particular region, IBV prevention considers inclusion of various serotypes in vaccination programs. This practice augments the likelihood of recombination among diverse genotypes even more. Indeed, numerous outbreaks of disease are currently being caused by vaccine-like viruses and wild-vaccine recombinant viruses (177, 179). Mass-type live vaccine viruses, same as every other IB coronavirus, have the ability to recombine with other IBV types. After initial reports of protection conferred by Mass-type vaccines in the early 1960s, Mass-type vaccines were registered and licensed worldwide. During years thereafter, other type-specific live vaccines were developed to protect against regionally emergent serotypes. Pharmaceutical companies made efforts to sell these vaccines in other regions with variable success as countries became more careful at allowing the introduction of foreign IBV strains/genes into their industry. In fact, several countries still only allow Mass-type vaccines to be used. Thus, because Mass wild and vaccine viruses are endemic worldwide, a combination of live Mass with a recombinant vaccine virus that enhances its cross-protection provides an advancement towards a better solution to the problem. The viral load and

tracheal histomorphometry results indicate that use of rLS/ArkSe.GMCSF with Mass in a prime/boost vaccination regime enhances the cross-protection capability of Mass, as this combination more effectively protected chickens against Ark challenge. Interestingly, based on viral load and tracheal histopathology, better protection was achieved when priming was performed with rLS/ArkSe.GMCSF followed by Mass boosting. The inverse approach, i.e. prime with Mass and boost with rLS/ArkSe.GMCSF, provided similar protection of the tracheal epithelium but reduction of viral load was less effective. Higher levels of Ark Se specific antibodies were elicited by the former combination, which may explain those better results. However, the immunological mechanism behind this difference cannot be explained in the current study. We also analyzed antibody avidity of both groups but were not able to detect any differences (data not shown). Using Mass and replacing attenuated type-specific booster vaccines by tailored type-specific S-ectodomain expressed from recombinant virus provides an opportunity to achieve enhanced cross-protection, and equally important, should reduce potential recombination and emergence of new IBV variants.

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Table 1**:** Biological assessments of the rLS/ArkSe.GMCSF virus

Virus	MDT[a]	ICPI[b]	HA[c]	EID_{50}[d]
LaSota	134h	0.15	2^9	3.16×10^9
rLS/ArkSe.GMCSF	>150h	0.18	2^8	5.62×10^8

[a] Mean death time in embryonating eggs.

[b] Intracerebral pathogenicity index in 1-day-old chickens

[c] Hemagglutinating titer

[d] 50% embryo infective dose

Figure 1. Expression of IBV Se and NDV HN proteins by IFA

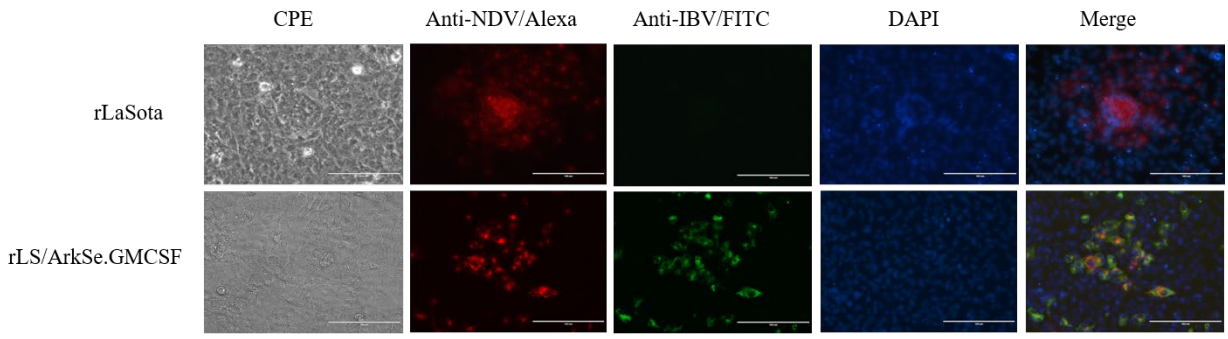

Fig. 1. Expression of IBV Se and NDV HN proteins by IFA. DF-1 cells were infected at 0.01 MOI with LS and rLS/ArkSe.GMCSF, respectively. At 24hrs post-infection, cells were fixed and stained with a mixture of chicken anti-Ark-type IBV serum and mouse anti-NDV HN Mab, followed by a mixture of FITC-labeled goat anti-chicken IgG and Alexa Fluor® 568 labeled goat anti-mouse IgG. Finally, the infected cells were stained with DAPI. Green, red and blue fluorescent images taken in the same field of virus-infected cells were merged into a single image. Bars represent 100µm in length.

