*IN OVO* IMMUNIZATION OF CHICKENS FOR NEWCASTLE DISEASE USING RECOMBINANT NEWCASTLE DISEASE VIRUSES

By

VALERIE CRISTINA MARCANO GOMEZ

(Under the Direction of Claudio Afonso and Robert Gogal, Jr.)

ABSTRACT

*In ovo* vaccination has been used commercially for over 20 years to control avian diseases due to its significant advantages over conventional vaccination. The advantages of *in ovo* vaccination include a significant reduction in costs, induction of earlier immunity, uniform vaccine delivery, and reduction of stress. *In ovo* vaccination programs currently available for Newcastle disease virus (NDV) have limitations, including interference by maternal antibodies (MatAbs) and the inability to induce protective immunity until four weeks post vaccination. Current biosecurity measures and vaccination protocols have been unable to stop the occurrence of outbreaks with virulent vNDV around the world particularly in developing countries. Thus, it is imperative to develop new effective vaccines able to overcome the maternal antibody inhibition and confer the earliest protection against vNDV. The poultry industry needs vaccines able to boost both cellular and humoral immune response and to achieve clearance of the challenge virus without compromising the flock's health.

In a first set of experiments, attenuated recombinant NDV vaccines containing either an antisense chicken IL-4 insert (ZJ1-L-IL4R), IL-2 expressing variant (-IL2), IL-10 expressing variant (-IL-10) or IFNγ expressing variant (-IFNγ) were tested as vaccine candidates against homologous challenge with a genotype VIId NDV. Results show that strain ZJ1-L-IL4R may serve as a viable vaccine candidate for *in ovo* vaccination against

NDV, as it compared favorably to the commercial strains regarding protection to challenge, yet displayed lower mortality rates compared with the *in ovo* live vaccine candidates for NDV.

In another set of experiments, we evaluated the modulatory effects of an attenuated recombinant Newcastle disease virus vaccine containing an antisense chicken interleukin 4 insert (IL4R) on the chicken adaptive immune response. Our results did not confirm that the improvement in survival and body weight seen in birds vaccinated with ZJ1-L-IL4R compared to ZJ1-L is due actions by the antisense RNA.

**INDEX WORDS:**

Avian paramyxovirus-1, APMV-1, Newcastle disease virus, NDV, In Ovo vaccines, Vaccine, Poultry, Immunohistochemistry, Recombinant vaccines, Antisense RNA, cytokines, IL2, IL10, IFNγ

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# CHAPTER 1

## INTRODUCTION

Newcastle Disease (ND) continues to be a major threat to the poultry industry world-wide. Current biosecurity measures and vaccination protocols have been unable to stop the occurrence of outbreaks with virulent Newcastle disease virus (vNDV) around the world particularly in developing countries [1-6]. It is imperative to develop an effective vaccine that is able to overcome maternal antibody inhibition and confer the earliest protection against vNDV. The poultry industry is in need of vaccines that are able to boost both cellular and humoral immunity, achieve clearance of the challenge virus and therefore, reduce horizontal transmission within flocks without compromising the flock's health.

Over 270 avian and non-avian species are affected by NDV, with chickens and turkeys being highly susceptible to disease and ducks and geese often showing few or no clinical signs [7, 8]. The causative agents, vNDV, belongs to the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genera *Avulavirus*, and species *avian paramyxovirus* (*APMV*), where it is synonymous with APMV serotype 1 (APMV1) [9]. The virus has a single stranded, non-segmented, negative sense RNA genome and is enveloped with a helical capsid. It has a genome with approximately 15186 nucleotides that translate into 6 structural proteins. From 3' to 5' these proteins are the nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin- neuraminidase (HN) and the RNA directed RNA polymerase (L).

There are four pathotype groups of NDV: velogenic, mesogenic, lentogenic, and asymptomatic enteric [7, 8]. Velogenic NDV (vNDV) strains cause acute and often lethal disease. There are two forms of vNDV, viscerotropic vNDV strains and neurotropic vNDV

strains. Doyle's form or viscerotropic vNDV strains cause gastrointestinal hemorrhagic lesions and neurological signs. Beach's form or neurotropic vNDV strains cause neurological signs and some respiratory signs without gastrointestinal lesions. Beaudette or mesogenic NDV strains are less pathogenic forms of neurotrophic vNDV. Mesogenic NDV strains can also cause neurological disease, but death is only seen in young birds. Hitchner's form or lentogenic NDV causes mild or unapparent respiratory infections. Asymptomatic enteric NDV is not thought to cause or contribute to clinical disease [7, 8].

Newcastle disease virus has been eradicated from commercial poultry in developed countries such as the United States, however it remains endemic in many developing countries. Vaccination programs against NDV include the use of recombinant, live attenuated and killed vaccines. Inactivated vaccines are given intramuscularly, while live attenuated vaccines are given through eye inoculation or through IM injection or mass applied in the water or as aerosol [10]. In the US, where ND is not endemic, broiler breeders receive at least three attenuated live lentogenic vaccines during their lifetime, broiler birds receive one vaccine at day of age and may or may not receive vaccine boosts and layers receive an inactivated vaccine and may receive up to 3 additional live vaccines and other boosts [8, 10, 11]. In countries where ND is endemic, vaccination protocols are much more rigorous, and the vaccines used are often more virulent, for example, they may use mesogenic strains instead of lentogenic ones [5, 12]. In some countries such as Israel, NDV vaccination is mandatory but still outbreaks continue to occur [13]. Despite these extensive vaccination programs, there are continuous outbreaks in these countries and many others including India [1], China [14], Pakistan [3], and Libya [4].

In the present research work we evaluated recombinant NDV (rNDV) vaccines as *in ovo* vaccine candidates. *In ovo* vaccination, or administration of the vaccine to the egg prior to hatching, has been explored as an option to confer early protection. *In ovo* vaccination has been used commercially for over 20 years to control avian diseases due

to its significant advantages over conventional vaccination. The advantages of *in ovo* vaccination include a significant reduction in costs, induction of earlier immunity, uniform delivery of vaccine, and reduction of stress [15, 16].

The recombinant NDV strains used in this study contain either an antisense chicken interleukin 4 or sense interleukins 2 or 10, or interferon gamma inserts in their genome. Cytokines can be used to enhance the protection induced by vaccines to create a stronger, faster and more specific immune response [17-19]. Cytokines such as interferon gamma (IFNγ) can directly mediate effector function, killing a variety of intracellular pathogens while others such as interleukin-2 (IL-2), IL-7, and IL-15 enhance effector and memory T-lymphocyte responses, which play a critical role in protection against intracellular pathogens [19, 20].

Antisense RNA transcripts are non-coding RNAs that are complementary to those of a sense RNA transcript that may or may not code a protein [21]. Antisense can affect transcription of the sense RNA through transcriptional interference [21]. It has been reported that antisense RNA can cause transcriptional interference through several mechanisms, thereby modulating gene expression by reducing it or completely silencing it [21-24]. Antisense vaccines have the potential to modulate the immune response within specific cells, limiting their negative impact. While cytokines such as IFNγ may have systemic impacts, a live attenuated NDV vaccine containing an antisense insert may have a subtler impact, as its effect is limited to the cell types infected with the virus. While no literature was found on the use of a recombinant antisense vaccine to protect against avian pathogens, antisense technology has proven successful in tumor and viral vaccines. Intracranial delivery of tumor vaccine and antisense TGF-β2 oligonucleotides to mice with brain tumors significantly enhanced their survival [25]. In human patients with non-small cell lung cancer, intradermal immunization with an TGF-β2 antisense tumor cell vaccine improves patient outcome [26]. A vaccine for foot and mouth disease containing a sense

protein of the virus in conjunction with an antisense sequence targeting the 5' untranslated region of the virus successfully protected cells against foot and mouth disease infection and improved survival of mice challenged against the disease [27]. These studies successfully used antisense technology to improve disease outcome however, they did not use live attenuated virus to simultaneously vaccinate and direct the immune response. This avenue should be further explored to improve vaccine and challenge outcomes against NDV.

In the first set of experiments, we evaluated the effect of dose and vaccination time on survival after vaccination, and protection after challenge. This was accomplished by vaccinating embryonated chicken eggs at different times *in ovo* with different doses of rNDV vaccines containing an antisense chicken interleukin 4, sense interleukins 2 or 10, or interferon gamma and tested them as vaccine candidates against challenge against homologous challenge with a genotype VIId NDV. In the second of experiments, we evaluated the effect of vaccination with an antisense chicken interleukin 4 on the development of acquired immunity by vaccinating embryonated chicken eggs with rNDV vaccines containing an antisense chicken interleukin 4.

Here we hypothesized that 1) vaccination *in ovo* with rNDV does not negatively impact hatchability or survival after hatch, 2) vaccination *in ovo* with rNDV will improve survival after vNDV challenge compared to non-vaccinated birds without negatively impacting survival compared to parental viral strains, and 3) rNDV containing antisense chicken interleukin 4 will improve vaccine efficacy by modulating adaptive immunity toward towards a predominantly cell-mediated response by inhibiting IL-4 production.

These hypotheses were evaluated through a series of vaccine trials in which we evaluated survival after vaccination, survival after challenge, differences in average body weight and humoral and cellular response to vaccination through immunological assays.

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# CHAPTER 2

# LITERATURE REVIEW

## *2.1. Newcastle Disease Virus*

Newcastle disease virus (NDV), recently renamed Avian Avulavirus 1, infects over 270 avian and non-avian species with chickens and turkeys being highly susceptible to disease and ducks and geese often showing few or no clinical signs [7, 8]. Virulent strains of NDV cause Newcastle Disease. The virus belongs to the from the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genera *Avulavirus*, and species *avian paramyxovirus* (*APMV*). There are 12 APMV serotypes and of these, NDV is synonymous with APMV serotype 1 (APMV1) [9].

NDV is a single stranded, non-segmented, negative sense, enveloped RNA virus with helical capsid symmetry. It has a genome with approximately 15186 nucleotides that translate into 6 structural proteins. From 3' to 5' these proteins are the nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin- neuraminidase (HN) and the RNA directed RNA polymerase (L).

### *2.1.1 NDV Pathotypes*

There are four pathotype groups of NDV: velogenic, mesogenic, lentogenic, and asymptomatic enteric [7, 8]. Velogenic NDV (vNDV) strains cause acute and often lethal disease. There are two forms of vNDV, viscerotropic velogenic NDV (vvNDV) and neurotropic velogenic NDV (nvNDV). Doyle's form or vvNDV cause gastrointestinal hemorrhagic lesions and neurological signs. Beaudette or mesogenic NDV (mNDV) are less pathogenic forms of nvNDV. mNDV also causes neurological disease, but death is only seen in young birds. Hitchner's form or lentogenic NDV (lNDV) causes mild or

unapparent respiratory infections. Asymptomatic enteric NDV is not thought to cause or contribute to clinical disease [7, 8].

Human infection with NDV can lead to self-limiting unilateral or bilateral conjunctivitis. Infection can result in ocular reddening, excessive lacrimation, edema of the eyelids, conjunctivitis and subconjunctival hemorrhage. People at risk are those who work closely with the virus or with poultry. These individuals can acquire the infection from direct contact with the virus on infected birds or vaccines through splashing and rubbing [7].

### *2.1.2 NDV Pathotyping and Classification*

The cleavability of the F protein has been directly correlated with virulence, although other factors are also involved. The F protein is first produced as the F0 precursor. F0 must be cleaved by host proteases into F1 and F2 for viral infection to occur. In strains of low virulence, F0 can only be cleaved by epithelia specific host enzymes. However, in virulent strains F0 can be cleaved by ubiquitous host proteases. Therefore, virulent velogenic viruses spread systemically throughout the host's body, with systemic infection usually being fatal. Lentogenic (low virulence) viruses, on the other hand, are restricted to the respiratory and intestinal tracts where the appropriate proteases it needs for cleavage are present [7, 8].

Strain classifications can be done by using virus neutralization (VN), agar gel diffusion assays, monoclonal antibodies or cross hemagglutination inhibition assays to determine antigenicity [7, 8]. Immunogenicity tests, molecular analysis, pathogenicity tests using intracerebral pathogenicity index ICPI, mean death times (MDTs) and plaque assays can also be performed to classify different NDV strains. The virus replicates readily in embryonated chicken eggs –which can be used to isolate and propagate it, and in many cell lines, where syncytia formation can be observed as the main cytopathic effect [7, 8].

The World Organization for Animal Health (OIE) has adopted intracerebral pathogenicity index (ICPI) as the *in vivo* test to define virulence [8]. By OIE Terrestrial Manual standards Newcastle disease is defined as an infection of birds caused by a virus that meets one of the following criteria: (1) it has an ICPI of 0.7 or higher OR (2) it has multiple basic amino acids at the c-terminus of the F2 proteins, at genomic positions 113, 115, and 116 and a phenylalanine at position 117, which is the N terminus of the F 1 protein.

### *2.1.3. NDV Transmission and Replication*

Natural routes of NDV infection include the nasal, oral, and ocular routes, although the virus has also been found to infect the host when inoculated through the intramuscular (IM), intravenous (IV) and intracerebral (IC) routes. Birds may also become infected by inhaling or ingesting contaminated dust or aerosolized virus [8]. After transmission, its replication begins with HN binding receptors on host cell membranes. The fusion protein then aids in fusion of the viral and cell membranes, after which the nucleocapsid complex enters the host cell's cytoplasm, where it replicates. RNA directed RNA polymerase produces the complementary positive sense strand of genome. Host cell's mechanism is then used to translate the positive strand into protein and viral genome.

The HN receptors of NDV bind the sialic acid receptors on the surface of RBCs. This allows for agglutination of red blood cells (RBCs) and can be used as a diagnostic tool in hemagglutination assays. The HN protein can also degrade sialic acid receptors, preventing self-attachment of viral particles and clumping. This is called neuraminidase activity and can also be used in diagnostics. Finally, NDV causes hemolysis of RBCs and pH independent fusion of cells. When the viral membrane fuses with the host cell membrane following receptor binding, neighboring cells may also fuse, and multi-nucleated cells or syncytia are formed.

*2.1.4. NDV Pathology and Clinical Disease*

Factors affecting disease with NDV include host species, host age, host immune status, coinfections, environmental stress, social stress, route of exposure, virus strain and virus dose. Infection routes may enhance clinical disease symptoms. In poultry, natural routes of infection (nasal, oral and ocular routes) enhance respiratory disease, whereas IM, IV, and IC routes enhance neurologic signs [7, 8, 28]. Non-vaccinated birds are susceptible to disease caused by velogenic strains of NDV. Mesogenic strains can cause mild disease in adult and young non-immunized birds. Lentogenic strains of NDV only cause disease in young non-immunized birds in the absence of maternal antibodies (further discussed in the immunity and vaccinations sections of this chapter).

NDV infects hundreds of bird species; however, most of the current literature focuses on poultry. Gross lesions and organs affected depend on the strain and pathotype of the virus and several host factors. These host factors include age and vaccination status, with younger animals having more severe clinical signs and vaccinated commercial poultry often lacking lesions [8]. The route of infection can also affect disease presentation; intravenous inoculation is more likely to elicit neurologic signs, and aerosolization of high viral doses tends to impact the upper respiratory tract preferentially [8, 28].

Velogenic viscerotropic Newcastle disease (vvND) can cause up to 100% mortality in chicken flocks. Early clinical signs seen in birds infected with vvNDV strains include conjunctival swelling, reddening of the lower eyelid, anorexia, weakness, tremors, ruffled plumage, prostration, and diarrhea [29]. In vvNDV, birds will often appear listless, depressed, weak, and have increased respiration, ending in prostration and death [7-9, 28-30]. Other clinical signs are severe respiratory signs, severe edema around the eyelids and the head, and green diarrhea (2-6). Typically, these birds will have a clear mucoid

discharge from their mouths [8]. The hallmark of vvNDV infection is the induction of intestinal hemorrhages and lymphoid depletion destruction in the eyelid, laryngeal tonsils, cecal tonsils, Peyer's patches, spleen, thymus, and bursa of infected birds [7-9, 28-30]. The hemorrhage and necrosis of mucosal-associated lymphoid tissue can be due to infection or to changes in the vascular system. Multifocal intestinal hemorrhages, multifocal necrosis and/or ulceration of the gut-associated lymphoid tissue, and disseminated foci of necrosis in the spleen are highly suggestive of vvNDV infection [29].

In chickens infected with vvNDV, there is severe necrosis of the lymphoid tissues throughout the body. Lymphoid depletion is most marked in spleen and gut-associated lymphoid tissue [30]. Macrophage infiltration is also common in lymphoid tissue during the later stages of disease [30]. In animals inoculated via the conjunctival route, eyelid edema and hemorrhage may develop [7, 30]. Multifocal hemorrhage and ulceration can be observed in the junction between proventriculus and gizzard [8, 29]. Hemorrhage, edema and necrosis of the cecal tonsils are also often observed [29, 30]. Necrosis in the laryngeal tonsils may lead to hemorrhage within the cranial portion of the trachea [8]. Severe atrophy of thymus and the bursa of Fabricious (bursa) are also typically observed [8, 9, 28, 29]. Perithymic hemorrhage and necrosis of the cortex of the thymus are commonly seen. Interestingly, the medulla of the thymus usually has less severe lymphoid depletion whereas in the bursa, severe cortical and medullary lymphocyte depletion is observed [7, 29]. The spleen may appear enlarged, congested and severely mottled, with multiple necrosis foci [7-9, 28-30]. During vvNDV infection, inflammatory infiltrate to the bursa, spleen and intestinal lymphoid tissue is mainly composed of heterophils and macrophages [29, 30]. Hyperplasia of macrophages with large vacuolated cytoplasm has also been observed [29].

Lesion formation in other organ systems of poultry infected with ND are highly variable. Regardless of NDV pathotype, no gross lesions are seen in the central nervous

system (CNS). Changes in the brain are minimal with perivascular cuffing occasionally described [30]. All NDV pathotypes have the potential to induce myocardial damage [4, 6], with necrotic myocarditis commonly found [8]. Vascular changes include hydropic degeneration of the media, hyalinization, and development of hyaline thrombosis [7, 8, 29]. Comb and/or wattle edema, congestion and petechia can be observed, along with hemorrhage and ulceration of the skin [29] . Multifocal necrosis can be observed in the pancreas, liver, and gall bladder [8, 30]. In the respiratory tract, mucosal hemorrhage and marked tracheal congestion can be seen. Tracheal hemorrhage and necrosis is limited to the lymphoid tissue at the upper pharynx. The lungs may also be affected, with marked congestion and edema of the parabronchi. Hemorrhage and erythrophagocytosis in alveolar areas of parabronchi may be seen [7, 8]. In the reproductive system, the greatest damage is seen in the uterus and shell forming portions of the oviduct. If chickens and turkeys are infected in lay, yolk peritonitis with macrophage infiltration may be observed. Flaccid and degenerative ovarian follicles with inflammatory cell infiltration will be seen [7, 8]. Lymphoid aggregates, hemorrhage and discoloration of other reproductive organs may also be present.

Velogenic neurotropic Newcastle disease (vnNDV) causes morbidity that often affects 100% of the flock, and mortality is usually around 50%, but can be much higher in young birds [7]. In poultry, nvNDV causes severe neurological signs. Birds may develop muscular tremors, torticollis, and paralysis of the legs and/or wings but not diarrhea [8, 9, 28-30]. The birds are often bright and alert, and death is usually associated with the bird's inability to reach food and water. Gross lesions are often absent although splenic or proventricular congestion may be seen in animals euthanized early [29]. Like vvNDV, there are no gross CNS lesions present. Histopathologic changes are restricted to the CNS. These changes consist of multifocal mononuclear perivascular cuffing, associated

with hypertrophy/hyperplasia of vascular endothelium, moderate gliosis, and multifocal necrosis of the Purkinje cells, mostly in the cerebellum [7-9, 28, 29]. Lymphoid depletion and myocarditis have also been reported.

Mesogenic Newcastle disease (mNDV) causes mild clinical disease such as, some respiratory signs, a slight drop in egg production and misshapen eggs [7, 29]. More severe morbidity can be caused by concurrent viral and secondary bacterial infections. Histiocytic airsacculitis can occur due to secondary bacterial infection. In rare occasions, neurologic signs, similar to those seen with vnND, but much milder may be observed. Gross lesions are minimal, with mild splenomegaly and some conjunctivitis observed when inoculated via the eye-drop route. Histologically, nonsuppurative encephalitis, myocarditis, and splenic and pancreatic necrosis can be observed [29]. These strains are used as vaccine strains in countries where NDV is endemic (further discussed in the vaccination section of this chapter).