Figure 2. NDV antibodies determined by hemagglutination inhibition (HI) assay

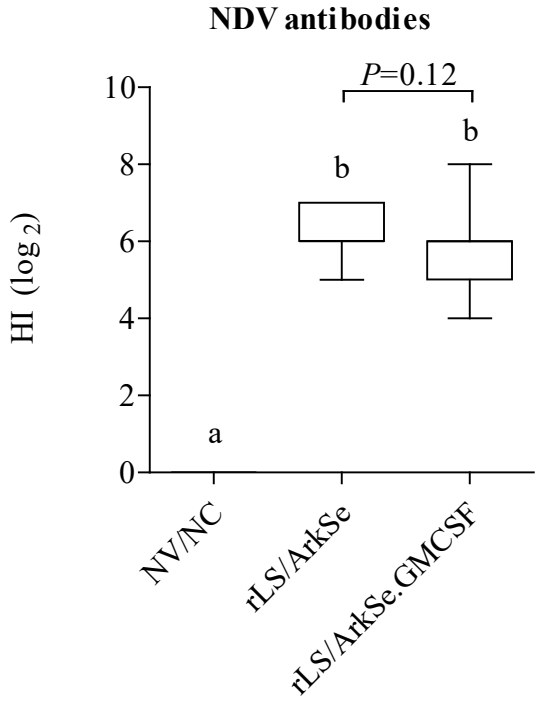

Fig 2. NDV antibodies determined by hemagglutination inhibition (HI) assay using chicken sera (n=19/group) collected 23 days after single vaccination with rLS/ArkSe or rLS/ArkSe.GMCSF. Data analyzed by ANOVA with Tukey's multiple comparison post-test. Different letters indicate significant differences at $P<0.05$. Exact P values between distinct groups determined by two-tailed *t*-tests.

Figure 3. Protection induced by single vaccination

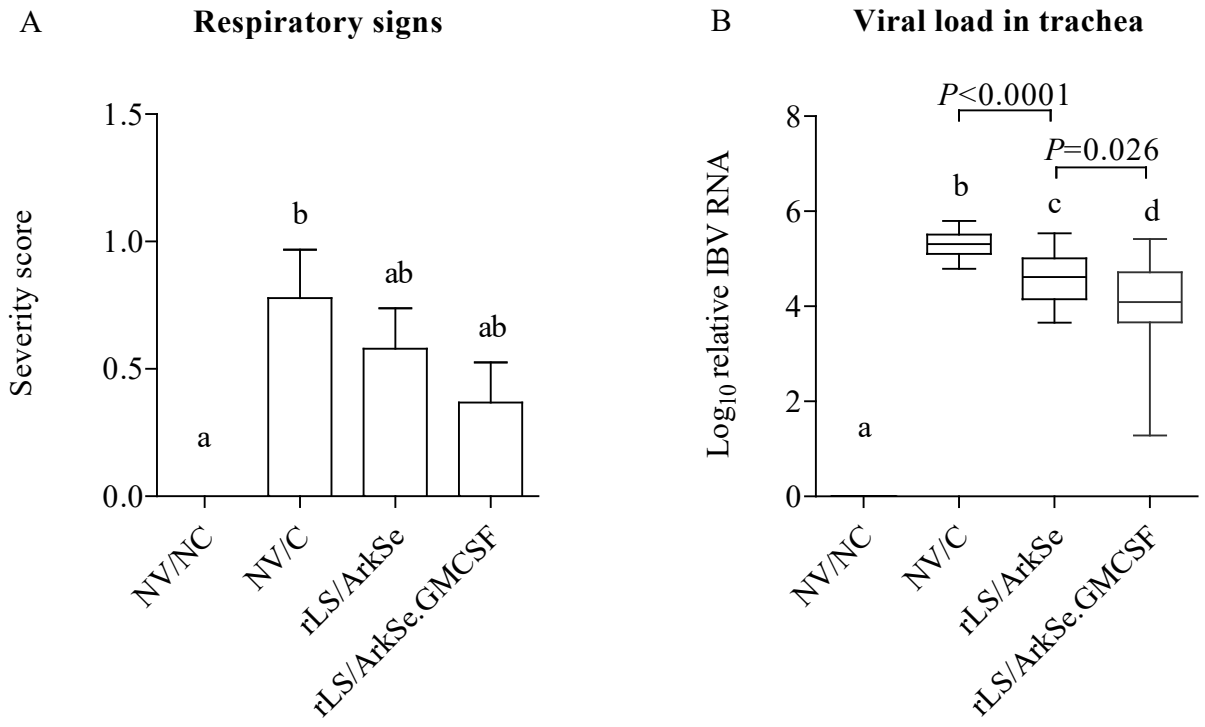

Fig. 3. Protection induced by single vaccination in chickens (n=19-20/group) at 7 days of age with 10^6 EID_{50}/bird of rLS/ArkSe or rLS/ArkSe.GMCSF. Chickens were challenged with 10^4 EID_{50}/bird of virulent Ark 24 days post-vaccination and respiratory signs and viral load (relative IBV RNA) determined 5 days after challenge. NV/NC = non-vaccinated/non-challenged; NV/C = NC/challenged controls. (A) Respiratory sign severity scores; means and SEM are shown for each group. Data analyzed by Kruskal-Wallis test and Dunn posttest. (B) Relative IBV RNA determined by quantitative RT-PCR in tracheas. Data analyzed by ANOVA and Tukey posttest (boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum). Different letters indicate significant differences at $P<0.05$. Exact P values between distinct groups determined by two-tailed t-tests.

Figure 4. Tracheal histomorphometry and histopathology

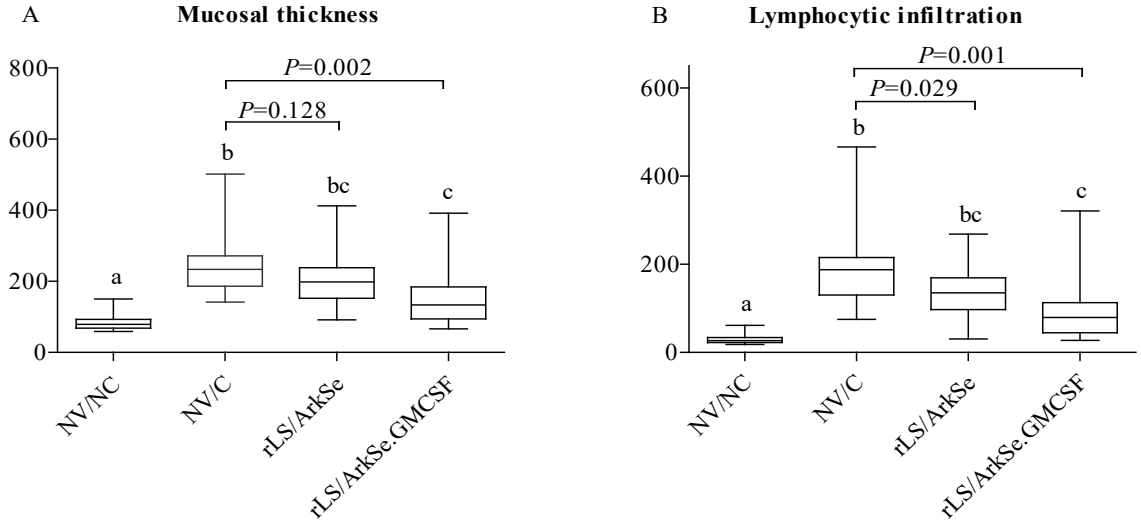

Fig 4. Tracheal histomorphometry and histopathology of chickens treated as described in legend of Fig. 2. NV/UC = non-vaccinated/non-challenged and NV/C = non-vaccinated/challenged controls. Tracheal histomorphometry (A) mucosal thickness and (B) lymphocytic infiltration presented in arbitrary units using ImageJ. Values analyzed by ANOVA and Tukey posttest (boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum). Different letters indicate significant differences at $P<0.05$. Exact P values between distinct groups determined by two-tailed *t*-tests.