Lentogenic Newcastle disease virus (lNDV) does not cause disease in adult chickens, and is often isolated from waterfowl, pet birds and live bird markets around the world [31, 32]. Young birds may develop respiratory disease, with mild signs such as histiocytic airsacculitis, if a secondary bacterial infection develops. There will be minimal to no gross lesions. Mild pulmonary hemorrhages and splenomegaly might be seen grossly. Histologically, hyperplasia of the lymphoid follicles in spleen and air sacs and lymphoid follicle proliferation in trachea may be seen [29]. These strains are commonly used as vaccine strains (further discussed in the immunity and vaccination sections of this chapter).

Enteric asymptomatic NDV (lNDV) strains replicate within the gastrointestinal tract without causing any sings of disease [8, 29].

## 2.2 Avian Immune system

The immune system acts to guard the host organisms against injury. This protection includes defense against microbial infection, tissue necrosis, tumors, trauma, and invasion by allergens and other toxins. This brief and broad review will focus on the immune response to microbial infection, specifically by viruses. The immune system is broadly divided into innate and adaptive immunity. Innate immunity is the earliest to respond during pathogen infection however, it possesses no memory. Activation of innate immunity leads to the production and release of immune molecules that ultimately lead to activation and modulation of adaptive immunity. Adaptive immunity takes longer to respond however, it has memory, which allows it to become more specific with additional exposure to the same pathogen.

### 2.2.1 Chicken Innate Immunity

Ciliated and non-ciliated epithelial and mucosal surfaces form a physical barrier, the host's first line of defense against pathogens. The cells at these surfaces produce mucus and antimicrobial compounds such as defensins and sustain commensal organisms (normal microflora), which provide an extra layer of protection through competitive exclusion [33]. As physical barriers are breached by pathogens, immune and non-immune cells of the innate immune system recognize signs of invasion. Non-immune cells such as epithelial cells send signals to activate innate immunity and local sentinel phagocytic immune cells become activated. Immune and non-immune cells release immune factors such as cytokines that travel through the circulation recruiting other immune cells, and cause vasodilation. Vasodilation increases permeability, allowing for the delivery of these immune cells and factors. In short, the host's defense mechanisms are activated and inflammation, consisting of heat, redness, swelling, pain and loss of function may ensue [33].

Acute Phase Proteins

Inflammation leads to an acute-phase response along with other metabolic, physical and chemical changes within the body. Inflammation triggers the release of acute phase proteins (APP), non-specific molecules released into the host's circulation in response to infection and other stressors [33-35]. Some of these molecules act to promote inflammation while others act as anti-inflammatory molecules. These APPs include fibrinogen, ceruloplasmin, serum amyloid A (SAA), C-reactive protein (CRP), and haptoglobin (Hp), and mannan-binding lectin (MBL) [33-35].

Fibrinogen activates of neutrophils and enhances their cytotoxicity and phagocytotic functions [34, 36]. Ceruloplasmin acts as a superoxide scavenger during neutrophil activation [34]. Both fibrinogen and ceruloplasmin are elevated in poultry serum after intraperitoneal inoculation with E. *coli* and oral inoculation with E. *tenella* [37]. The APPs fibrinogen and ceruloplasmin were measured at 4 and 8 days after inoculation with E. *Coli* and E. *tenella*, respectively. There was 4 times more fibrinogen and 2.5 times more ceruloplasmin production in birds infected with E. *coli* compared to controls. Birds infected with E. *tenella* produced double the amount of both serum fibrinogen and ceruloplasmin compared to the controls. Interestingly, chickens suffering from mild and severe infection with infectious bursal disease virus (IBDV) exhibit a significant decrease in ceruloplasmin levels that correlates with the severity of the disease, and fibrinogen levels do not change based on disease status or severity of IBDV-related disease [38].

Mammalian CRP has been described to aid in protection against infection and regulation of the inflammatory response [33-35]. Serum CRP of 21-day-old chickens has been shown to increase within 48 h after infection with IBV [39]. Mammalian SAA aids in the prevention of oxidative tissue damage, in the recruitment of immune cells to the site of infection and in the inhibition of inflammation [33, 34, 40]. Significant increases in serum SAA have been observed in chickens referred to the avian diseases clinic at Shiraz

University during IBV and IBDV field outbreaks [38, 41]. The serum SAA levels of birds infected in the field with IBV and IBDV were 1.5 and 2 times higher respectively, compared to the serum SAA levels of healthy birds. Serum SAA levels of chickens experimentally infected with IBV at 21 days of age were 2.2 times higher by 2 days post infection, compared to the levels observed prior to IBV infection [39].

The main function of Hp is to reduce oxidative damage to tissues by binding hemoglobin [34, 42]. The chicken Hp counterpart is PIT54 and was identified through biochemical analysis, rather than genomic similarities with mammalian Hp [43, 44]. It acts as an antioxidant and becomes elevated during pathogenic infections, such as infection with IBV and E. *coli* [37, 39, 41, 44]. Intraperitoneal infection of 16-day-old chickens with E. *coli* leads to a significant increase in Hb serum levels [37]. In the same study, oral inoculation of 12-day-old chickens with E. *tenella*, did not lead to a significant increase although Hb levels trended upwards [37]. Inoculation of 21-day-old chickens with IBV leads to a 2 fold increase in serum Hb by two days post infection [39]. In separate study, 24-day old chickens that had become infected with IBV were referred to avian diseases clinic at Shiraz University. Serum Hp was measured and found to be significantly higher than that of age matched clinically healthy 24-day-old chickens of the same flock from 0.09 in healthy animals to 0.12 g/l diseased ones [41]. In birds infected in the field with IBDV however, no differences in Hp production were observed [38].

MBL activates complement and inhibits viral replication of IBV [45, 46]. Complement activation and serum MBL are directly associated [47]. In chickens, MBL has been shown to increase 2-3-fold during experimental infection with infectious laryngotracheitis virus (ILTV), IBDV, and IBV [47, 48]. Chickens of different lines and haplotypes were inoculated with IBDV at 21 days of age and while no differences were observed based on genetics, a 2-fold increase in MBL production was observed in chickens infected with IBDV compared to those that were not infected [48]. Similarly, 7-week-old chickens were

infected intratracheally with IBV or two different strains of ILTV and significant increases in serum MBL were observed at 3 days post inoculation [49]. Chicken lines with lower serum base levels of MBL have higher disease severity associated with IBV, *Pasteurella multocida* and E. *coli* infection [46, 50, 51]

The Complement System and Pattern Recognition Receptors (PRR)

The complement system enhances cell phagocytic activity, B and T cells responses and cytolysis of target cells [33]. Three different pathways of complement activation have been described, the classical, lectin and alternative pathways, all leading to the activation of complement factor C3 [33]. Ultimately, C3 is converted into C3a and C3b. The C3a component leads to increase in inflammation while C3b leads to enhancement of phagocytosis and increased inflammation [33, 52, 53]. The classical complement pathway is activated by antibody-bound antigen, the lectin complement pathway is activated by binding of lectins such as MBL to pathogen associated molecular pattern (PAMPs) and the alternative complement pathway is activated by microbial surfaces [33].

Immune cells express pattern recognition receptors (PRR) that recognize PAMPs molecules present in microbes. Examples of PRRs include soluble and cell-bound molecules such as toll like receptors (TLRs), scavenger receptors, lipopolysaccharide (LPS) receptors and complement receptors, which are expressed in many cell types [33]. Although there is no memory, these germline- coded molecules have some specificity. For example, different TLRs recognize different aspects of foreign molecules. Double stranded RNA is recognized by TLR3, while single stranded RNA is recognized by TLR7 [33, 54-57].

Binding of foreign pathogens to these receptors leads to the production and release of anti-microbial and signaling molecules such as cytokines and chemokines. Both immune and non-immune cells will release these molecules, which travel through the circulation

recruiting other immune cells, and vasodilation occurs to increase permeability to those tissues to increase delivery of these cells and immune factors [33]. Cytokines are broadly classified into interleukins (IL), interferons (IFN), tumor necrosis factor superfamily (TNFSF), transforming growth factor-β (TGFβ) and colony stimulating factor (CSF) [33]. Cytokines of innate immunity include type I IFNs, which have anti-viral activity and IL-10, which has anti -IFN activity [58, 59].

Cells of Innate Immunity

Cells of innate immunity at the site of infection include natural killer (NK) cells, heterophils, monocyte derived macrophages, thrombocytes, and dendritic cells (DCs) [33]. Macrophages and DCs are especially important, as they bridge innate and adaptive immunity. NK cells are lymphocytes of innate immunity that mediate cytotoxicity, causing lysis of tumor cells and cells infected with viruses [33]. They are not to be confused with cytotoxic T cells –to be further discussed in the adaptive immunity section, as they are part of innate immunity due to their lack of specific recognition molecules on their surface [60-62]. Chicken heterophils are the equivalent of mammalian polymorphonuclear neutrophils (PMNs). They are initial unspecific responders during pathogenic infection, where they phagocytize and kill pathogens [33]. However, unlike mammalian neutrophils, heterophils lack certain granule contents, such as myeloperoxidase, defensins, catalase and alkaline phosphatase [33, 63, 64]. Heterophils use cathepsin, lysosome, acid phosphatase, β-defensins, β-glucuronidase and α-glucosidase in their granules to kill invading pathogens [64].

Monocytes originate in the bone marrow and travel in the blood [33]. Macrophages are mononuclear phagocytic cells that differentiate from monocytes when they migrate into tissue. Macrophages engulf dying cells and foreign invaders into a phagosome that fuses internally with a lysosome, killing the invading microorganism [33]. Macrophages

then present peptides from the invading microorganisms to B and T cells, inducing their activation [65, 66]. Besides lymphocyte activation, macrophages can be polarized to express cytokines that direct the immune response into a helper T cell (Th) type 1 or 2 predominant response [67-69]. Classical activation of mammalian macrophages (M1), induced by IFN gamma (IFNγ) in the presence of microbial molecules, results in a microbicidal and pro-inflammatory state consisting of production of reactive oxygen species (ROS), nitric oxide (NO) and pro inflammatory cytokines such as IL1β, IL12, TNF, and IFNγ [67-70]. Alternative activation of macrophages (M2) on the other hand, results in the secretion of anti-inflammatory cytokines, leading to wound healing, angiogenesis, and inhibition of T cell proliferation [71, 72]. M2 synthesize NO in the absence of inflammatory agonists, while inhibiting it in the presence of microbial agonists such as LPS, a component of the outer cell wall of gram negative bacteria [67].

While macrophages can function as APCs, their main role is phagocytosis while DCs serve as professional APCs. Immature DCs are efficient at processing antigen and maturing into cells optimal for antigen presentation to naïve T cells [33, 73]. Stimulated DCs induce the proliferation of T cells and produce an array of cytokines, such as IL-1β, IL-18, IL-6, TNF-a, IFN-γ and IL-12 after treatment with LPS, IFN-γ and IL-12α after treatment with IBDV, and IL-8, IFN-a, and IFN- γ after treatment with avian influenza [73-77].

Major Histocompatibility Complex (MHC)

Among the many receptors expressed by cells on their surface, major histocompatibility complex (MHC) stand out in pathogen recognition. There are two main classes of MHC proteins, class I and class II. Class I molecules are expressed by all cells and present microbial proteins found in their cytoplasm upon becoming infected with intracellular pathogens [33]. Class I MHC molecules are recognized by cytotoxic T cells of

adaptive immunity, which proceed to kill the infected cell. Class II molecules on the other hand, are expressed by APCs and bind microbial proteins present in vesicles that APCs have taken up from the extracellular space [33]. Class II MHC molecules are recognized by helper and regulatory T cells of adaptive immunity.

### 2.2.2 Chicken Adaptive Immunity

The diversity within innate immunity is coded in the germline and therefore limited. Adaptive immunity of the other hand, has plasticity which allows for immunological memory to pathogens after first exposure. The two branches of adaptive immunity are humoral and cell-mediated, which are B cell and T cell dependent, respectively [33]. In avian species, B cells develop in the bursa, which mammals lack. In mammals, B cells develop in the bone marrow. In both mammals and avian species, T cells develop in the thymus.

#### Humoral Immunity

The bursa is the primary organ of B cell lymphopoiesis in avian species [78-81]. B cells produce a large repertoire of soluble and membrane bound immunoglobulins (Ig), also known as antibodies, to protect the host against infection with a wide array of pathogens. B cells must be activated through their B cell receptors (BCR) in combination with additional signals. Two proteins make up the BCR, a heavy chain and a lambda or kappa light chain, each encoded by different genes within the genome. Both the light and heavy chains contain hypervariable regions that are mixed and matched through splicing of the genes (Ig gene rearrangement) in mammals to create diversity [82-84]. Each individual B cell can produce one kind of antibody, a random assortment of these hypervariable regions that create unique Igs [82]. The variability of the Ig repertoire is immense. In humans for example, the Ig heavy chain region alone contains 2 constant, 44 variable (V), 27 Diversity (D) and 6 Joining (J) gene segments [85-87]. Isotypes of

antibodies are based on the Ig heavy chain. In mammals there are 5 types, generating the IgA IgD, IgE, IgG and IgM subclasses of antibodies. Somatic hypermutation, further discussed below, and mutations are minor means of Ig diversity in mammals.

In chickens, as well as in other avian species such as the zebra finch and the ostrich, there is only one V and one J gene in the lambda-like Ig light chain compared to numerous copies in mammalian species [88, 89]. This means that in chickens only one rearrangement event takes place in the light chain, providing very little diversity [90-96]. Chickens have one V, one J, 15 D genes and three different heavy chain isotypes, IgA, IgG and IgM [33, 88, 97]. These isotypes serve similar functions to their mammalian counterparts, with avian IgM as the main surface Ig and the first antigen specific antibody after pathogen exposure, avian IgA as the predominant Ig isotype in secretions and IgG as the predominant isotype in secondary antibody responses [33]. Unlike mammals, which have two classes if IgG isotypes, chickens only have only one IgG equivalent (IgY) compared to mammals and lack IgE and IgD altogether [98, 99].

Gene rearrangement in the chicken generates limited Ig diversity; however, chickens have a wide array of antibodies. In chickens, most antibody diversity occurs through somatic gene conversion in the bursa of Fabricious, a process in which the rearranged V segment of the light chain and the rearranged V and D segments of the heavy chain encoded sequences are replaced with sequences derived from non-functional pseudo genes located upstream [88, 91, 93, 97]. Bursectomized chickens can produce IgA, IgG and IgM; however, B cell diversity is severely impaired, specifically within the light chain. Although these bursectomized chickens have normal levels of circulating B cells, they are unable to mount specific antibody responses, despite hefty immunization [100-102]. The authors concluded that although the bursa is not necessary for antibody production, it is crucial for the diversification of the antibody repertoire [88, 103].

The function of B cells is to secrete antibodies in response to microbial invasion, and these antibodies bind microbes and their products. B cell protection against microbial invasion happens through neutralization of microbes, opsonization, which makes phagocytosis and destruction easier, and activation of complement which enhances opsonization or directly kills microbes and infected cells [33]. Naïve B cells pick up antigen using their BCR and present it to T cells in the context of MHC class II. T cells in turn, activates the B cell through the interaction of the B cell's CD40 surface protein with the CD40 ligand protein on the surface of B cells [33, 104]. As a result of this interaction both naïve B and T cells become activated. Activation of B cells results in the formation of germinal centers (GCs), which are follicles that form in secondary lymphoid tissue, and in these follicles B cells interact with T cells and DCs and proliferation of specific B cells takes place [33, 105]. At this point, B cells mature, switch antibody classes, and plasma and memory cells are generated [33]. Plasma cells secrete large amounts of IgA, IgG and IgM [104, 106].

Cellular Immunity

The main component of cellular immunity are T cells. However, it should be noted that T cells require interactions with both innate and humoral immunity for a proper immune response to occur. T lymphocytes and cytokines are critical in orchestrating specific immune responses. Cytokines are naturally derived regulatory proteins and immunologic effectors that play a crucial role in controlling the immune system and host response to invading pathogens. The production of these cytokines guides the immune response towards a pro- or anti-inflammatory state.

Similar to B cells, T cells have T cell receptor (TCR) on their surface that are essential for in antigen recognition [90]. These receptors can be made up of 2 out of 4 different chains, alpha, beta, delta and gamma. TCR are made up of either alpha and

beta chains (αβ T cells), or gamma and delta chains (γδ T cells). Diversity of the T cell repertoire is accomplished through recombination identical to that described in mammals. The Ig components of the beta and delta TCR are made up of V, D, and J segments, while the alpha and gamma chains have V and J segments [90]. αβ T cells can be further divided into CD8+ cytotoxic T cells and CD4+ helper T cells, which recognize antigen in the context of MHC class I and II, respectively [90]. As previously mentioned, class I molecules are expressed by all cells in response to infection with intracellular pathogens such as viruses and intracellular bacteria which are recognized by cytotoxic T cells while class II molecules are expressed by APCs and lead to activation of helper and regulatory T cells of adaptive immunity [33].

The CD4+ T lymphocytes that can differentiate into at least two functionally distinct helper subsets (Th1 and Th2) which secrete different effector cytokines. These CD4+ T cells are necessary as helpers to promote B cell antibody production and are often required for generation of memory and cytotoxic CD8+ cells [107, 108]. Th1 biased immune responses are associated with inflammation and are most efficient at fighting invasion by intracellular organisms such as viruses while Th2 biased responses are associated with helminth infections and allergic responses [33, 109]. The induction and maintenance of specific T helper cell responses is dependent on the secretion of key cytokines, which are important in affecting the direction of an immune response toward either cell-mediated (Th1) or humoral (Th2) immunity. IFN-γ, IL-2, and/or IL-12 cytokines are secreted by Th1 cells, while IL-4, IL-5, IL-6, and/or IL-10 are secreted by Th2 cells [110-114].

When an immune response is biased toward Th1, IL2 and/or IFN-γ secretion by Th1 cells results in in the selective expression of the IgG subtype of immunoglobulins, activation of cytotoxic T cells, and armed mononuclear phagocytes. When the response shifts toward Th2, secretion of IL-4, IL-5, IL-6, and/or IL-10 by Th2 cells results in the

selective expression of different immunoglobulin isotypes, such as IgA [115-126]. Th1 and Th2 are inhibitory towards one another [114].

It is important to note that more recently, several Th cell subtypes have been identified in mammals, including regulatory T (Treg) cells, Th17 cells, follicular helper T (Tfh) cells, Th22 cells and Th9 cells. There is evidence of the presence of Th1, Th2, Tregs, and Th17 cells in chickens. However, there is no current evidence for the presence of the Tfh, Th9 and Th22 lineages [127-129]. Regulatory T (Tregs) cells secrete TGFβ, IL35 and IL10 and function in immune tolerance, lymphocyte homeostasis and regulation of immune responses [130-132]. Th17 cells produce IL17A, IL17F, IL21 and IL22 and perform important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces [133]. Tfh cells regulate maturation of B cell responses and produce a wide array of cytokines, including IL-4, IFN-γ, IL-5 and IL-13 and IL-17 [134, 135].

Mammalian IFN-γ is normally produced by Th1 cells and NK cells, acting on macrophages to induce MHC class I and II molecule presentation and pathogen elimination, while IL-2 increases T cell responses, stimulates the growth, differentiation, and survival of antigen-selected cytotoxic T cells, and is necessary for the development of T cell immunological memory. IL-10 expression by DCs blocks the Th1 (IL-12 and IFN-γ) response, generating an anti-inflammatory Th2 response phenotype that stimulates macrophages to differentiate into regulatory macrophages [136, 137]**.**

Cytokines have been found to have similar effects in chickens. Chicken IFN-γ behaves similarly in chickens in that it activates macrophages inducing nitric oxide (NO) production, inhibits viral replication and induces a Th1 response [138, 139]. Chicken IL-2 also behaves as its mammalian counterpart in that it activates natural killer cells and clears intracellular pathogens, aiding in viral clearance [140, 141]. Chicken IL-10 also functions as an anti-inflammatory molecule that inhibits pro-inflammatory cytokines [68, 142].

In mammals, interleukin-4 (IL-4) is an immunoregulatory cytokine involved predominantly in Th2 cell immunity, a component of humoral immunity. IL-4 has a wide array of functions most of which ultimately stimulate humoral immunity. IL-4 can regulate B cell growth, immunoglobulin subtype switching, and growth of T cell and mast cells [143]. Further, IL-4 can influence muscle regeneration [144] adipocyte differentiation [145], as well as learning and memory [146]. Cells that produce IL-4 include Th2 cells, NK T cells[147], basophils [148], mast cells [149, 150] and eosinophils [151, 152]. IL-4 has a positive feedback on stimulation of Th2 cell differentiation and its receptors are present in a variety of cells, including T cells, mast cells and macrophages [143]. IL-4 has been found to suppress antiviral cell-mediated immunity, reduce viral clearance, and downregulate Th1 cytokines such as IL-2, IL-12 and IFN-γ [153, 154]. IL-4 down regulates the Th1 cell response through two mechanisms; through the inhibition of Th1 cell differentiation and reversion to the Th2 lineage and through inhibition of IFN-γ translation [155]. Regardless of its perceived importance, studies on chicken IL-4 (chIL4) are lacking.

### *2.3. NDV Immunity*

Innate and adaptive branches of the immune system play an important yet understudied role in NDV infection. Innate immunity to NDV is composed of physical and chemical barriers, complement proteins and mediators of inflammation, phagocytic cells such as macrophages and cytokines [156]. Cytokines are proteins that play a fundamental role in both innate and adaptive immunity. NDV-infected chicken heterophils and mononuclear cells produce nitric oxide (NO) [156] and splenic cells begin producing alpha and beta interferon (IFN-ά and IFN-β respectively), interferon gamma (IFN-γ), and interleukin 6 (IL-6) within a few hours [8, 30]. Natural killer (NK) cells begin secreting IFN-γ, which activates macrophages and induces cell-mediated immunity (CMI) [8].

CMI is detectable as early as 2-3 days post infection, although it may or may not protect against NDV challenge by itself [157]. Some studies have deduced that NDV-specific CMI is unable to provide protection against NDV challenge alone [158], whereas other studies have found some protection [159]. Reynolds and Maraqa (2000) used denatured NDV to induce CMI but not humoral immunity [158]. They identified anti-NDV antibodies through Western blot but not through virus neutralization or hemagglutination inhibition tests and all vaccinated birds succumbed to challenge with vNDV. However, SDS denaturing NDV may have also inhibited uptake and presentation of NDV through MHC II by DCs and macrophages, making T cells unable to properly differentiate and protect birds against infection. In a second part of their study, they bursectomized chickens and vaccinated with either live or UV inactivated NDV. In this portion of their study they saw that 12 out of 12 mock vaccinated birds succumbed to disease with NDV compared to 9 out of 12 bursectomized ones, showing some protection in the absence of humoral immunity [158]. Marino and Hanson (1986) bursectomized chickens in ovo and vaccinated them with live NDV at 45 days of age [159]. The survival rate of bursectomized chickens was significantly lower than that of non-bursectomized chickens and were smaller during their first weeks of life [159]. These chickens did not produce detectable antibodies after vaccination, had significantly lower viral shedding amounts compared to non-vaccinated challenged birds, and they survived challenge [159]. However, there was not a complete elimination of antibodies, as there were very low levels of antibody present in the bursectomized birds. Protection with CMI alone has been demonstrated with other viruses, such as IBV, demonstrating the need for better studies on the role of CMI during NDV infection.

Production of IFN-γ by macrophages is characteristic of the T helper 1 (Th1) response, providing signals for humoral immunity activation [8, 158, 160]. Studies have

also demonstrated a protective role of IFN-γ [156]. Early production of high levels of IFN-γ during vNDV replication can decrease pathogenicity, tissue damage and viral load [156].

The host's humoral immunity can protect the host across all age groups. Protective antibodies secreted by B cells are directed against either the HN or F glycopolypeptides and block binding of the Hn protein to the receptor on the cell membrane or fusion of the viral and host cell membrane, respectively [7]. These antibodies are detectable within 6-10 days post infection and are produced by differentiated B-lymphocytes known as plasma cells [8, 11]. Three types of antibodies are produced in avian species: Immunoglobulins A, Y and M (IgA, IgY and IgM, respectively) [8, 161]. IgY is the analogue of the mammalian immunoglobulin IgG. Local protection in the respiratory and intestinal tracts is induced by IgA. When inoculated with inactivated vaccines, which are normally given subcutaneously or intramuscularly, only IgG and IgM are detected while live vaccination has been found to stimulate production of IgA in addition to IgM and IgG [11]. Although both vaccine types induce full protection against challenge, birds vaccinated with live strains shed less virus through the oropharyngeal and cloacal routes [162] .

## 2.4 Vaccination Strategies for Newcastle Disease Virus

ND continues to be a threat to the poultry industry world-wide. Current biosecurity measures and vaccination protocols have been unable to stop the occurrence of outbreaks with virulent Newcastle disease virus (vNDV) around the world particularly in developing countries [1-6]. It is imperative to develop an effective vaccine that can overcome the maternal antibody inhibition and confer the earliest protection against vNDV. The poultry industry needs vaccines that can boost the immune response, both cellular and humoral, to achieve clearance of the challenge virus and therefore, reduce horizontal transmission within flocks without compromising the flock's health.

Live NDV vaccines are inexpensive to produce and are easily mass administered in water and through spray. Live vaccines replicate within the host, therefore no adjuvants are required, less antigen is needed to induce an immune response, and they are able to induce immunity faster [163]. Available NDV vaccines induce a strong cell- mediated and humoral immune response, providing complete immunity within two weeks of oral or ocular delivery and induce mucosal immunity [158, 164-166]. However, these live NDV vaccines can be easily killed by chemicals, emphasizing the importance of the cold chain and their replication within the host may lead to adverse reactions [163]. For example, some live vaccine strains (LaSota strain), may cause mild to moderate respiratory disease and reduce productivity [10].

Inactivated vaccines do not replicate in the host and therefore, do not induce clinical disease and do not elicit a strong cell-mediated immune response [163, 167]; however, they do induce long-lasting antibody responses [168]**.** They are more stable than live attenuated vaccines, but are more expensive to produce, more labor intensive to apply and often require adjuvants, higher levels of antigens and boosters to induce appropriate immunity [163].

Several attempts have been made to develop improved vaccines, including development of vaccines homologous to the vNDV strains. Vaccination with homologous viruses primarily impacts shedding of the challenge virus [169-172]. Unfortunately, not many of these vaccines are available on the market, as efficacy is measured through survival and clinical signs, and the main impact of these vaccines is in viral shedding.

Vaccination programs for Newcastle disease (ND) vary greatly between countries. Newcastle disease virus has been eradicated in commercial poultry in developed countries such as the United States, however it remains endemic in many developing countries, and there are many theories as to why. Vaccination programs against NDV include the use of recombinant, live attenuated and killed vaccines. Inactivated vaccines

are given intramuscularly, while live attenuated vaccines are given through eye inoculation or through IM injection or mass applied in the water or as aerosol [10]. To control ND, extensive vaccination programs have been put in place. Both inactivated and live vaccines are used for control of ND virus (NDV). The most common lentogenic live vaccine strains include LaSota, B1, Hitchner, Ulster, QV4, VG/GA, and I2. QV4 and I2 are thermostable [8].

In the US, where ND is not endemic, broiler breeders receive at least three live vaccines during their lifetime [11]. Broiler birds only receive one vaccine at one day of age and may also receive vaccine boosts [8, 10]. Layers receive an inactivated vaccine and may receive up to 3 additional live vaccines and other boosts [8, 10].

In countries where ND is endemic, vaccination protocols are much more rigorous, and the vaccines used are often more virulent. In Venezuela for example, the first line of protection is achieved through vaccination of breeders with several live and killed vaccines followed by booster vaccines to increase the level of maternal antibody titers. Production birds receive both live and killed vaccines *in ovo* and at day of hatch, then at days 7 and 17 in the field [12]. A study of several farms in West Malaysia found that all the farms were vaccinated for NDV using lentogenic vaccines, a third also used mesogenic vaccines and half used inactivated vaccines [5]. In some countries such as Israel, NDV vaccination is mandatory but still outbreaks continue to occur [13]. Despite these extensive vaccination programs, there are continuous outbreaks in these countries and many others including India [1], China [14], Pakistan [3], and Libya [4].

The rationale for these practices is that vaccination with more virulent viruses plus intense vaccination programs enhances immunologic memory, measurable through antibodies titers. Speculations for vaccine failure in the field include inappropriate application of the vaccine, immunosuppression by other agents, repeated exposure to the agent, interference by maternal antibodies in young birds (further addressed in the *in ovo*

vaccine section), antigenic diversity and improper biosecurity measures [169, 173, 174]. Previous studies in our laboratory have demonstrated that daily exposure to NDV for 10 days in vaccinated birds does not cause mortality, and therefore, as long as the vaccine is applied properly the birds should be protected [173]. While some studies have shown that homologous vaccination can improve vaccine response to NDV by enhancing the humoral response, reducing clinical signs and reduced mortality levels however, other studies have not shown a difference in survival when vaccines are matched to the field strains [169, 174].

### *2.4.1. NDV in ovo vaccination*

*In ovo* vaccination, or administration of the vaccine to the egg prior to hatching, has been explored as an option to confer early protection. It was first described in 1982 as a vaccination method against Marek's disease [15]. *In ovo* vaccination has been used commercially for over 20 years to control avian diseases due to its significant advantages over conventional vaccination. The advantages of *in ovo* vaccination include a significant reduction in costs, induction of earlier immunity, uniform delivery of vaccine, and reduction of stress [15, 16]. *In ovo* vaccination has proven successful for diseases such as Marek's disease and infectious bursal disease [15, 16, 175]. However, current vaccine options for NDV possess significant limitations. Commercially available vaccines against NDV such as LaSota, I2, Hitchner B1 and NDV substrain of Ulster 2C cause significant mortality when used *in ovo* [176-178].

One limitation of early vaccination for NDV is the presence of anti-NDV maternal antibodies (MatAbs). MatAbs are transferred from the hen to the egg through the yolk and can interfere with the establishment of early and persistent immunity after a single vaccination [179, 180]. MatAbs are detectable for up to 4 weeks of life, although protection is only provided during the first two weeks of life [180]. IgY is the predominant Ig isotype

transferred to the chick [181]**,** and the levels transferred are directly related to the circulating levels of IgY in the dam [181]. The transfer percentage of IgY from the dam to the chick has been estimated to be 30%, while that of IgM and IgA is less than 1% [181]. In the egg, most IgY is present in the egg yolk, and IgA and IgM are predominantly present in the egg white [181, 182]. MatAbs can interfere with the establishment of early immunity through epitope masking and by inhibition of B cell response through binding with FcγR [183-188]. T helper cell immunity has not been found to be suppressed by maternal antibodies in neonates vaccinated with paramyxoviruses [189, 190]. MatAbs interfere with the development of the humoral response but not T cell proliferation and IFN-γ secretion. In this study, we will induce the differentiation of T helper cells, specifically IFN-γ producing Th1 cells, in order to induce immunity despite the presence of maternal antibodies.

Another limitation of *in ovo* vaccination is the inefficiency of current vaccine strains to develop high levels of protective antibodies in short periods of time. Currently, there are two vaccines that can be administered *in ovo*. The first option is a live NDV conjugated with an antibody. The antibody is slowly released from the virus over time, which prevents the usual mortality observed when using a live NDV vaccine administered *in ovo* [191]. However, results from this platform are inconsistent. The second option is a recombinant herpes virus from turkey (rHVT) expressing the F protein from NDV. This rHVT confers long-lasting protection against challenge with vNDV after a single application without causing clinical signs**.** Unfortunately, it takes over 4 weeks to mount a protective immune response [192, 193], and there is no reliable test to determine and monitor the antibody response of vaccinated flocks. In 1982 it was reported that HVT, which by then was already used widely as a vaccine strategy for prevention of Marek's disease, provided protection in chickens vaccinated at 18 days of embryonation (DOE)[15]. The herpesvirus can replicate in chickens, is non-pathogenic and inoculation of chickens with HVT has been found to prevent tumors normally caused by virulent Marek's disease virus both in

chickens with and without maternal antibodies. HVT has a large double stranded DNA genome and the ability to replicate efficiently without causing embryo pathogenesis which makes it a good candidate as a recombinant vaccine. Several HVT recombinants have been developed since 1982, including one expressing the fusion (F) gene of ND virus (NDV) [193]. When given at 18 DOE, this recombinant vaccine was able to provide significant protection in specific pathogen free (SPF) and commercial eggs after 4 and 5 weeks-of-age, respectively. Studies with the HVT vaccine, but not *in ovo*, have found that HVT vaccination induces a strong cell-mediated and humoral immune response denoted by increased activation of natural killer cells [194], cell-mediated cytotoxicity of lymphocytes [195], and production of antibodies [196].

### *2.4.2. Cytokines as vaccine adjuvants*

Cytokines can be used to enhance the protection induced by vaccines to create a stronger, faster and more specific immune response [17-19]. Cytokines such as interferon gamma (IFN-γ) can mediate effector function, killing a variety of intracellular pathogens while others such as IL-2, IL-7, and IL-15 enhance effector and memory T-lymphocyte responses. These cytokines are known to play a critical role in protection against intracellular pathogens [19, 20]. Their primary role in modulating immune responses makes them useful components in vaccine formulations to improve protection against disease [17]. Studies in our laboratory have shown that recombinant virulent NDV strains containing IL-2 and IFN-γ can decrease viral load and decrease the pathogenicity of the virus in chickens [140, 156].

Cytokines have been used as intranasal vaccine adjuvants as an alternative to non-specific and more toxic agents [20]. For example, IL-2 is involved in T cell proliferation as well as enhancing the production of serum antibodies. In one study in chickens, the authors evaluated the impact of IL-2 expression on an avian influenza vaccine. Chickens

immunized with the virus plus IL-2 exhibited significantly increased levels of IgA, IgG, and antibody-secreting cells at weeks 3, 5, and 7 following vaccination as compared to the controls vaccinated with virus alone [197]. Other studies focused on the immunomodulatory effects of IFN-γ or IL-4 individually or co-delivered in chickens. Sawant and colleagues used a DNA vector to deliver NDV protective antigens in combination with the selected cytokines [198]. While the DNA vaccine alone only conferred 10% protection, this was increased to 40% when the IL-4 gene construct was administered with the vaccine. The use of single cytokines for immune modulation are limited. The immune system is interconnected and while small changes can positively impact disease outcome, studies driving the immune response using a single cytokine can have detrimental downstream effects. Since the immune response also has great self-regulation, there may not be a great impact on disease outcome.

The widespread use of cytokines as adjuvants has not been possible because of the high production costs, technical difficulties involved in delivering them to sites of infection, lack of information about the optimal time of delivery to achieve a maximal effect, and the potential side effects associated with each cytokine. Recently, the delivery of cytokines by live viruses has been experimentally demonstrated in birds [68, 69, 140, 156, 199, 200]. Turkeys vaccinated *in ovo* with recombinant fowl pox viruses expressing NDV's F and HN glycoproteins in addition to type II IFN (IFNγ) were better protected against NDV challenge [199]. While previous studies have found that IFNγ attenuated infection with vNDV, other experiments have found no enhancement in protection against NDV infection when recombinant NDV containing IFNγ is delivered after hatch or in ovo [69, 156]. Similarly, another recombinant paramyxovirus (respiratory syncytial virus) was able to deliver murine IFN-γ (Th1) or IL-4 (Th2) and affect the response to subsequent infections [201-203]. Delivery of IFNγ with RSV induced protective immunity. The results of these studies emphasize the need for further studies emphasizing the dose and timing of

delivery of cytokines such as IFN-γ in conjunction with vaccine and challenge times. Cytokines such as IFN-γ are attractive vaccine adjuvants for viral infections as they induce Th1 immunity, which is more efficient at fighting viral infections.

*2.3. Antisense RNA*

Antisense ribonucleic acid (asRNA) transcripts are a class of non-coding RNAs (ncRNAs) that are complementary to those of a sense RNA transcript that may or may not encode a protein [21]. Antisense technology takes advantage of the hydrogen bonds that form between complementary bases of nucleotides to silence sense RNA through binding of antisense RNA. Antisense can affect transcription of the sense RNA through transcriptional interference [21]. It has been reported that antisense RNA can cause transcriptional interference through several mechanisms, thereby modulating gene expression [21]. Transcription of the sense RNA can be affected through promoter competition, DNA methylation, or histone modifications [21]. These modifications can reduce gene expression or completely silence partner genes [22-24].

Antisense vaccines have the potential to modulate the immune system within specific cells, limiting their impact. While cytokines such as IFNγ may have systemic impacts, a live attenuated NDV vaccine containing an antisense insert may have a more subtle impact, as its effect is limited to the cell types infected with the virus. While no literature was found on the use of a recombinant antisense vaccine to protect against avian pathogens, antisense technology has proven successful in tumor and viral vaccines. Intracranial delivery of tumor vaccine and antisense TGF-β2 oligonucleotides to mice with brain tumors significantly enhanced their survival [25]. In human patients with non-small cell lung cancer, intradermal immunization with an TGF-β2 antisense tumor cell vaccine improves patient outcome [26]. A vaccine for foot and mouth disease containing a sense protein of the virus in conjunction with an antisense sequence targeting the 5’ untranslated

region of the virus successfully protected cells against foot and mouth disease infection and improved survival of mice challenged against the disease [27]. These studies successfully used antisense technology to improve disease outcome however, they did not use live attenuated virus to simultaneously vaccinate and immunomodulate immunological memory. This avenue should be further explored to improve vaccine and challenge outcomes against NDV.

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# CHAPTER 3

## RECOMBINANT NDV VIRUSES FOR *IN OVO* VACCINATION IMPROVE SURVIVAL POST-VACCINATION WHILE MAINTAINING PROTECTION AGAINST VIRULENT NDV CHALLENGE

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3.1 Abstract

In ovo vaccination has been used commercially for over 20 years to control avian diseases. Unfortunately, current NDV vaccines have significant limitations for in ovo use, including embryo mortality and the inability to induce full protection during the first two weeks of life. In this study, recombinant Newcastle disease viruses (NDV) expressing IL-10 (ZJ1-L-IL10), or IFNγ (ZJ1-L-IFNγ), or containing a “reverse” (R) antisense chicken IL-4 insert (ZJ1-L-IL4R) were administered in ovo to naïve specific pathogen free (SPF) embryonated chicken eggs (ECEs) and evaluated against a homologous challenge with a genotype VIId virulent NDV. Controls included the ZJ1-L backbone, the LaSota vaccine, and diluent alone. We hypothesized that combining two technologies, in ovo vaccination and cytokines as vaccine adjuvants, would facilitate the development of a more effective vaccine for NDV. In the first experiment, ECEs were vaccinated at 18 days of embryonation (DOE) using either $10^{4.5}$ or $10^{3.5}$ 50% embryo infectious dose (EID50/egg) and chickens were challenged at 21 days post hatch (DPH). In the second experiment, $EID_{50}$/egg of each vaccine was administered at 19 DOE with chickens challenged at 14 DPH. Chickens vaccinated with $10^{3.5}$ $EID_{50}$/egg of ZJ1-L-IL4R had hatch rates comparable to the sham vaccine, whereas other groups had significantly lower hatch rates. All vaccinated chickens survived challenge without displaying clinical disease, had protective antibody titers, and had comparable levels of viral shedding. In summary, the recombinant ZJ1-L-IL4R vaccine yielded lower post-vaccination mortality rates compared with the other in ovo NDV live vaccine candidates and provided strong protection post challenge.

3.2. Introduction

Newcastle disease virus (NDV), recently re-named Avian Avulavirus 1 (AAvV-1) (http://doi.org/10.1007/s00705-017-3311-7), infects over 236 species with chickens and turkeys being highly susceptible to disease, and ducks and geese often showing few or

no clinical signs [1]. Infections of poultry species with virulent strains of NDV cause Newcastle disease (ND). The NDV is a single stranded, non-segmented, negative-sense, enveloped RNA virus with helical capsid symmetry. It has a genome with approximately 15kb that encode at least 6 proteins – the nucleocapsid protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin- neuraminidase (HN) and the RNA directed RNA polymerase (L).

NDV has been eradicated in commercial poultry in certain developed countries, including the United States; however, it remains endemic in commercial and domestic poultry in many developing countries throughout the world [2]. Vaccination programs in countries where ND is endemic are very extensive, but outbreaks still occur [3-9]. Both inactivated and live vaccines are used to control of ND [10]. The most common lentogenic live vaccines include LaSota (LS), Hitchner B1, Ulster, QV4, VG/GA, and I2 strains [1, 10]. Live vaccines tend to be inexpensive to produce, induce a strong cell-mediated immune response, provide neutralizing immunity via the oral or ocular route and induce mucosal immunity [11, 12]. Further, the poultry industry is familiar with their use. However, some live vaccines such as LS, may cause mild to moderate respiratory disease and reduce productivity [13]. Inactivated vaccines do not replicate in the host, and therefore, do not elicit a strong cell-mediated immune response [14]; however, they can induce long-lasting antibodies [15]. An ideal vaccine should be able to confer strong cell-mediated and strong humoral immune responses to decrease virulent virus replication and shedding to a level that inhibits or decreases horizontal transmission, which is hard to achieve without compromising the bird's health and productivity.