Figure 5. Protection induced by prime and booster vaccination

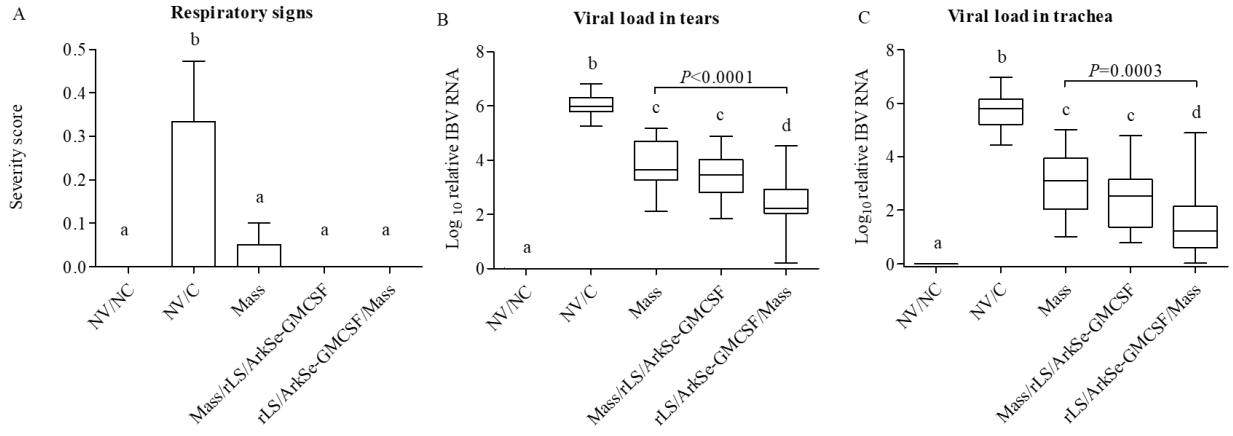

Fig 5. Protection induced in chickens (n=20/group) by prime and booster vaccination regime with rLS/ArkSe.GMCSF and a commercial live-attenuated Massachusetts (Mass)-type vaccine. Chickens were prime vaccinated at 1 day of age with either 10^6 EID_{50}/bird of rLS/Ark.Se.GM-CSF or a commercial live Mass vaccine at recommended dose. Booster vaccination was performed at 14 days of age. Mass/rLSArkSe.GMCSF = primed with Mass and boosted with recombinant virus. rLSArkSe.GMCSF/Mass = primed with recombinant virus and boosted with Mass. Controls include a group vaccinated with Mass only as well as NV/NC = non-vaccinated/non-challenged and NV/C = non-vaccinated/challenged. Challenge performed ocularly at 16 days post-boost with 10^4 EID_{50}/bird of virulent Ark. Statistical analysis of respiratory signs and relative IBV RNA levels 5 days after challenge performed as described in the legend of figure 3. Different letters indicate significant differences at $P<0.05$. Exact P values between distinct groups determined by two-tailed t-tests.