*In ovo* vaccination was first described in 1982 as a vaccination method against Marek's disease [16]. The advantages of *in ovo* vaccination include a significant reduction in costs, induction of earlier immunity, uniform delivery of vaccine, and reduction of stress [16, 17]. *In ovo* vaccination has proven successful for preventing diseases such as

Marek's disease, and infectious bursal disease [16-18]. Unfortunately, current *in ovo* vaccine options for NDV possess significant limitations, including high mortality rates to the embryo caused by available live attenuated vaccines against NDV such as LS, I2, Hitchner B1 and Ulster 2C [19-21] Thus these vaccines can only be used post hatching. Currently, a recombinant Meleagrid alphaherpesvirus 1 (commonly known as herpesvirus of turkeys, (rHVT) expressing the F protein from of NDV is commercially available for *in ovo* vaccination against ND. This rHVT confers long-lasting protection against challenge with vNDV after a single application without causing clinical signs. Unfortunately, it takes over 4 weeks to mount a protective immune response and prevents the use of other rHVT-vectored vaccines subcutaneously in the same chickens after hatch, as the immunity that is induced from the first vaccine will neutralize the viruses from the second application after it is administered [22-24].

Cytokines can be used to enhance the protection induced by vaccines to create a stronger, faster and more specific immune response [25-27]. Cytokines such as IFNγ can mediate the killing of a variety of intracellular pathogens while others such as IL-2, IL-7, and IL-15 enhance effector and memory T-lymphocyte responses, which play a critical role in protection against intracellular pathogens [27, 28]. The cytokine IL-10 inhibits the Th1 response, allowing for an increase in Th2 cytokine response and antibody production [29][30]. IL-4 has been found to suppress antiviral cell-mediated immunity, reduce viral clearance, and downregulate Th1 cytokines such as IL-2, IL-12 and IFN-γ [31, 32]. Cytokines' primary role in modulating immune responses makes them useful components in vaccine formulations to improve protection against disease [25]. Studies in our laboratory have shown that recombinant virulent NDVs containing IL-2 and IFNγ can decrease viral load and decrease the pathogenicity of the virus in chickens [33, 34], thus demonstrating an active role of cytokines in modulating viral infection and clinical signs.

In this study, we evaluated three attenuated recombinant NDV vaccines containing a reverse (R) or antisense chicken IL-4 insert (ZJ1-L-IL4R), a sense IL-10 insert or a sense IFNγ insert. They were tested as *in ovo* vaccine candidates against challenge with a genotype-matched (VIId) NDV. We hypothesize that combining two technologies, *in ovo* vaccination and cytokines as vaccine adjuvants, would facilitate the development of a more effective vaccine for NDV while providing protection earlier in the life of the bird.

## 3.3. Materials and Methods

### *3.3.1. Viruses*

Virulent NDV (vNDV) ZJ1 (Goose/China/ZJ1/2000; GeneBank accession number AF431744.3), a genotype VIId virus, was used as a challenge virus in the *in ovo* vaccination experiment. Recombinant ZJ1-L (ZJ1-L) is an attenuated version of ZJ1 previously generated at the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) through reverse genetics to generate a recombinant virus of low virulence. LaSota (LS) is employed worldwide as a live or inactivated vaccine and was used here as a control vaccine in the immunization-challenge experiments. All viruses were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) virus stocks or repository, propagated and titrated in 9–11 day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). The recombinant viruses ZJ1-L-IL4R, -IFNγ, and -IL10 were generated as previously described [35].

### 3.3.2. Chickens and Embryonated Chicken Eggs

All 9–11-day-old SPF ECEs and adult chickens were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) SPF flocks. Chickens were provided food and water *ad libitum*.

### 3.3.3. Hatchability and Post-Hatch Survival of SPF Chickens Vaccinated at 18 DOE with $10^{3.5}$ or $10^{4.5}$ $EID_{50}$/egg of Various NDV Vaccines.

Less than 24 hr old ECEs were obtained collected, washed, and incubated until 18 days of embryonation (DOE). At 18 DOE, SPF ECEs were randomly divided into 11 groups and 22 eggs per group were inoculated with viral titers of $10^{4.5}$ or $10^{3.5}$ $EID_{50}$/egg of LS, ZJ1-L, or one of three recombinant NDV viruses (ZJ1-L-IL4R, -IL10, and - IFNγ). One group received only inoculum, brain heart infusion (BHI). All ECEs received a final volume of 100 µl. Vaccination doses were selected based on survival of embryos and chickens in previous experiments (not shown). ECEs were incubated until hatch as previously described [35]. Chickens were monitored daily for post- hatch survival and clinical signs until 21-days post hatch (DPH), at which time they were individually identified, blood was collected, and chickens moved into a biosafety level-3 (BSL3) laboratory for challenge with $10^5$ $EID_{50}$/bird vZJ1 by the oculo-nasal route (100 µl). Challenged chickens were monitored daily for clinical signs (depression, swelling of the head, conjunctivitis, and neurological signs), and mortality for 14 days post challenge (DPC). Oropharyngeal (OP) and cloacal (CL) swab samples were collected at two and four DPC. At 14 DPC, serum samples were collected from the remaining chickens, after which they were humanely euthanized. Pre-challenge and post-challenge antibody titers were determined by hemagglutination inhibition (HI) assay following standard procedures [36, 37].

### 3.3.4. Hatchability and Post- Hatch Survival of SPF Chickens Vaccinated at 19 DOE with $10^{3.5}$ of Various NDV Vaccines.

A second experiment was conducted using the same conditions described above except that ECEs were incubated until 19 DOE, at which date they were inoculated with $10^{3.5}$ $EID_{50}$/ egg of LS, ZJ1-L, one of three recombinant NDV viruses (ZJ1-L-IL4R, -IL10, and - IFNγ) or brain heart infusion (BHI) control. Post hatch, chickens were monitored daily for

survival and clinical signs until 14 DPH. Chickens were weighed at 1, 8 and 14 DPH. At 14 DPH, 12 chicks in each group were individually identified, bled and moved into BSL3 for challenge with $10^5$ $EID_{50}$/ bird vZJ1 by the oculo-nasal route (100 µl). Post challenge care and sampling was carried out as in experiment one.

### 3.3.5. Isolation and Quantification of Viral RNA in Swabs

To assess the degree of viral shedding, OP and CL swab samples were obtained from each bird at two and four DPC and placed in separate tubes containing 1.5 mL BHI with antibiotics (2000 U/mL penicillin G, 200 mg/mL gentamicin sulfate, and 4 mg/mL amphotericin B; Sigma Chemical Co., St. Louis, MO). Total RNA was extracted from swab medium and quantified as previously described [38]. Briefly, RNA was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX, USA). Quantitative real time RT-PCR (qRT-PCR) targeting the NDV M gene was performed using previously described primers [39], the AgPath-ID one-step RT-PCR Kit (Ambion, Austin, TX, USA) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, USA). The qRT-PCR lower detection limit for NDV was between $10^{1.5}$ and $10^{2.3}$ $EID_{50}$/mL.

### 3.3.6. Statistical analysis

HI antibody and virus titers were expressed as arithmetic means plus or minus the standard error of the mean for each vaccine group. Group means were analyzed by ANOVA and Tukey's test for multiple comparisons when appropriate, and using Student's *t*-test when comparing only two groups at a time. The survival curves were analyzed using the Log-rank test. The level of significance used to determine statistical differences among groups was 5% ($\alpha = 0.05$). The data was analyzed using Prism software version 6.0 (GraphPad Sofware, La Jolla, CA, USA).

3.3.7. Animal use and care:

All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee and were conducted with appropriate measures to maintain biosecurity and biosafety. General care of chickens was provided in accordance with the procedures reviewed and approved by the SEPRL Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

## 3.4. Results

### 3.4.1. SPF Chickens Vaccinated at 18 DOE with $10^{3.5}$ or $10^{4.5}$ $EID_{50}$/egg of Various NDV Vaccines.

#### 3.4.1.1. Hatchability and Post-Hatch Survival

Hatchability was defined as the number of ECEs completely hatched by 22 DOE compared to the number of inoculated ECEs. Chickens vaccinated with $10^{3.5}$ $EID_{50}$/egg of ZJ1-L-IL4R had mean hatchability rates of 91.0%, the same as BHI sham vaccinated chickens (Table 1). Chickens that received the LS vaccine mean hatchability rates that were significantly lower compared to those of the sham vaccinated control, 45.5% and 27.3% for $10^{3.5}$ and $10^{4.5}$ $EID_{50}$/egg, respectively ($p = 0.001$). The ZJ1-L-IL10 recombinant vaccine $10^{3.5}$ $EID_{50}$/egg group had a mean hatchability that was significantly lower compared to the sham vaccinated control, 59.1% ($p = 0.016$).

Post hatch survival was defined as the number of live chicks completely hatched from the egg, beginning at 21 DOE. The BHI vaccinated chickens and the ZJ1-L-IL10 $10^{3.5}$ $EID_{50}$/egg vaccinated chickens had mean post-hatch survivals that were not significantly different from one another –85.0% and 61.5%, respectively (Table 1). The post-hatch survival of chickens in all other groups was 55.0% or below, and was significantly different from that of the BHI sham vaccinated chickens ($p \leq 0.05$).

Hatchability and post-hatch survival data were combined into a total post-vaccination survival curve (Figure 1). Chickens vaccinated with all live vaccines except with $10^{3.5}$ $EID_{50}$/egg ZJ1-L-IL4R had total post-vaccination percent survival means that were significantly lower compared to the BHI sham vaccinated chickens (*p ≤ 0.05)*. Chickens vaccinated with the LS virus had significantly lower bird total post-vaccination survival compared to all recombinants at both doses, with 4.5% and 0.0% survivals for the $10^{3.5}$ and the $10^{4.5}$ $EID_{50}$/ egg vaccine doses, respectively (*p ≤ 0.05)*.

3.4.1.2. Humoral immune response

All vaccinated groups had HI titers that were significantly higher than the sham-vaccinated group (*p ≤ 0.05)* (Figure 2a and b). Pre-challenge, chickens vaccinated with $10^{4.5}$ $EID_{50}$/egg of ZJ1-L-10 had average HI titers that were significantly higher than ZJ1-L-IL4R and ZJ1-L-IFNγ $10^{3.5}$ and ZJ1-L $10^{4.5}$ $EID_{50}$/egg vaccinated chickens (*p ≤ 0.05)*. The HI titers 14 DPC was not significantly different between groups (*p = 0.367*) (Figure 2b). When pre-challenge HI titers were compared to post-challenge titers, the ZJ1-L-IL10 $10^{4.5}$ $EID_{50}$/egg vaccinated group had a significantly decreased HI titer from $2^{9.7}$ before challenge to $2^{7.8}$ 14 DPC (*p = 0.011)*.

3.4.1.3. Viral Shedding and Survival Post Challenge

The detection limit for the assay was estimated to be $10^{1.5}$ $EID_{50}$/mL. Chickens with viral levels below the limit of detection were recorded as shedding just below the limit of detection ($10^{1.4}$ $EID_{50}$/mL). Sham vaccinated-challenged chickens shed significantly higher amounts of virus via the OP route at 2 DPC and via both routes at 4 DPC compared to all vaccinated groups (*p = 0.000)* (Figure 3a-d). Post challenge survival was 100% for all vaccinated groups, compared to 0% for non-vaccinated chickens (Table 2). No adverse

clinical signs were observed in vaccinated chickens. Sham vaccinated chickens were depressed, as noted by their ruffled feathers and reluctance to move when approached.

### 3.4.2. SPF Chickens Vaccinated at 19 DOE with $10^{3.5}$ of Various NDV Vaccines.

To evaluate the impact of delaying vaccination for 24 hr and challenging at an earlier time, chickens were vaccinated at 19 DOE and challenged at 14 DPH.

#### 3.4.2.1. Hatchability and Post-Hatch Survival

The survival post hatch of chickens vaccinated with ZJ1-L-IL10, ZJ1-L-IFNγ, and LS was significantly lower than of chickens vaccinated with the BHI sham vaccine or the ZJ1-L-IL4R vaccine ($p \leq 0.05$) (Table 3). When hatch and post-hatch survival rates were combined as total post-vaccination bird survival, the total post-vaccination bird survival of the chickens vaccinated with ZJ1-L-IL10, ZJ1-L-IFNγ, and LS was significantly lower compared to the total post-vaccination survival of chickens that received the ZJ1-L-IL4R or BHI sham vaccine ($p \leq 0.05$) (Figure 4). Chickens vaccinated with the ZJ1-L vaccine had total post-vaccination survival rates that were significantly lower compared to the total post-vaccination survival rates of ZJ1-L-IL4R vaccinated chickens. Chickens vaccinated with ZJ1-L-IL4R had post hatch ($p = 0.43$) and total post-vaccination survival ($p \leq 0.65$) rates that were not significantly different from those of the sham vaccinated chickens.

#### 3.4.2.2. Body Weights

Average body weights were not significantly different between chickens that received the ZJ1-L-IL4R or BHI sham vaccine at 1, 8 or 14 DPH ($p \geq 0.05$). At 1 DPH, average body weights for all groups were not significantly different between groups (Table 4). At 8 DPH, the average body weights of chickens vaccinated with ZJ1-L-IL10, ZJ1-L and LS were significantly lower than those of chickens that received the BHI sham vaccine

($p \leq 0.016$). At 14 DPH, the average body weights of chickens vaccinated with ZJ1-L and LS were significantly lower than those of chickens that received the BHI sham vaccine ($p \leq 0.034$).

3.4.2.3. Vaccine Shedding

Chickens vaccinated with LS had significantly higher viral titers than all other vaccinated groups through the OP route ($p \leq 0.000$) (Figure 5a). Chickens vaccinated with ZJ1-L-IL4R shed significantly lower viral levels compared to ZJ1-L-L10, ZJ1-L and LS vaccinated chickens through the OP route ($p \leq 0.011$). Further, ZJ1-L-IL4R vaccinated chickens shed significantly less virus through the CL route compared to the ZJ1-L vaccinated chickens ($p = 0.041$) (Figure 5b).

3.4.2.4. HI Titers

Chickens vaccinated with ZJ1-L-IL4R had HI titers at 14 DPH that were significantly higher than those of chickens vaccinated with ZJ1-L-IL10 and LS ($p \leq 0.019$) (Figure 6a). Chickens vaccinated with ZJ1-L had HI titers at 14 DPH that were significantly higher than those of chickens vaccinated with LS ($p \leq 0.019$). Post challenge, HI titers of vaccinated chickens were not significantly different between groups (Figure 6b). All groups had significantly higher antibody titers post-challenge compared to their pre-challenge titers ($p \leq 0.05$), except ZJ1-L-IL4R vaccinated chickens, which had HI titers of $2^{6.6}$ before challenge compared to $2^{7.3}$, at 16 DPC ($p = 0.404$).

3.4.2.5. Viral Shedding, Body Weights Post Challenge and Survival Post Challenge

The detection limit for the assay was $10^{1.5}$ $EID_{50}$/mL. Chickens with viral levels below the limit of detection were recorded as shedding just below the limit of detection ($10^{1.4}$ $EID_{50}$/mL). There were no significant differences in viral titers between the

vaccinated groups via either the OP or CL routes at 2 or 4 DPC ($p \geq 0.337$) (Figure 7a-d). Chickens that received the BHI sham vaccine shed significantly higher viral titers compared to all vaccinated chickens at 2 and 4 DPC through the OP and CL route ($p \leq 0.005$). Average body weights of chickens from the vaccinated groups were not significantly different from one another and were 197.6–209.1 g at 7 DPC and 295.0–307.3 g at 14 DPC ($p \geq 0.810$) (Table 5).

Post challenge survival and clinical signs were recorded for 14 DPC. Post challenge survival was 100% for all vaccinated groups, compared to 0% for non-vaccinated chickens. No adverse clinical signs were observed in vaccinated chickens. Sham vaccinated chickens were depressed, as noted by their ruffled feathers and reluctance to move when approached.

### 3.5. Discussion

While *in ovo* vaccination has been a successful tool for diseases such as Marek's disease and infectious bursal disease (IBD), an *in ovo* vaccine protocol that is effective, consistent and that provides early immunity without clinical signs for against ND is not currently available. The results herein indicate that live vaccination with a variety of NDV viruses *in ovo* at 18 and 19 DOE confers 100% protection against homologous NDV challenge, consistent with previous reports Furthermore, vaccination at 19 DOE with a lower dose can greatly improve post-vaccination survival. This was especially noticeable with the ZJ1-L-IL4R recombinant vaccine.

Vaccination in in the first experiment was performed at 18 DOE since traditionally, *in ovo* vaccination for Marek's disease and IBD are performed at 18 DOE [16, 17]. It was hypothesized that *in ovo* vaccination with cytokine-containing NDV recombinants would facilitate the development of a more effective vaccine for NDV while providing protection earlier in the life of the bird. The ZJ1-IFNγ and ZJ1-L-IL10 recombinants did not

significantly improve post-vaccination survival when compared to either dose of the ZJ1-L; however, the surviving chickens were protected against challenge with velogenic NDV at 14 and 21 DPH. The post-challenge survival suggests that the replication of the vaccine generated a rapid immune response.

The results from the present study are supported by previous studies with ZJ1-IFNγ, which reported that IFNγ expression during infection may not increase mortality, or affect immune responses to NDV [35, 40].However, other studies using vNDV recombinants expressing IFNγ have shown that expression of IFNγ can decrease viral pathogenicity during NDV infection [33]. The discrepancies in the results from these previous studies highlights the need for further investigation of the effect of IFNγ on the development of immunity against NDV.

While the ZJ1-L-IL10 recombinant did not improve post-vaccination survival, it did increase antibody production prior to challenge with vNDV compared to the other vaccine groups. This was expected, as a primary function of IL10 is the downregulation of the Th1 response, which shifts immune responses toward Th2 response and antibody production [29, 41, 42]. This increase in antibody production, however, was only observed with the higher dose of ZJ1-L-IL10 recombinant, which unfortunately was also associated with higher levels of bird mortality. This may have been due to an increase in viral load in conjunction with an increase in IL10 production.

Lowering the vaccine dose at 18 DOE improved post-vaccination survival; however, the levels of mortality were very high. The study focus shifted toward a lower dose ($10^{3.5}$ $EID_{50}$/egg) of the vaccine and evaluated the effect of vaccinating a day later. Post-vaccination survival was enhanced by vaccinating at 19 DOE compared to 18 DOE. However, the trends observed with all ZJ1-L recombinants remained constant, with ZJ1-L-IL4R having enhanced post-vaccination survival rates in comparison to other viruses,

without negatively impacting survival post challenge. All chickens vaccinated at 19 DOE were protected against challenge at 14 DPC.

The high mortality observed post vaccination with the ZJ1, ZJ1-L-IFNγ, ZJ1-L-IL10, and LS suggests that they are not viable *in ovo* vaccine candidates. However, the absence of mortality observed with the ZJ1-L-IL4R recombinant vaccine accompanied by 100% protection post challenge makes the ZJ1-L-IL4R recombinant an excellent candidate for *in ovo* vaccination. Not only did these ZJ1-L-IL4R recombinant-vaccinated chickens display no adverse clinical signs but they also had robust weight gains, a powerful index of production.

A possible yet still undetermined cause for the reduced mortality of -IL4R may be attributed to the existence of the IL4R antisense message that is produced by the virus; however, the mechanism of action of the antisense RNA is yet to be determined. Antisense RNA transcripts are non-coding RNAs that are complementary to those of a sense RNA transcript that may or may not encode a protein [43]. Antisense can affect transcription of the sense RNA through transcriptional interference [43]. It has been reported that antisense RNA can cause transcriptional interference through several mechanisms, thereby modulating gene expression by reducing it or completely silencing it [43-46]. While currently no literature exists on the use of a recombinant antisense vaccine to protect against avian pathogens, antisense technology has proven successful as viral vectors targeting cancer cells and as viral vaccines including antisense TGF-β2 oligonucleotides in mice with brain tumors [47], TGF-β2 antisense tumor cell viral vector to humans with non-small cell lung cancer [48] and a sequence targeting the 5' untranslated region of foot and mouth disease virus [49]. Thus, based on our preliminary results with our ZJ1-L-IL4R recombinant vaccine, this antisense RNA avenue needs to be further explored to improve vaccine and challenge outcomes against NDV.

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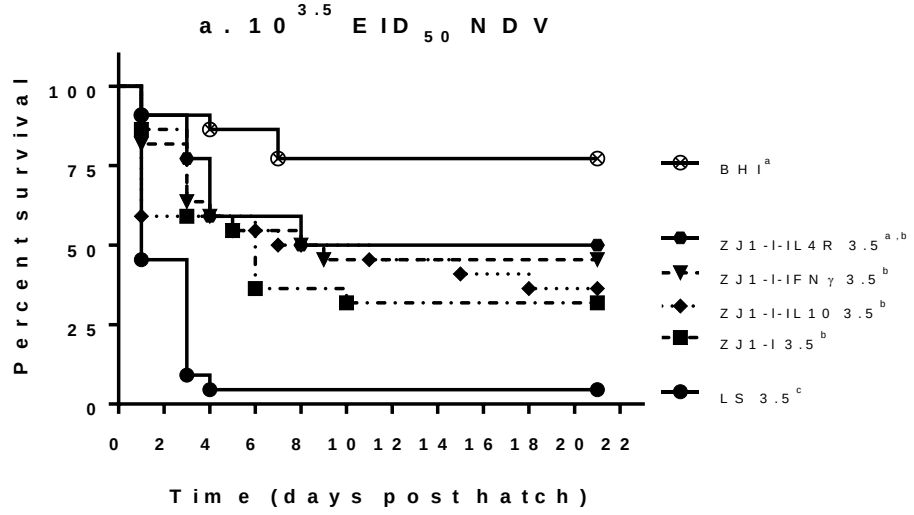
**Table 3.1. Hatchability and post-hatch survival of SPF ECES vaccinated at 18 days of embryonation from NDV with live recombinant NDV vaccines.**

| Vaccine Group | Hatched[a] | % Hatchability | Survival Post Hatch[b](%) |
|---|---|---|---|
| **BHI** | 20 | 90.9 | 85.0 |
| **LS $10^{3.5}$** | 10 | 45.5[c] | 10.0[d] |
| **LS $10^{4.5}$** | 6 | 27.3[c] | 0.0[d] |
| **ZJ1-L $10^{3.5}$** | 19 | 86.4 | 36.8[d] |
| **ZJ1-L $10^{4.5}$** | 19 | 86.4 | 26.3[d] |
| **ZJ1-L- IFNγ $10^{3.5}$** | 18 | 81.8 | 55.6[d] |
| **ZJ1-L- IFNγ $10^{4.5}$** | 17 | 77.3 | 23.5[d] |
| **ZJ1-L- IL10 $10^{3.5}$** | 13 | 59.1[c] | 61.5 |
| **ZJ1-L- IL10 $10^{4.5}$** | 18 | 81.8 | 33.3[d] |
| **ZJ1-L- IL4R $10^{3.5}$** | 20 | 90.9 | 55.0[d] |
| **ZJ1-L- IL4R $10^{4.5}$** | 15 | 68.2 | 53.3[d] |

[a]Total number of chicks hatched. A total of 22 viable eggs were inoculated.
[b] Survival between 1 and 21 days post hatch.
[c] Hatchability differences were analyzed using a using a Long Rank Test. Statistical significance was considered $p<0.05$. Significant differences between vaccinated eggs and BHI groups are marked by [c].
[d] Differences in post hatch survival were analyzed using a using a Long Rank Test. Statistical significance was considered $p \leq 0.05$. Significant differences between vaccinated eggs and BHI groups are marked by [d].

**Figure 3.1. Post-vaccination percent survival of SPF ECEs vaccinated with live recombinant ND vaccines at 18 days of embryonation (DOE).** At 18 DOE, SPF embryonated chicken eggs (n = 22) were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg or $10^{4.5}$ $EID_{50}$/ egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. After hatch, chickens were housed in negative pressure isolators and their survival was checked daily for 21 days. The survival curves were analyzed using the Long-rank test ($p \leq 0.05$). Significant differences are denoted by different superscript letters.



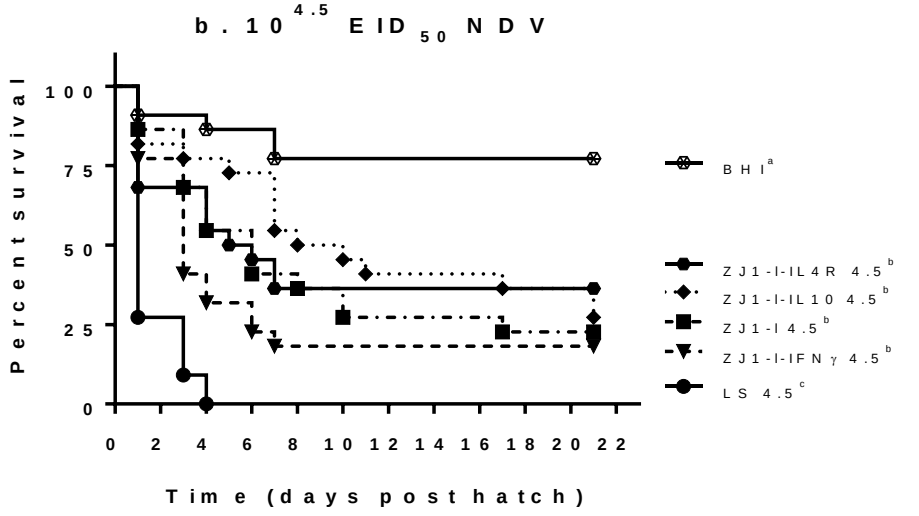



**Figure 3.2. Pre- and post-vNDV challenge serum HI titers of 18 DOE vaccinated SPF chickens challenged at 21 DPC.** At 18 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/egg or $10^{4.5}$ $EID_{50}$/egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. Serum was collected at 21 DPH pre (**A**) or at 14 DPC (**B**) with $10^5$ $EID_{50}$ vZJ1. One-way ANOVA followed by a multiple comparisons Tukey's test was done for statistical analysis. Significant differences are denoted by different letters, $p \leq 0.05$. The horizontal line indicates protective antibody levels.

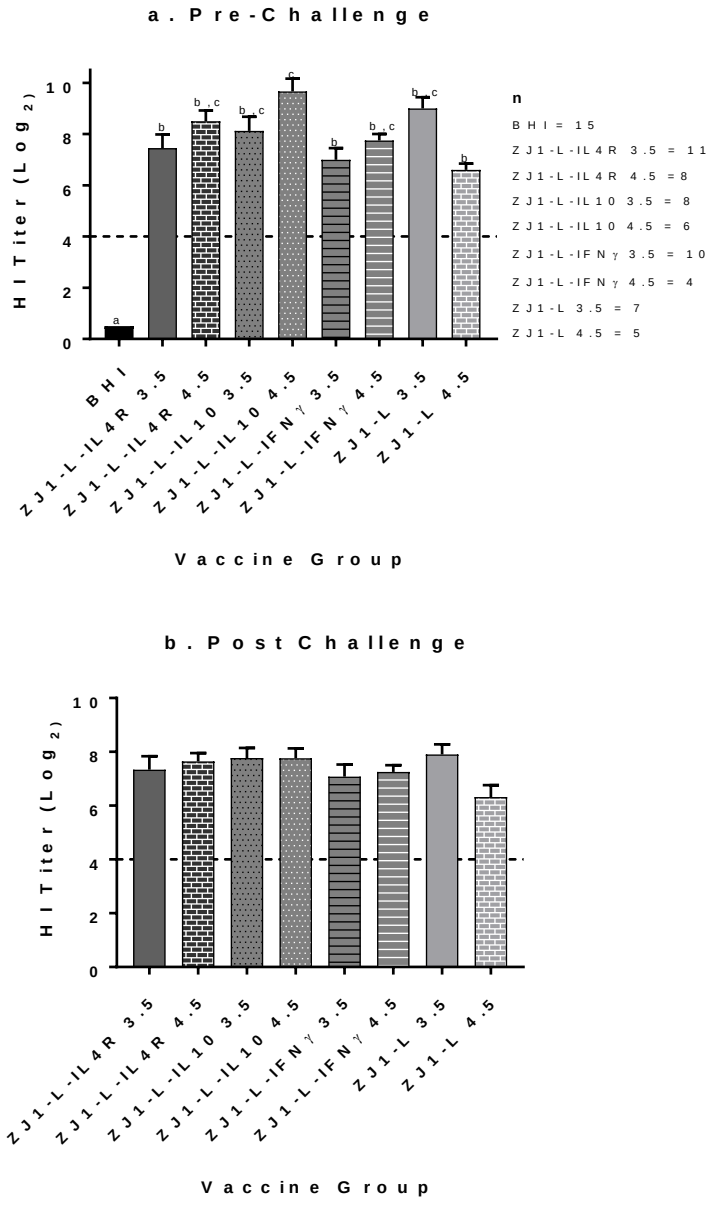


**Figure 3.3. Viral shedding in 18 DOE NDV-vaccinated chickens challenged at 21 DPH with vNDV.** At 18 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/egg or $10^{4.5}$ $EID_{50}$/egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. At 21 DPH, chicks were challenged with $10^5$ $EID_{50}$ vZJ1 via the oculo-nasal route. Viral titers from the oropharyngeal (OP) (a, c) and cloacal (CL) (b, d) routes were measured at 2 (a-b) and 4 (c-d), days DPC. Significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test. Reference line denotes the average limit of detection.

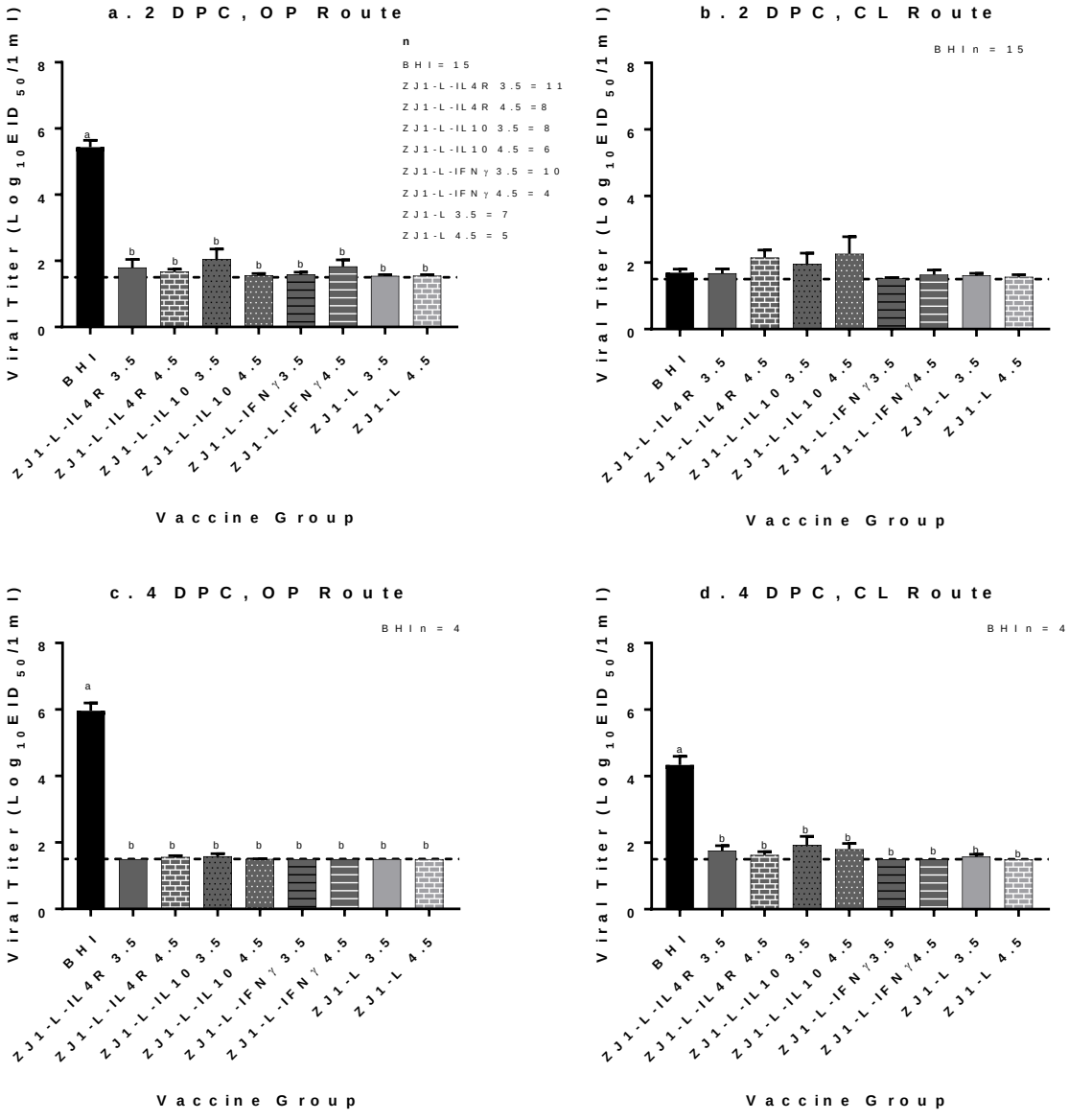


**Table 3.2. Survival of 18 DOE *in ovo* NDV or sham vaccinated chickens challenged at 21 days post hatch (DPH) with vNDV**

| Vaccine Group | Chickens Challenged | Survival (%) |
|---|---|---|
| BHI | 15 | 0[a] |
| ZJ1-L $10^{3.5}$ | 7 | 100[b] |
| ZJ1-L $10^{4.5}$ | 5 | 100[b] |
| ZJ1-L- IFNγ $10^{3.5}$ | 10 | 100[b] |
| ZJ1-L- IFNγ $10^{4.5}$ | 4 | 100[b] |
| ZJ1-L- IL10 $10^{3.5}$ | 8 | 100[b] |
| ZJ1-L- IL10 $10^{4.5}$ | 6 | 100[b] |
| ZJ1-L- IL4R $10^{3.5}$ | 11 | 100[b] |
| ZJ1-L- IL4R $10^{4.5}$ | 8 | 100[b] |

[a-b]Survival differences were analyzed using a using a Long Rank Test. Statistical significance was considered $p \leq 0.05$. Groups with different letters are significantly different.

**Table 3.3. Survival of specific pathogen free (SPF) embryonated chicken eggs (ECEs) inoculated at 19DOE with $10^{3.5}$ EID50 of NDV or a mock until 14 days post hatch (DPH).**

| Group | n[a] | Hatched | Hatchability (%) | Survival Post Hatch[# b](%) |
|---|---|---|---|---|
| **BHI** | 26 | 24 | 92.3 | 100.0 |
| **ZJ1-L-IL4R** | 40 | 39 | 97.5 | 97.4 |
| **ZJ1-L-IL10** | 40 | 34 | 85.0 | 82.4[c] |
| **ZJ1-L-IFNγ** | 40 | 37 | 92.5 | 73.0[c] |
| **ZJ1-L** | 27 | 25 | 92.6 | 80.0[c] |
| **LS** | 25 | 22 | 91.7 | 63.6[c] |

[a] = Number of viable eggs inoculated
[b] Survival between 1 and 21 days post hatch.
[c]Differences in post hatch survival were analyzed using a using a Long Rank Test. Statistical significance was considered $p \leq 0.05$. Significant differences between vaccinated eggs and BHI groups are marked by.

**Figure 3.4. Survival proportions of chickens vaccinated at 19DOE with $10^{3.5}$ $EID_{50}$ of different NDVs.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. Post hatch, chickens were housed in negative pressure isolators and their survival was checked daily for 14 days. Survival curves were analyzed using the Long-rank test ($p \leq 0.05$). Significant differences are denoted by different letters.

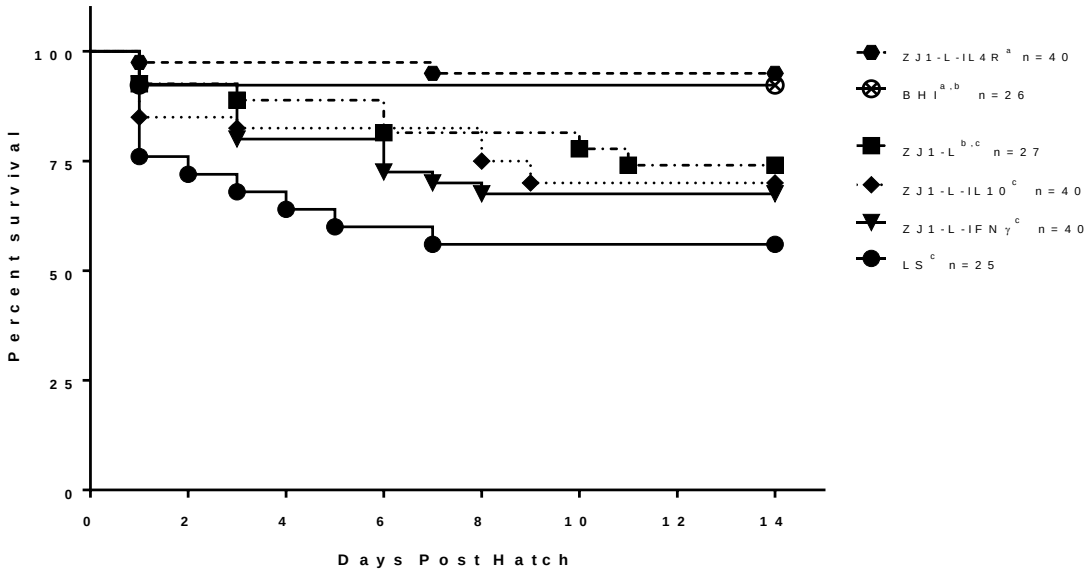
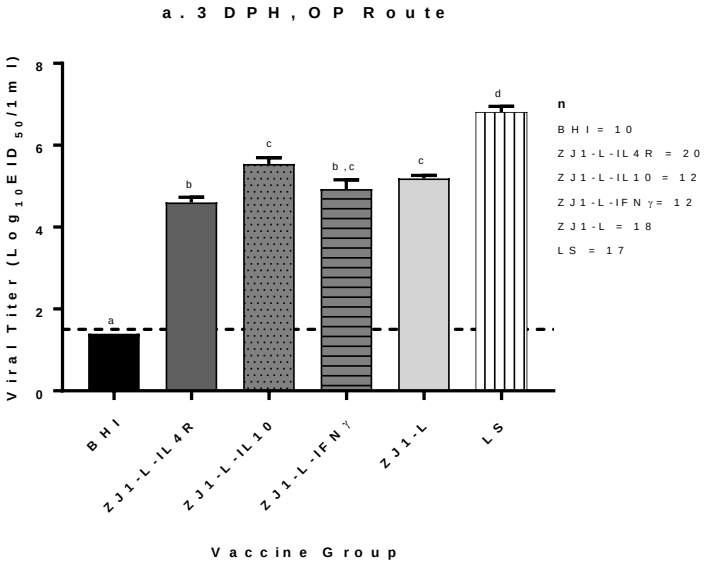



**Table 3.4. Body weight of chickens vaccinated at 19 DOE with NDV at 1, 8 and 14 days post hatch**

| Days Post Hatch | BHI (Mean ± SEM)# | ZJ1-L-IL4R (Mean ± SEM) # | ZJ1-L-IL10 (Mean ± SEM) # | ZJ1-L- IFNγ (Mean ± SEM) # | ZJ1-L (Mean ± SEM) # | LS (Mean ± SEM) # |
|---|---|---|---|---|---|---|
| **1** | 34.4 ± 0.9 | 33.8 ± 0.9 | 32.9 ± 0.8 | 34.0 ± 0.8 | 34.7 ± 0.8 | 33.7 ± 0.8 |
| **8** | 66.1 ± 1.2$^{a}$ | 56.8 ± 1.4$^{a,b}$ | 52.4 ± 3.3$^{b}$ | 57.6 ± 2.0 $^{a,b}$ | 50.6 ± 3.0$^{b}$ | 54.4 ± 2.8$^{b}$ |
| **14** | 125.9 ± 2.7$^{a}$ | 111.2 ± 2.6$^{a,b}$ | 112.4 ± 5.4 $^{a,b}$ | 113.0 ± 2.9 $^{a,b}$ | 107.1 ± 4.0$^{b}$ | 108.1 ± 5.3$^{b}$ |

#body weight in grams, *p ≤ 0.05*, one-way ANOVA followed by a multiple comparisons Tukey's test. Significant differences are denoted by different letters, n = 14-20 chickens/ vaccine group.

**Figure 3.5. Viral titer of 3-day-old 19 DOE NDV vaccinated chickens.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. Vaccine shed titers through the oropharyngeal (OP) (A) and cloacal (CL) (B) routes were measured at 3 DPH. Significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test. Reference line denotes the average limit of detection.