Figure 6. Tracheal histomorphometry and histopathology

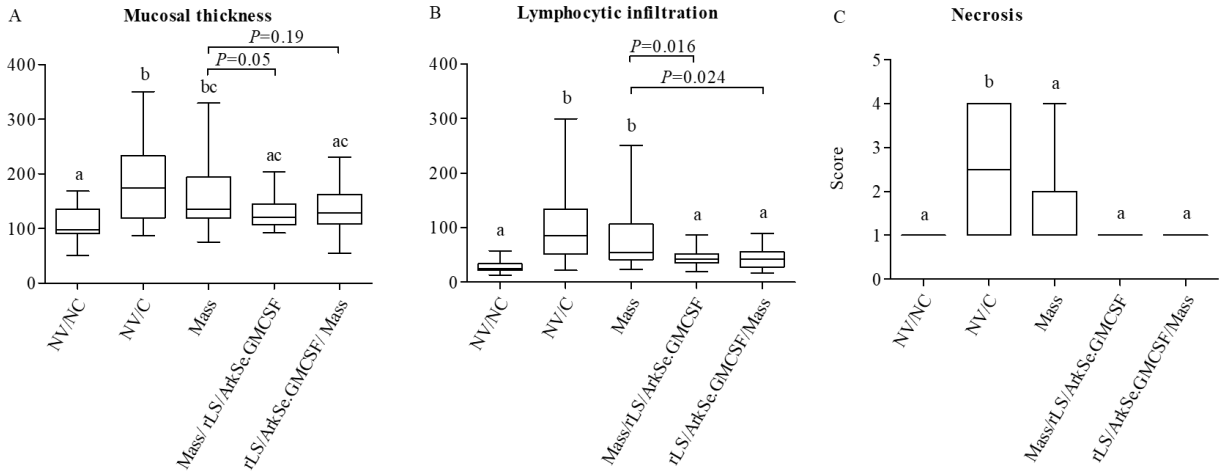

Fig 6. Tracheal histomorphometry and histopathology of chickens treated as described in legend of Fig. 5. Tracheal histomorphometry: (A) mucosal thickness and (B) lymphocytic infiltration presented in arbitrary units using ImageJ were analyzed by ANOVA and Tukey posttest. (C) Necrosis scores analyzed by Kruskal-Wallis test and Dunn posttest. NV/NC = non-vaccinated/non-challenged and NV/C = non-vaccinated/challenged controls. Boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum. Different letters indicate significant differences at $P<0.05$. Exact P values between distinct groups determined by two-tailed t-tests.

Figure 7. Ark Se antibody levels

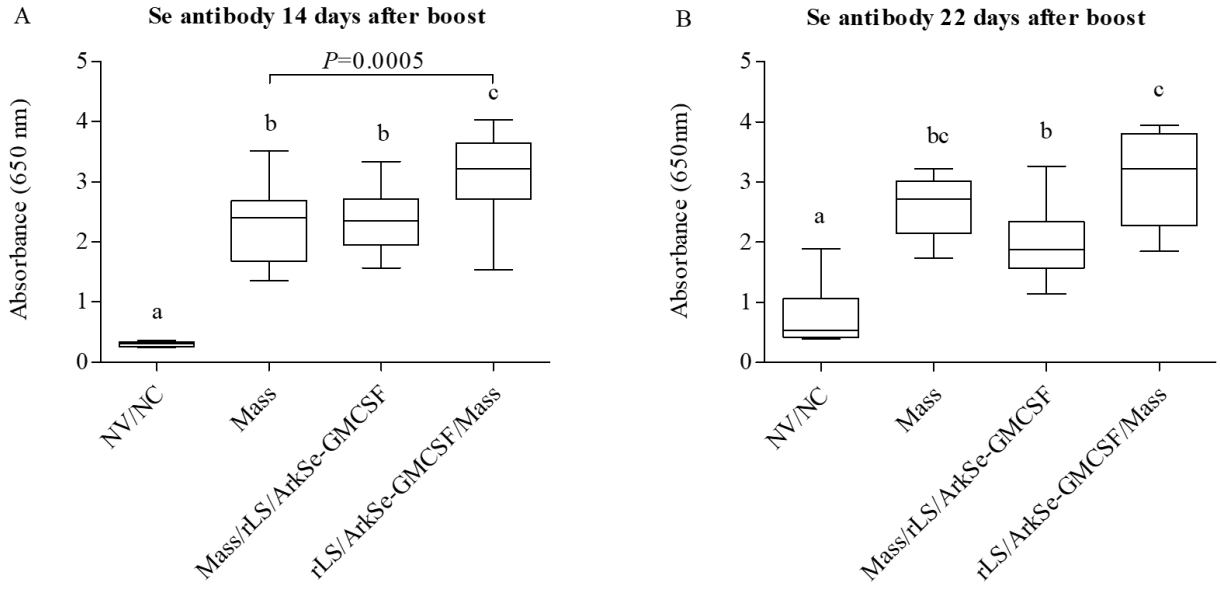

Fig 7. Ark Se antibody in chickens treated as described in the legend of Fig. 5. Ark Se antibody determined by ELISA using recombinant Ark Se protein coated plates. Data analyzed by ANOVA and Tukey posttest. Se antibody measured (A) 14 and (B) 22 -days post boost. NV/NC = non-vaccinated/non-challenged controls. Different letters indicate significant differences at $P<0.05$.