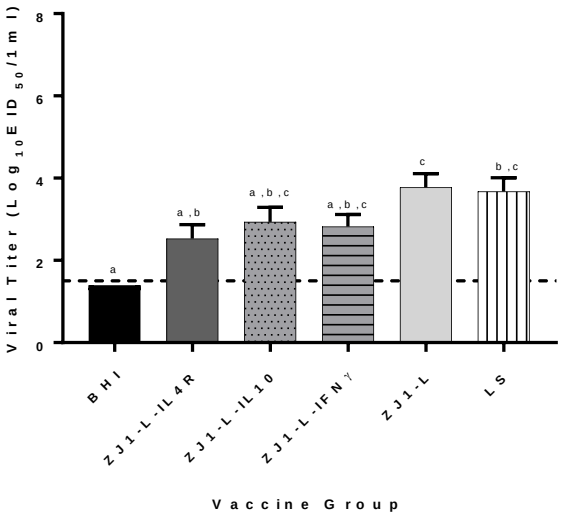



**Figure 3.6. Pre-and Post vNDV challenge serum HI titers of 19 DOE vaccinated chickens.** At 19 DOE, SPF embryonated chicken eggs (ECEs) were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/egg of ZJ1-L-IFNγ*, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L*, LS* or brain heart infusion (BHI) control. Serum was collected at 14 DPH pre (a) or at 14 DPC post (b) challenge with $10^5$ $EID_{50}$ vZJ1. Significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test, n= 11-12 chickens/vaccine group. The horizontal line indicates protective antibody levels. *These data were previously published (Cardenas et al. 2016) and has been included for completion.

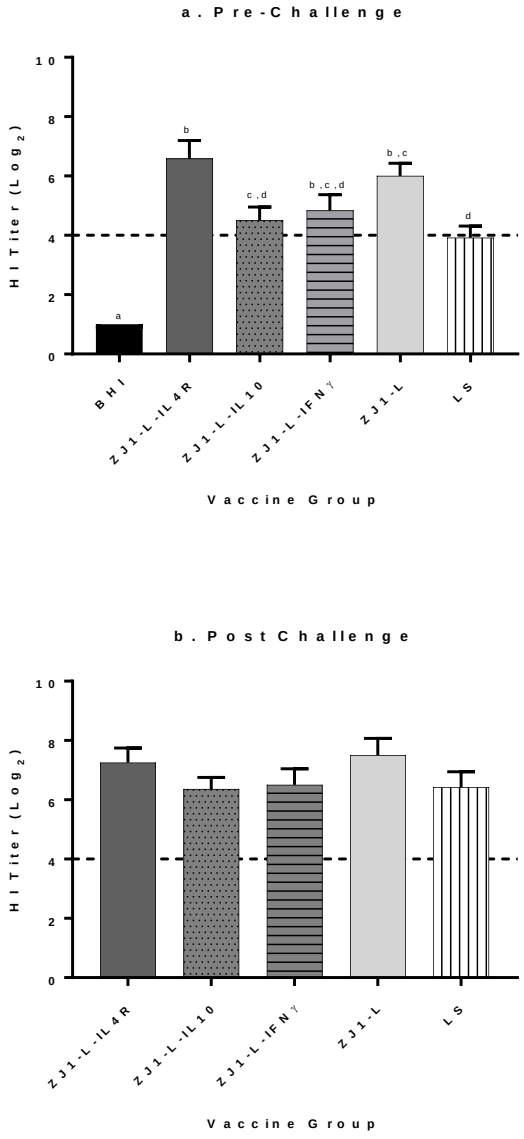
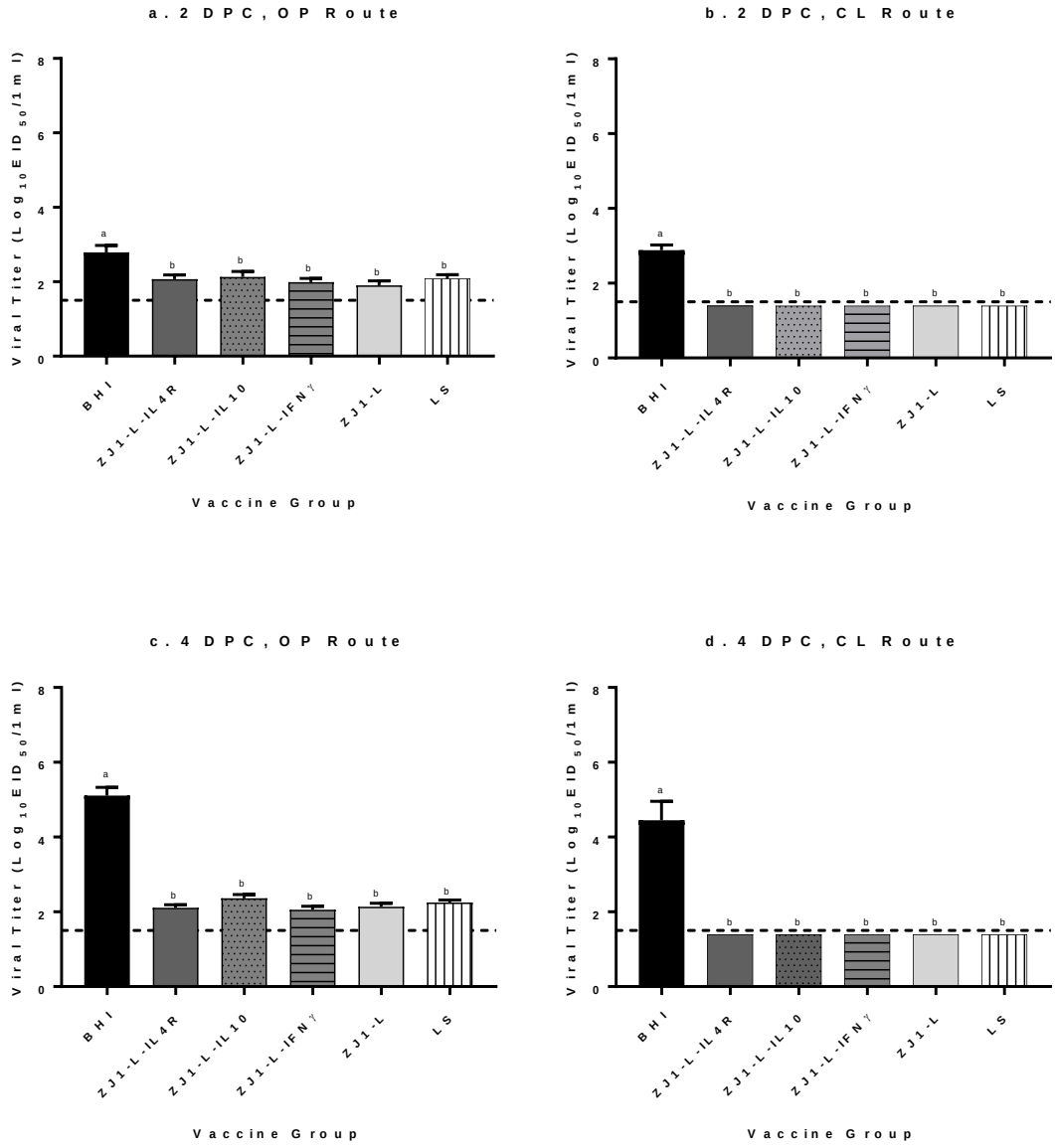


**Figure 3.7. Viral shed titers of 19 DOE NDV vaccinated chickens challenged at 14 DPH with vNDV.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IFNγ, ZJ1-L-IL10, ZJ1-L-IL4R, ZJ1-L, LS or brain heart infusion (BHI) control. At 14 DPH, 12 chicks from each group were challenged with $10^5$ $EID_{50}$ vZJ1 via the oculo-nasal route. Viral shed titers through the oropharyngeal (OP) (a, c) and cloacal (CL) (b, d) routes were measured at 2 (a-b), 4 (c-d), days post challenge (DPC). Significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test, n= 12 chickens/vaccine group except for the BHI sham vaccine group at 4 DPC, in which only 2 chickens remained alive.



| **Table 3.5. Body weights of 19 DOE NDV vaccinated chickens at 7 and 14 days post challenge with vNDV** | | | | | | |
|---|---|---|---|---|---|---|
| **Days Post Challenge** | **BHI** (Mean ± SEM)[#] | **ZJ1-L-IL4R** (Mean ± SEM) | **ZJ1-L-IL10** (Mean ± SEM) | **ZJ1-L-IFNγ** (Mean ± SEM) | **ZJ1-L** (Mean ± SEM) | **LS** (Mean ± SEM) |
| **7** | N/A | 197.5 ± 5.5 | 209.1 ± 11.0 | 203.3 ± 6.9 | 196.7 ± 5.9 | 199.2 ± 9.7 |
| **14** | N/A | 295.0 ± 8.5 | 307.3 ± 16.7 | 305.8 ± 9.3 | 298.3 ± 9.0 | 297.3 ± 14.0 |

[#]body weight in grams, significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test, n= 11-12 chickens/vaccine group.

# CHAPTER 4

# INTRACELLULAR FIZATION BUFFER INACTIVATED NEWCASTLE DISEASE VIRUS IN CHICKEN ALLANTOIC FLUID, MACROPHAGES AND SPLENOCYTES[1]

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[1]Marcano, V., Cardenas-Garcia S., Gogal, Jr, R.M., Afonso, C. L. 2018 Journal of Virological Methods. Reprinted here with permission of the publisher.

4.1 Abstract

Inactivation of Newcastle disease virus (NDV) has been routinely achieved with heat, β-propiolactone, binary ethylenimine, ultraviolet light and formalin. However, these strategies have not been tested for cell surface ligand or receptor phenotype in viral-infected chicken immune cells. To study the capacity of fixation buffers to preserve surface markers while inactivating NDV, a primary splenocyte culture was infected with NDV and incubated with a commercial intracellular fixation buffer (ICB), formulated with 4% formaldehyde. Splenocytes were fixed with a 1:2 dilution of ICB in phosphate buffered saline (PBS) for 45 minutes (min) at 23˚C or 4˚C, and inactivation of NDV was tested in addition to recognition of antigens by antibodies in fixed and non-fixed splenocytes via flow cytometric analysis. The binding and percentage of splenic CD4+ and CD8+ cells were not affected. In addition, NDV titers as high as $10^{9.5}$ and $10^{7.6}$ $EID_{50}$ in allantoic fluid (AF) and macrophages, respectively, were successfully inactivated after 45 min at 23˚C and 4˚C, confirming the ICB's effectiveness in inactivating high concentrations of NDV. In conclusion, high concentrations of NDV in AF, chicken splenocytes, and macrophages can be inactivated using ICB. Additionally, this method did not compromise cell phenotyping of enriched chicken splenocytes.

4. 2. Introduction

Newcastle disease virus (NDV) can infect over 270 avian and non-avian species [1]. In poultry, virulent strains can lead to 100% mortality and morbidity of the flock [2]. Velogenic or virulent forms of NDV (vNDV) are considered biological select agents, as they can pose a severe threat to poultry health and safety. Since current biosecurity measures and vaccination protocols have been unable to prevent outbreaks worldwide, especially in developing countries, it is critical to ensure the safe handling of NDV in the laboratory setting [3-8]. While research efforts have focused on developing improved vaccines

against NDV [9-12], mechanistic research into characterizing the avian immune response to viral pathogens is still needed to optimize vaccines that are highly effective. Specifically, continued studies focusing on the role of and type of humoral and cellular immune response associated with NDV infection at multiple stages of exposure are needed.

Experiments to specifically study innate and adaptive immune responses to NDV require timed infection exposures with the activated and inactivated virus. To conduct vaccination experiments that allow evaluation of the bird's immune system, samples need to be transported from a biosafety level (BSL) 2 facility to a BSL1 facility to perform several immune-based assays, such as flow cytometric analysis. It is therefore crucial for the virus to be completely inactivated. In the present study, we evaluated a fixation procedure that would eliminate the risk of transferring live NDV to lower biosafety levels and minimize release of the virus into the environment, while allowing cellular preservation for select immune studies.

Inactivation of NDV can be achieved using heat, β-propiolactone (BPL), binary ethylenimine, formalin or ultraviolet (UV) light [13-16]. However, currently available methods for NDV inactivation have not being designed or tested for use in immunological studies and some may compromise the structure of the plasma membrane of the host cells, while others have not been tested for their efficacy against high viral titers. In addition, the noted shortcomings of these inactivation methods include inactivation inconsistency, inactivation of low NDV titers, incomplete confirmation of inactivation through several passages in eggs that extend at least 5 days per passage and lack of testing with splenic lymphocytes and flow cytometric analysis. *Sutton et al* (2013), successfully inactivated NDV using UVB light; however, other studies have been unable to inactivate NDV using UVC light demonstrating that further research needs is needed to ascertain whether this method can reliably be used or even proposed as an effective protocol [15, 16].

Qayyum *et al* (1999) achieved successful inactivation of NDV using heat for 45 min, at a pH of 1 and 13 for 6 hr, in 0.48% formalin for 30 min, and several concentrations of disinfectants such as Phenol crystals, iosan and bromosept for 15 and 30 min [15]. Nevertheless, they were unable to successfully inactivate the virus with UV light. However, their evaluation was not as sensitive as the one performed in the present study as only 2 HA units were used for inactivation, only one passage of eggs was performed to verify viral inactivation and the eggs were only incubated for 48 h.

Previous studies by *King* (1991) showed that inactivation of NDV was possible using 0.04 and 0.1% formalin for 16 h at 37˚C, 0.025% and higher concentrations of BPL for 18 h at 4˚C, and heat (60˚C) in less than 30 min [13]. In comparison, the current protocol in this study used 4% formalin. This increase in concentration greatly reduced the inactivation time from 16 h to 45 min, allowing for same day analysis of samples, more efficient use of personnel time and faster data acquisition. *King* (1991) was unable to inactivate high concentrations of NDV in 120 min at 56˚C, but achieved inactivation in less than 30 mins at 60˚C. Swayne *et al.* also achieved inactivation of NDV using high temperatures ranging from 55˚C and 63˚C [14]. Although very successful in inactivating NDV, heat inactivation is not suitable for immune phenotyping via flow cytometry. This can lead to protein denaturing, which negatively impacts antibody cell surface protein recognition binding during the immune phenotyping procedure. These conformational changes can be prevented at least short term with formaldehyde fixation [17].

The aim of the present study was to test the effectiveness of a commercial intracellular fixation buffer (ICB), which is based on a 4% formaldehyde solution, as an appropriate inactivation procedure for NDV. Formalin is often used as a cellular fixative for flow cytometric analysis and as a tissue fixative for histopathology [18]. We hypothesized that ICB can inactivate high concentrations of NDV without compromising phenotype identification using flow cytometry. For this purpose, we modified a fixative

protocol normally used for fixation of lymphocytes for flow cytometric analysis using ICB [18]. This procedure was adapted to inactivate NDV from allantoic fluid (AF), infected chicken macrophage HD11 cells and infected splenocytes isolated from specific pathogen free (SPF) chickens.

## 4.3. Materials and Methods

### 4.3.1 Viruses

All viruses used were lentogenic NDV strains, or strains of high pathogenicity that have been attenuated by a change in the fusion protein cleavage site. Recombinant ZJ1-L is an attenuated version of ZJ1 (Goose/China/ZJ1/2000; GB AF431744.3) that was previously generated in our laboratory through reverse genetics. Recombinant ZJ1-L has a mean death time (MDT) of over 175 h and an intracerebral pathogenicity index (ICPI) between 0.3 and 0.43. The NDV strain LaSota (LS) is used worldwide as a live or inactivated vaccine. The LS vaccine strain has a MDT of 110 hours (h) and an ICPI of 0.15 [19]. Recombinant LS with the red fluorescent protein (LS-RFP) was generated in our laboratory as previously described [19]. It has a MDT of 127 h and an ICPI of 0.00. All viruses were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) stocks and propagated in 9-11-day-old SPF embryonated chicken eggs (ECEs).

### 4.3.2 Eggs and cells

SEPRL White Leghorn SPF flocks were the source of all adult chickens and ECEs. All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee (IBC) and were conducted with appropriate measures to maintain biosecurity and biosafety. All protocols were carried out in accordance with the procedures reviewed and approved by the SEPRL Institutional

Animal Care and Use Committee (IACUC), as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. The HD11 cells, a chicken macrophage cell line, were grown and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 media (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 Mm L-Glutamine (Gibco), 100 U/mL of Penicillin (Gibco) and 100 µg/mL of Streptomycin (Gibco), and incubated at 37°C under 5% CO2 atmosphere.

4.3.3 Lymphocyte Isolation

Lymphocyte isolation from spleen was modified from a protocol previously described [18, 20]. Briefly, three adult SPF White Leghorn hens were euthanized through cervical dislocation and the spleens were aseptically removed and placed into 50 mL conical tubes containing 15 mL of PBS. Spleens were passed through a 70 µm cell strainer (Fisher) into a sterile petri dish containing 6 mL PBS, using the plunger of a 20mL syringe. The cell suspension was mixed and then transferred into a 50-mL conical tube to be centrifuged at 290xg for 7 min at RT. The supernatant was discarded, and the cell pellet was re-suspended with 14 mL PBS. The 14 mL of cell suspension was overlaid onto 7mL of Histopaque 1.077 (Sigma) in a 50-mL centrifuge tube and centrifuged at 450 xg for 30 min at RT. Following centrifugation and using a glass Pasteur pipette, the opaque interface containing the lymphocytes was removed and washed 3 times in 10 mL PBS, centrifuging at 450 xg for 10 min at 18 ºC. Following the final wash, the cells were re-suspended in RPMI-1640 supplemented with 10% FBS, 2 mM of L-Glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cells were enumerated on a cellometer Auto T4 (Nexcelom, Lawrence, MA) and adjusted to 5.0 x $10^6$/mL, in complete media.

4.3.4 Inactivation Protocols

A commercially available ICB, formulated with 4% formaldehyde was employed for all the inactivation protocols (eBiosciences, Cat. # 00-8222). It is a component of the Fixation and Permeabilization Kit (eBiosciences, Cat. # 88-8823), which is designed to prepare cells for staining for use in flow cytometric analysis. ICB is a cross-linking and stabilizing reagent which is designed to fix the cell's membrane.

4.3.4.1 Inactivation of NDV suspended in AF via fixation at 23ºC and 4ºC

Three different dilutions of three NDV strains were tested to determine the capacity of the ICB to inactivate live viruses. The ZJ1-L, LS or LS-RFP viral strains were diluted in ICB at a 1:2, 1:4 or 3:4 dilution. Each virus came from infected AF stocks with titers ranging between $10^{9.3}$ and $10^{9.5}$ embryo infectious dose 50% ($EID_{50}$) per mL. Dilutions were made in 1.5 mL microcentrifuge tubes, with a final volume of 200 μl. The first dilution contained 100 μL virus and 100 μl ICB (1:2), the second contained 50 μl virus and 150 μl ICB (1:4), and the third contained 150 μl virus and 50μl ICB (3:4). After the buffer and virus were mixed together, all groups were incubated for 45 min at 23ºC or at 4°C in the dark. After incubation, 10-fold dilutions from each AF-ICB mixture were prepared, using brain heart infusion (BHI) containing penicillin (2000 IU/mL), gentamicin (200 μg/mL), and amphotericin B (4 μg/mL). Of these dilutions, 100 μL was inoculated into each of five 9-day-old ECEs per dilution.

In addition, 5 ECEs were inoculated with 100 μL of a 10-fold dilution of non-ICB exposed LS, ZJ1-L or LS-RFP virus, to be used as a positive control. As negative controls, 5 ECEs were inoculated with 100 μL of a 10-fold dilution of non-infected AF mixed with ICB, and 5 more ECEs were inoculated with 100ul of BHI. Every 24 h for up to 96 h, ECEs were candled and mortality was recorded. After death or at the end of the experimental

period (96 h), AFs were harvested from chilled eggs, and viral presence was then determined by hemagglutination assays (HAs) as previously described [21, 22].

Five hundred microliters of the AF collected from each egg were pooled with the AF collected from the other eggs from the same group. The pooled AF was then inoculated into 5 ECEs (100 μL per egg) as passage 2. The second passage ECEs were candled every 24 h for 96 h and mortality was recorded. After death or at the end of the experimental period (96 h), AFs were harvested from chilled eggs, and viral presence was then determined by HA.

4.3.4.2 Inactivation of NDV via 4ºC fixation in HD11 inoculated cells

Macrophage- HD11 cells were seeded in 6-well plates at a density of 5 x $10^6$ cells per well, in 2 mL of media. The next day, the cells were infected with a multiplicity of infection (MOI) of 10 of the LS NDV strain, in 500 µL of media, in triplicates. Four wells were left uninfected. Of the uninfected cells, one well was fixed for 45 min, one was fixed for 45 min and lysed by freezing and thawing at -80 degrees 3 times, one was fixed for 45 min but not lysed, and one was not fixed nor lysed. The infected cells were incubated for 1 h, rocking the plates every 15 min after which the inoculum was removed, and the cells were washed once with phosphate buffered saline (PBS) to remove the excess virus. Thereafter, 2 mL of RPMI-1640 media supplemented with 10% FBS, 2 mM of L-Glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin were added to each well, and the cells were incubated at 37°C under a 5% $CO_2$ atmosphere. The cell culture supernatant was removed at 24 h of culture and the cells were washed once with 2mL PBS.

Cells were fixed by adding 100 µL of ICB to each well and incubated for 45 min, 4ºC in the dark. A 10-fold dilution was done of the fixed samples, and 100 µL/replicate were inoculated in each of 5 ECEs per group (15 ECEs total). Three wells of cells were fixed for 45 min, 3 wells were fixed for 45 min and then lysed, 3 wells were lysed without

being fixed for back-titer calculation, 3 wells were not lysed nor fixed, also for back-titer calculation.

Ten-fold dilutions from $10^{-1}$ to $10^{-7}$ were generated from the non-fixed wells to get a viral titer. Each dilution from $10^{-2}$ to $10^{-7}$ was inoculated into 5 ECEs (100 µL each). Five hundred microliters of the AF collected from each egg were pooled with the AF collected from the other eggs from the same group. The pooled AFs were inoculated into 5 ECEs (100 µL per egg) as passage 2. The second passage ECEs were candled every 24 h for 96 h and mortality was recorded. After death or at the end of the experimental period (96 h), AFs were harvested from chilled eggs, and viral presence was then determined by HA.

4.3.4.3 Inactivation of NDV via fixation in splenocytes

Splenocytes were isolated as described below in section 2.4 and were seeded, infected and fixed as described in section 2.3.2. with minor adjustments. Briefly, lymphocytes were seeded at a density of $5 \times 10^5$ cells per well in 96-well plates, and were infected with a MOI of 10 of the LS strain, in triplicates. After 24 h of culture, the cell culture supernatant was removed, the cells were washed, and three wells were fixed for 45 min at 23°C, 3 wells were fixed for 45 min at 4°C, 3 wells were left uninfected, and cultured and fixed for 45 min at 23°C, 3 wells were left uninfected and cultured and fixed for 45 min at 4°C, 3 wells were infected but not fixed, to be used for back-titer calculation. Splenocytes were inactivated by adding 100 µL of ICB to 100 µL of PBS and incubating together for 45 min, at 23˚C or 4ºC. A 10-fold dilution was done of the fixed samples, and 100 µL /replicate were inoculated in each of 5 ECEs and two passages were tested for HA activity. Ten-fold dilutions from the non-fixed wells were also inoculated into 5 ECEs.

4.3.5 Flow Cytometric Analysis

Determination of lymphocyte subpopulations in spleens from SPF birds was conducted as previously described [18]. (i) Splenocytes were seeded in 96-well plates at a density of 5 x $10^5$ cells per well, in 100 µL of media. (ii) Splenocytes (5.0 x $10^5$/sample) were then stained with phycoerythrin (PE) conjugated anti-chicken CD4 (0.4 µg/sample) and/or fluorescein isothiocyanate (FITC) conjugated anti-chicken CD8 (0.5 µg/sample) antibodies (Southern Biotech, Birmingham, AL) for 30 min at 4°C in the dark. (iii) The cells were washed with PBS and centrifuged at 200 xg for 10 min at 4°C and re-suspended with 100 µL of PBS. All samples were stained in duplicate, half were fixed with 100 µL of 4% formaldehyde, and the other half of the samples were left unfixed. (iv) Samples were stained by adding 100 µL of ICB (eBiosciences) to each well and incubating for 45 min, at 4◦C, in the dark. (v) Samples were evaluated on a CyAn flow cytometer (Beckman Coulter, Brea, CA) measuring 10,000 events per sample.

4.4. Results

*4.4.1 Inactivation of NDV in AF using ICB.*

All the AF samples from eggs inoculated with the 1:4 and a 1:2 dilution of virus in ICB, at both 23ºC and at 4°C were negative for HA activity (**Table 4.1**). The second passage in the eggs was also negative for HA activity. However, a 3:4 dilution of virus in ICB was positive for HA activity at both 23ºC and at 4°C, in the first passage. A second passage from this treatment group was not performed.

4.4.2 *Inactivation of NDV in macrophage HD11 supernatants using ICB*

Macrophage HD11 cells were inoculated with the LS strain at an MOI of 10 and the virus was inactivated using 100 µL ICB for 45 min at 4°C. All AF collected from ECEs inoculated with media control and inactivated virus was negative for HA activity (**Table 4.2**). Five

eggs were inoculated per group. The AFs were collected and inoculated into another 15 eggs as a second passage. The ECEs inoculated from cells that were not inactivated with ICB had viral titers of $10^{7.6}$ $EID_{50}$/ mL for the lysed, non-fixed group. Lysing the cells by freeze thaw did not affect the viral titers, as non-lysed non-fixed cells has NDV titers of $10^{7.5}$ $EID_{50}$/ mL.

4.4.3 *Inactivation of splenocyte supernatants using ICB*

Splenocytes were inoculated with the LS strain at an MOI of 10 for one h, and the virus was thereafter inactivated using 100 µL ICB for 45 min at 23˚C or 4°C. All AF from media control and virus inoculated ECEs was negative for HA activity (**Table 4.3**). Five eggs were inoculated per group. The AFs were collected and inoculated into another 15 eggs as a second passage. The ECEs inoculated from cells that were not inactivated with ICB had viral titers of $10^{4.9}$ $EID_{50}$/ mL.

*4.4.4 Fixation did not affect T cell monoclonal binding*

Lymphocytes from a healthy adult SPF chicken were isolated and stained with anti-chicken CD4 and CD8 antibodies, both or neither. A set of them were fixed with ICB while another set was left unstained**.** Phenotypic analysis of the T helper and T cytotoxic populations in the spleen revealed that cell fixation did not impact cell surface binding **(Figure 4.1**). The percentage of $CD4^+$ splenocytes ranged between 32.0 and 34.4% (**Table 4.4**). For $CD8^+$ splenocytes, the range was between 35.5 and 36.5% (**Table 4.4**). All results were run in triplicate and were non-significant based on a Student's *t* test ($p<0.05$).

## 4.5. Discussion

In this study, a novel protocol was employed to inactivate two different strains of lentogenic NDV (LS and LS-RFP) and one strain of virulent NDV (ZJ1-L) attenuated by a mutation of the cleavage site of the fusion protein. The viruses were inactivated in AF and the LS strain was also inactivated in avian splenocytes and in a chicken macrophage cell line. The results demonstrate that the fixation protocol used in this study can inactivate actively replicating viruses inside chicken immune cells. In unfixed avian macrophages, the backtiters demonstrated viral loads of $10^{7.5}$ $EID_{50}$/ mL, whereas in the unfixed splenocytes, they were $10^{4.9}$ $EID_{50}$/ mL. In addition, this fixation method was also able to achieve inactivation of $10^{9.5}$ $EID_{50}$/ mL of NDV in AF.

Although the viruses used in this study had the characteristics of lentogenic NDV, their origins and genotype groupings were different. The ZJ1-L for example, was derived from the vZJ1 virus, which is a genotype VIId virus, whereas the LS strain and its recombinant LS-RFP both belong to the genotype II [23, 24]. The only difference between ZJ1-L and vZJ1 is the fusion cleavage site, as it was attenuated through site directed mutagenesis [18]. Therefore, it is reasonable to predict that our current inactivation protocol would be just as efficient with the other vNDV strains. Further studies still need to be performed to definitively confirm this issue.

In summary, ICB incubated for 45 min was effective at inactivating very high concentrations of NDV. Based on the results presented in Table 1, we were able to inactivate up to $10^{8.5}$ $EID_{50}$ of NDV (100 µL of $10^{9.5}$ $EID_{50}$/ mL) using 100 µL ICB. However, we were unable to inactivate $10^{8.66}$ $EID_{50}$ (150 µL of $10^{9.5}$ $EID_{50}$/ mL) using 50 µL ICB. Further, ambient or refrigerated temperature (23°C vs 4°C) did not seem to influence inactivation of NDV using 4% ICB for 45 min. Overall, high concentrations of NDV in AF, chicken splenocytes, and macrophages can be inactivated using 100 µL ICB.

Further, this method did not compromise cell phenotyping of enriched chicken splenocyte cell suspensions.

## 4.6. References


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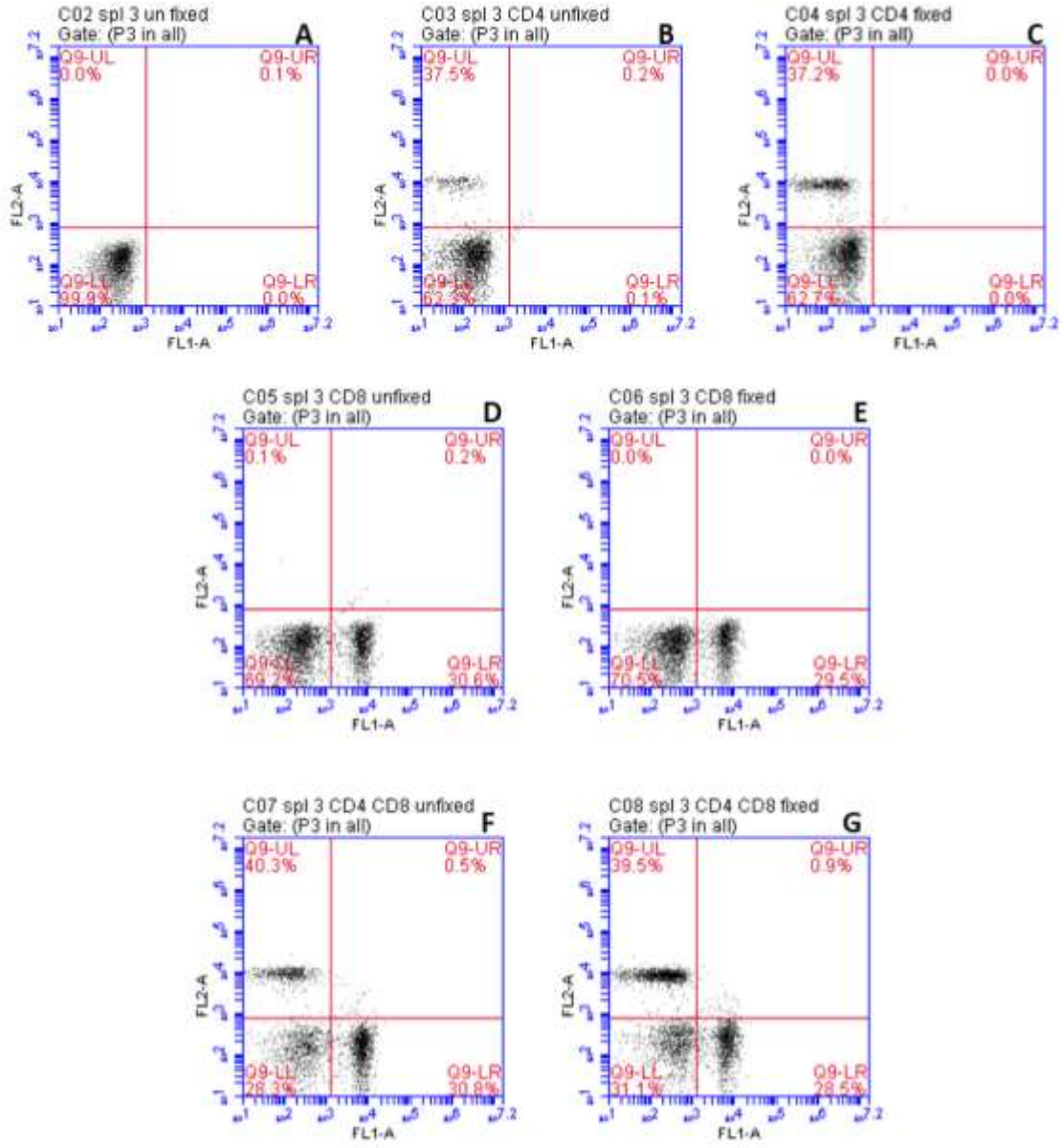
**Figure 4.1. Effect of fixation on splenic T cell phenotype.** Representative histograms of splenic lymphocytes from a healthy adult specific pathogen free chicken were isolated and stained with anti-chicken CD4 (**B, C)** and CD8 (**D, E**) antibodies or both (**F,G**). Histograms **C, E** and **G** were from ICB-fixed cells and Histograms **B, D** and **G** were unfixed. Histogram **A** are of unstained and unfixed cells.



| Table 4.1. Effect of 23ºC and 4°C fixation on inactivation of three lentogenic NDV strains in AF. | | | | | |
|---|---|---|---|---|---|
| | | Incubated at RT | | Incubated at 4°C | |
| | | Total Dead | HA + | Total Dead | HA+ |
| Negative Controls | BHI | 0 | 0/3 | 0 | 0/3 |
| | BHI + ICB | 0 | 0/3 | 0 | 0/3 |
| 100µl virus 100µl ICB | LS + ICB | 0 | 0/5 | 0 | 0/5 |
| | rZJ1*L + ICB | 0 | 0/5 | 0 | 0/5 |
| | LS-RFP + ICB | 1* | 0/5 | 0 | 0/5 |
| 50µl virus 150µl ICB | LS + ICB | 0 | 0/5 | 0 | 0/5 |
| | rZJ1*L + ICB | 0 | 0/5 | 0 | 0/5 |
| | LS-RFP + ICB | 0 | 0/5 | 0 | 0/5 |
| 150µl virus 50µl ICB | LS + ICB | 0 | 0/5 | 0 | 0/5 |
| | rZJ1*L + ICB | 0 | **3/5** | 0 | 1/5 |
| | LS-RFP + ICB | 0 | **1/5** | 0 | 0/5 |
| Virus alone | LS | 3 | 3/3 | 3 | 3/3 |
| | rZJ1*L | 3 | 3/3 | 3 | 3/3 |
| | LS-RFP | 2 | 3/3 | 2 | 3/3 |
| Passage 2 | | | | | |
| 100µl virus 100µl ICB | LS + ICB | | 0/5 | 1 | 0/5 |
| | rZJ1*L + ICB | | 0/5 | | 0/5 |
| | LS-RFP + ICB | 2 | 0/5 | 1 | 0/5 |
| 50µl virus 150µl ICB | LS + ICB | 1 | 0/5 | | 0/5 |
| | rZJ1*L + ICB | | 0/5 | 1 | 0/5 |
| | LS-RFP + ICB | | 0/5 | | 0/5 |
| | **LS-RFP + ICB | | 0/5 | | |

*The mortality of this egg was unexpected; therefore, this individual egg was passed onto another 5 eggs.

| Table 4.2. Effect of 4°C fixation on inactivation of LaSota (LS) strain-infected HD11 cells | | | | |
|---|---|---|---|---|
| **Passage 1** | | **Eggs Inoculated** | **Total Mortality** | **HA+** |
| **Media Controls** | **Not fixed or lysed** | 5 | 0 | 0/5 |
| | **Lysed Cells** | 5 | 0 | 0/5 |
| | **Fixed and Lysed cells** | 5 | 0 | 0/5 |
| | **Fixed cells** | 5 | 0 | 0/5 |
| **Virus Inoculated Cells** | **Fixed, Lysed cells** | 15 | 0 | 0/15 |
| | **Fixed, Not lysed** | 15 | 0 | 0/15 |
| | **Lysed, Non- Fixed cells** | Calculated titer of virus was $10^{7.6}$ $EID_{50}$/ mL | | |
| | **Non- Lysed, Non-Fixed cells** | Calculated titer of virus was $10^{7.5}$ $EID_{50}$/ mL | | |
| **Passage 2** | | | | |
| **Virus Inoculated Cells** | **Fixed and Lysed cells** | **15** | 1 | 0/15 |
| | **Fixed, Not lysed** | **15** | 0 | 0/15 |

| Table 4.3. Effect of RT or 4°C fixation on inactivation of LaSota (LS) strain-infected splenocytes isolated from adult SPF White Leghorns. | | | |
|---|---|---|---|
| **Passage 1** | **Eggs Inoculated** | **Total Mortality** | **HA+** |
| **RT Media Controls** | 5 | 0 | 0/5 |
| **4°C Media Control** | 5 | 0 | 0/5 |
| **RT Fixed** | 5 | 0 | 0/5 |
| **4°C Fixed** | 5 | 0 | 0/5 |
| **Passage 2** | | | |
| **LS Fixed Cells 4°C** | 15 | 0 | 0/15 |
| **LS Fixed Cells RT** | 15 | 0 | 0/15 |

| **Table 4.4. Comparing lymphocyte subpopulations before and after fixation with ICB at 4°C.** | | | |
|---|---|---|---|
| | % CD4+CD8- (Mean ± SEM) | % CD4-CD8- (Mean ± SEM) | % CD4-CD8+ (Mean ± SEM) |
| Unstained Unfixed Cells | 0.14 ± 0.10 | 99.59 ± 0.18 | 0.18 ± 0.10 |
| Unstained Fixed Cells | 0.00 ± 0.00 | 99.93 ± 0.02 | 0.03 ± 0.01 |
| CD4 Stained Unfixed | 32.63 ± 4.28 | 67.17 ± 4.33 | 0.08 ± 0.02 |
| CD4 Stained fixed | 32.01 ± 4.24 | 67.91 ± 4.22 | 0.02 ± 0.01 |
| CD8 Stained Unfixed | 0.03 ± 0.02 | 64.39 ± 2.58 | 35.46 ± 2.62 |
| CD8 Stained Fixed | 0.00 ± 0.00 | 63.94 ± 3.65 | 36.03 ± 3.64 |
| CD4 + CD8 Stained Unfixed | 34.42 ± 5.36 | 29.26 ± 3.78 | 35.79 ± 2.62 |
| CD4 CD8 Stained Fixed | 33.42 ± 4.40 | 29.48 ± 2.51 | 36.49 ± 4.06 |

n= 3 replicate experiments, *$p$< 0.05, Student's *t* test.

# CHAPTER 5

# IMMUNOLOGICAL RESPONSE AND PROTECTION OF CHICKENS USING AN ATTENUATED RECOMBINANT NEWCASTLE DISEASE VIRUS VACCINE, *IN OVO*[1]

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Marcano, V., Cardenas Garcia, S., Gogal Jr., R.M., Miller, P. J., Brown, C. C., Latiff Butt, S., Stanton, J., Goraichuk, I., Dimitrov, K. M., Taylor, T. L., Afonso, C. L. To be submitted to Vaccine.

5.1. Abstract

In ovo vaccination has been used commercially for over 20 years to control avian diseases. Unfortunately, current NDV vaccines have significant limitations for in ovo use, including embryo mortality and the inability to induce full protection during the first two weeks of life. Live attenuated recombinant Newcastle disease virus vaccines containing an a “reverse” (R) antisense encoding region of the chicken interleukin 4 inserted (IL4R) into the viral genome were evaluated as chicken vaccine candidates against a homologous challenge done with a genotype VIId virulent NDV via the *in ovo* route. The IL4R insert was traduced into the backbone of the ZJ1-L or LaSota strains. The effects of infection with ZJ1-L-IL4R or its parent virus, ZJ1-L on T cell proliferation and IFNγ gamma production in lymphocytes from naïve specific pathogen free (SPF) chickens were assessed. SPF embryonated chicken eggs (ECEs) were inoculated with $10^{3.5}$ $EID_{50}$/ ECE each vaccine or vehicle-alone at 19 days of embryonation (DOE). Survival post vaccination and post challenge, differences in body weights, vaccine shed titers, and antibody titers, cell-mediated immunity (CMI) recall response in vaccinated birds and the percentages of IgM+, Bu1+, CD4+CD8- and CD4-CD8+ cells were measured. Lymphocytes from SPF naïve birds infected with ZJ1-L-IL4R produced significantly lower levels of IFNγ compared to infected with ZJ1-L. However, lymphocytes isolated from birds vaccinated with ZJ1-L-IL4R, LS-IL4, and LS-RFP did not produce IFNγ levels that were significantly different. No significant differences between the percentages of IgM+, Bu1+, CD4+CD8- and CD4-CD8+ cells of different vaccine groups were observed. Our results indicate that ZJ1-L-IL4R is an excellent candidate for *in ovo* vaccination, as it compared favorably to the commercial strains in terms of protection without negatively impacting survival body weight gain. However, our results do not confirm that the improvement in survival and body weight seen in birds vaccinated with ZJ1-L-IL4R compared to ZJ1-L is due the insert of the antisense RNA and further work must be done to resolve this.

## 5.2. Introduction

Newcastle disease (ND) infects over 270 avian species with chickens and turkeys being highly susceptible to disease and ducks and geese often showing few or no clinical signs [1, 2]. ND continues to be a threat to the poultry industry world-wide. The causative agents are virulent (velogenic and mesogenic) strains of Newcastle disease virus (NDV) from the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, genera *Avulavirus*, and species *avian paramyxovirus* (*APMV*). Limited biosecurity in combination with vaccination has been unable to stop the occurrence of outbreaks with virulent Newcastle disease virus (vNDV) around the world particularly in developing countries [3-8].

Live NDV vaccines are inexpensive to produce and are easily mass administered in water and through spray. Live vaccines replicate within the host, therefore no adjuvants are required, less antigen is needed to induce an immune response, and they are able to induce immunity faster [9]. Available live NDV vaccines induce a strong cell- mediated and humoral immune response, providing complete immunity within two weeks of oral or ocular delivery and induce mucosal immunity [10-13]. However, these live NDV vaccines can be easily killed by chemicals or high temperature, emphasizing the importance of maintaining a cold chain for the survival of the vaccine virus. In addition, their replication within the host may lead to adverse reactions [9]. For example, some live vaccine strains (LaSota strain), may cause mild to moderate respiratory disease and reduce productivity [14]. Inactivated vaccines do not replicate in the host and therefore, do not induce clinical disease nor elicit a strong cell-mediated immune response [9, 15]; however they can induce a protective and long-lasting antibody response [16]. They are more stable than live attenuated vaccines, but are more expensive to produce, more labor intensive to apply, require the use of adjuvants, higher levels of antigens and boosters to induce appropriate immunity [9].

*The in ovo* vaccination route was first described in 1982 for use against Marek's disease and since then, has been used commercially to control select avian diseases (i.e. Marek's disease and infectious bursal disease) [17]. The advantages of *in ovo* vaccination over the conventional route include a significant reduction in costs, induction of earlier immunity, uniform delivery of vaccine, and reduction of stress [17, 18]. In poultry, however, *in ovo* vaccination for ND has been less efficacious. Current commercially available vaccines against NDV such as LaSota, I2, Hitchner B1 and NDV substrain of Ulster 2C can cause significant mortality when used *in ovo* [19-21]. Additionally, *in ovo* vaccination using the current vaccine strains have failed to develop adequate levels of protective antibodies. Currently, there are only two commercial ND vaccines approved for use *in ovo*. The first vaccine is a live NDV conjugated with an antibody. The antibody is slowly released from the virus over time, which lowers the mortality risk observed when using a live NDV *in ovo* vaccine [22]. However, results with this vaccine have been inconsistent, with ~50% of vaccinated birds displaying adverse clinical signs and mortality post challenge with NDV, despite significantly higher antibody levels. The second vaccine is a recombinant herpes turkey virus (rHVT) expressing the F protein from NDV. This rHVT confers long-lasting protection against challenge with vNDV after a single application without causing clinical signs. Unfortunately, it takes over 4 weeks to mount a protective immune response and currently, that there is no reliable test to determine and monitor the antibody response of vaccinated flocks [23, 24]. In 1982, it was reported that HVT, which by then was already used widely as a vaccine for prevention of Marek's disease, provided protection in chickens vaccinated at 18 days of embryonation (DOE) [17]. The herpesvirus can replicate in chickens, is non-pathogenic and inoculation of chickens with HVT has been found to prevent tumors normally caused by virulent Marek's disease virus both in chickens with and without maternal antibodies. HVT has a large double stranded DNA genome and the ability to replicate efficiently without causing embryo pathogenesis which

makes it a good candidate as a recombinant vaccine. Several HVT recombinants have been developed since 1982, including one expressing the fusion (F) gene of ND virus (NDV) [24]. When given at 18 DOE, this recombinant vaccine was able to provide significant protection in specific pathogen free (SPF) and commercial eggs after 4 and 5 weeks-of-age, respectively. Studies with the HVT vaccine, but not *in ovo*, have found that HVT vaccination induces a strong cell-mediated and humoral immune response denoted by increased activation of natural killer cells [25], cell-mediated cytotoxicity of lymphocytes [26], and production of antibodies [27].

In this study, we combined *in ovo* vaccination with antisense cytokine technology to improve current vaccination strategies against NDV, with the goal of minimizing adverse clinical signs and lowering bird mortality. Cytokines have been employed as intranasal vaccine adjuvants as an alternative to non-specific and more toxic agents [28]. Sawant and colleagues used a DNA vector to deliver NDV protective antigens in combination with the selected cytokines [29]. While the DNA vaccine alone only conferred 10% protection, this was increased to 40% when the IL-4 gene construct was administered with the vaccine. While previous studies have found that IFNγ attenuated infection with vNDV, other studies have reported no enhanced protection against NDV infection when recombinant NDV vaccines containing type II IFNs were administered post hatch or *in ovo* [30, 31].

Antisense ribonucleic acid (asRNA) transcripts are a class of non-coding RNAs (ncRNAs) that are complementary to those of a sense RNA transcript that may or may not code a protein [32]. Antisense technology takes advantage of the hydrogen bonds that form between complementary bases of nucleotides to silence sense RNA through binding of antisense RNA. Antisense can affect transcription of the sense RNA through transcriptional interference [32]. It has been reported that antisense RNA can cause transcriptional interference through several mechanisms, thereby modulating gene

expression [32]. Transcription of the sense RNA can be affected through promoter competition, DNA methylation, or histone modifications [32]. These modifications can reduce gene expression or completely silence partner genes [33-35].

In the present study, we investigated the ability of an attenuated recombinant Newcastle disease virus vaccine containing an antisense chicken interleukin 4 insert (ZJ1-L-IL4R) to modulate adaptive immunity. We hypothesized that antisense IL4 would inhibit the production of IL4 during the establishment of primary immunity during NDV vaccination. This inhibition could possibly shift the TH1/Th2 balance resulting in a subtle increase in IFNγ production, improving recall response and allowing for earlier protection against NDV challenge.

## 5.3. Materials and Methods

*5.3.1. Viruses:* Velogenic NDV (vNDV) ZJ1 (Goose/China/ZJ1/2000; GB AF431744.3), a genotype VIId virus, was used as a challenge virus in the *in ovo* vaccination experiment. NDV strain LaSota (LS) is used worldwide as a live or inactivated vaccine and will be used here as a control vaccine in the immunization-challenge experiments. Recombinant ZJ1-L (rZJ1-L) is an attenuated version of ZJ1 that was previously generated in our laboratory through reverse genetics from a backbone originated from China and modified at the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA). Recombinant LS with the red fluorescent protein (LS-RFP) was generated in our laboratory as previously described and is used as a control in these studies [36]. The recombinant viruses ZJ1-L-IL4R was generated following a protocol previously described in our laboratory [31, 37]. This virus was included as a control virus for all the characterization and immunization experiments reported here. All viruses were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) virus stocks or repository,

propagated and titrated in 9-11 day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs).

5.3.2. LS-IL4R construction: The LS green fluorescent protein (GFP) plasmid (pLS-GFP) was used as a backbone to construct a recombinant virus (pLS-IL-4R) expressing chicken IL-4 (chIL-4) antisense through a two-step approach using the infusion cloning of PCR products that were generated using Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies, La Jolla, CA). pLS-GFP was propagated in Stbl2 cells at 30°C for 24 h and purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). First, a cDNA fragment of the 6963-6242 region was amplified from pLS-GFP, removing the GFP ORF (6243-6962), using primers pLSUP (GGTGGCTACAACTATCAACTAAACT) and pLSdown (GTGTGTAACTACCGTGTACTAAGC); additionally, the chIL-4 gene was amplified from the Y2880K-mRNA-N411 plasmid using the primers Anti IL4F (AGTTGTAGCCACCTCACTTATTTTTAGCTAGTTGG) and Anti IL4R (ACGGTAGTTACACACGTCATGAGCTCCTCACTGCCCAC). Subsequently, the PCR product containing the chIL-4 ORF was cloned antisense, through the design of the primers, into the noncoding region downstream of the P gene and upstream of the M gene of the pLS vector, as an additional transcription unit, using the In-Fusion PCR cloning kit (Clontech, Mountain View, CA). The resultant plasmid was designated pLS –IL-4R. The recombinant virus was rescued by reverse genetic techniques from pLS-IL-4R as described elsewhere [31], using Hep-2 cells grown and maintained in Dulbeco's Modified Eagle Medium (DMEM) (Corning cellgro, Invitrogen), supplemented with 5% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), at 37°C with a 5% CO2 atmosphere. The virus was designated rLS-IL-4R and further subjected to RNA extraction, RT-PCR and sequencing to confirm its identity. Low virulence was confirmed through intracerebral pathogenicity index (ICPI) and mean death time (MDT).

*5.3.3. Intracerebral pathogenicity index (ICPI) assay*: One-day-old SPF chicks were inoculated intracerebrally with 50 µl of a 1:10 dilution of allantoic fluid (AF) harvested from ECEs infected with ZJ1-L-IL4R or LS-IL4R. Birds were monitored every 24 hr for 8 days and scored as follows: 0 = normal, 1 = sick or 2 = dead. An equation was used to calculate the ICPI and any virus with a value ≥ 0.7 was considered virulent [38, 39]. The ZJ1-L, LS and LS-RFP strains have an ICPI of 0.3-0.45, 0.15 and 0.00, respectively [36].

*5.3.4. Mean death time (MDT):* Nine to eleven day-old SPF ECEs were inoculated as preciously described [40, 41] with ZJ1-L-IL4R or LS-IL4R. Following death or at the termination of the experiment (6 days post-inoculation), AFs were harvested from chilled ECEs to determine virus titers by HA test using the Spearmann-Karber method to calculate the $EID_{50}$ / ml [42]. The ZJ1-L, LS and LS-RFP strains have a MDT of over 175 h, 110 h and 125h, respectively [36].

*5.3.5. Chickens, ECEs and cells:* All adult chickens, fresh and 9-11-day-old ECEs were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) SPF flocks except the fresh ECEs used in vaccine experiment 1. For vaccine experiment 1, fresh SPF ECEs were obtained from Charles River Laboratories (Norwich, CT). Birds were provided food and water *ad libitum*. The HD11 cells, a chicken macrophage cell line, were grown and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), 2 Mm L-Glutamine, 100 U/mL of Penicillin and 100 µg/mL of Streptomycin, and incubated at 37°C under 5% CO2 atmosphere.

*5.3.6. Growth curves in SPF ECEs*

Nine-day old SPF ECEs were inoculated in the allantoic cavity with 100µl total of $10^{2.5}$ $EID_{50}$/ 0.1 mL of ZJ1-L, ZJ1-L-IL4R, LS-IL4R LS-RFP, or LS. At 1, 6, 12, 24, 36 48, 72 and 96 h post inoculation 3 ECEs from each group were chilled and allantoic fluid from was collected. Virus titers from allantoic fluid were determined as embryo infectious dose 50 % ($EID_{50}$) per mL using quantitative reverse transcriptase polymerase chain reaction (qRT PCR).

*5.3.7. Isolation of splenic lymphocytes:* Lymphocytes were isolated using as previously described [31, 37, 43]. Briefly, spleens were aseptically removed from euthanized White Leghorn chickens and placed in cold 1X PBS (HyClone) for transport back to the laboratory. For the following steps, all reagents and incubation periods were performed at 23 ± 3ºC. Spleens were gently passed through a 70 µm cell strainer (Fisher) into a sterile petri dish containing 1X PBS using the barrel of a 20-cc syringe. The strain was then rinsed with 1X PBS, and the suspension was centrifuged at 450 x G for 7 min at 18ºC. Supernatants were discarded, and the cell pellets were re-suspended with 8 mL of sterile 1X PBS. The cell suspensions were then layered over 5 mL of Histopaque 1.077 (Sigma) in a 15-mL sterile centrifuge tube and centrifuged at 450 x G for 30 min. Following centrifugation, the buffy coat interface was removed with a pipette transferred to a 15-mL sterile centrifuge tube and washed in 10 mL of sterile 1X PBS, then twice in incomplete media, centrifuging at 450 x G for 10 min. After the final wash, the cells were re-suspended in RPMI-1640 (HyClone) supplemented with 10% FBS (Gibco), 100 U/mL of penicillin, 100 µg/mL of streptomycin (Gibco) and 2 mM of L-glutamine (Gibco), and counted using an AutoT4 cell counter (Nexcelom Bioscience, Lawrence, MA), and diluted to a concentration of 5 x $10^6$ cells/mL.

*5.3.8. Effect of primary infection of lymphocytes with NDV on lymphocyte proliferation and cytokine production:* Lymphocytes from the spleens of six adult SPF chickens were isolated as described above and diluted to a concentration of 5 x $10^6$ cells/mL. To each well, 5 x $10^5$ cells in 100 µl were plated in a 96 well plate with 200 µl reduced-serum media. Cells from each bird were plated into 18 individual wells. Six wells were suspended in 200 µl media alone, 6 with ZJ1-L-IL4R and with ZJ1-L alone. Plates were set up twice, once with ZJ1-L-IL4R and ZJ1-L, at an MOI of 1.0 and once at an MOI of 0.1. Cells were incubated for 1 h, shaking briefly every 15 min. After 1 h, cells were washed once with 1x PBS and RPMI media (with 10 % FBS with 1 % streptomycin/penicillin at 41 °C with 5 % CO2). Supernatants were collected from the wells and replaced 100 µl fresh media. For each group of 6 wells, three were incubated with 5 µg/ mL ConA –a T cell mitogen, and 3 were incubated with media alone. Each plate was run in triplicates. At 24 h of culture, 2 plates were selected, and supernatants were collected for IFNγ and IL10 cytokine level determination by enzyme-linked immunosorbent assay (ELISA). Quantification was done using a commercially ELISA kit for the detection of chIL10 (Cat. # MBS2509095, MyBiosource, San Diego, CA), and IL4 (cat. # SEA077Ga, Cloud Clone Corp.) and an antibody pair ELISA kit to detect chIFNγ (Cat.# CAC1233, Invitrogen). The third plate was used for measurement of T cell proliferation by adding 20 µL of AlamarBlue® (BioRad) at 48 h post inoculation and comparing to the me. Plates were read at 72 h post-stimulation in a microplate reader using excitation and emission wavelengths of 530 and 590, respectively. The readings from triplicate wells were averaged and the average relative fluorescence units (RFU) of the media wells for each bird was subtracted from the treatment groups to calculate the delta mean (Δ mean).

*5.3.9. Vaccine experiment 1: Cell-mediated immunity (CMI) recall response of chickens vaccinated at 19 DOE with $10^{3.5}$ of various NDV vaccines:* Fresh (less than 24 h -old) SPF White Leghorn chicken purchased from Charles River Laboratories (Norwich, CT) were incubated until 19 DOE. At 19 DOE, these SPF ECEs were inoculated with $10^{3.5}$ $EID_{50}$/ ECE of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. ECEs (n = 45/ virus vaccine) were manually inoculated with 100 µl through the amniotic route, using 1mL syringes with 24 G x 1/2". After vaccination, each vaccine group was placed in an incubator (2362E Turbofan Hova-Bator Incubator by GQF Manufacturing Company Inc. Savannah, GA). Each incubator was placed inside of an BSL2 isolator and the temperature and humidity were monitored until 21 DOE. Temperatures remained between 99.0-100.0 °F and the humidity remained between 65 and 70% for all incubators. After hatch, chicks were monitored daily for survival and clinical signs until 28 DPH. Birds were weighed at 1, 7, 14 and 21 DPH. At 7 DPH 12 chicks per group were individually identified, bled and moved into BSL3 for challenge with $10^5$ $EID_{50}$/ bird of vZJ1 by the oculo-nasal route (100µl). At 28 DPH, spleens were collected for cytometric analysis and proliferation assays from non-challenged vaccinated birds.

*5.3.10. Lymphocyte proliferation assay:* Lymphocyte proliferation was assayed as previously described [31, 44]. Briefly, lymphocytes isolated from spleens were aseptically seeded into Corning tissue culture round bottom 96-well plates (Corning, MA) at a density of 5 x$10^5$ cells/well in 100 µL of supplemented growth media, and stimulated with Con A (5 or 10 µg/mL) (Sigma), $10^{6.5}$ or $10^{5.5}$ $EID_{50}$/well (titer before inactivation) of purified BPL-inactivated rZJ1*L, or media only. One hundred µL of each treatment were added to triplicate wells. Cells were incubated at 41 ºC in a 5% $CO_2$ atmosphere. Forty-eight hours later, 20 µL of AlamarBlue® (BioRad) were added to each well. Plates were read at 72 h post-stimulation as described above.

5.3.11. Flow cytometric analysis: Lymphocytes ($5x10^5$/sample) from vaccinated birds were stained with CD4, CD8, or a combination of CD8 and CD8, IgM, Bu-1 or a combination of IgM and Bu1, and examined with flow cytometry using a previously published method [43]. Briefly, 100 µL of cells ($5x10^5$ cells/ 100 µL) were added to each well of a 96 well plate. A standardized concentration of PE-anti-CD4, FITC-anti-CD8, PE-anti-Bu1, FITC-anti-IgM monoclonal antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL.) were added to the cells and incubated at 4ºC on an orbital mixer at a slow speed in the dark. After 30 min, cells were washed with 100µL of PBS at 250 x g, 10 min, supernatant removed via aspiration. One hundred microliters of IC Fixing buffer (ICB, eBiosciences) was added to each well and incubated for 45 min, 4ºC in the dark. Analysis was performed with a BD flow cytometer, enumerating 10,000 events and the data analyzed by the FlowJo program.

*5.3.12.* Vaccine Experiment 2: *In ovo* vaccination and challenge*:* Fresh (less than 24 h old) SPF White Leghorn chicken ECEs from the SEPRL flock were collected and washed, then incubated until 19 DOE. At 19 DOE SPF ECEs were inoculated with $10^{3.5}$ $EID_{50}$ of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or vehicle alone as described above (n = 70 - 90 ECEs/ virus vaccine). Average body weights were recorded at 0, 1, 2 7, 14, 23 and 30 DPH. Vaccine shed titers through the oropharyngeal (OP) and cloacal (CL) routes were measured at 0, 1, 2 and 7 DPH. Serum was collected at 8, 14 and 30 DPH. Thymus, spleen, pharynx, intestine and bursas were collected at 0, 1, 2 and 7 DPH for immunohistochemistry and histopathological analysis. Spleens were collected at 7, 14 and 30 DPH for flow cytometric analysis of T and B lymphocytes.

At 7 and 14 DPH 10 chicks in each group were individually identified, bled and moved into BSL3 for challenge with $10^5$ $EID_{50}$ vZJ1 by the oculo-nasal route (100µl). Challenged chickens were monitored daily for clinical signs (depression, swelling of the

head, conjunctivitis, and neurological signs), and mortality until 14 DPC. They were weighted at 7 and 14 days after challenge. OP and CL swab samples were collected at 2 and 4-days post-challenge (DPC). At 14 DPC, the remaining birds were bled for post-challenge serology and euthanized. Pre-challenge and post-challenge antibody titers were determined by hemagglutination inhibition (HI) assay [38, 39].

*5.3.13.* Vaccine experiment 3: *In ovo* vaccination with antisense chIL4 using two different viral backbones*:* Fresh (less than 24 h-old) SPF White Leghorn chicken ECEs from the SEPRL flock were collected and washed, then incubated until 19 DOE. At 19 DOE, SPF ECEs were inoculated with $10^{3.5}$ $EID_{50}$ of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS, or vehicle alone as described above (n = 30 - 35 ECEs except for the LS vaccinated group, which had 70 ECEs). Hatchability and survival were recorded until 28 DPH. Average body weights were recorded at 7, 14, and 21 DPH. Vaccine shed titers through the OP and CL routes were measured at 2, 4, 7, 9, 11 and 14 DPH. Serum was collected at 14 and 28 DPH. Spleens were collected at 35 DPH for proliferation assays.

*5.3.14. Virus isolation and titration of swabs*

To assess viral shed titers, OP and CL swab samples were obtained from each bird at 2 and 4 DPC and placed in separate tubes containing 1.5 mL of brain-heart infusion broth (BHI) with antibiotics (2000 U/mL penicillin G, 200 mg/mL gentamicin sulfate, and 4 mg/mL amphotericin B; Sigma Chemical Co., St. Louis, MO). Viral RNA was extracted and quantified as previously described [45]. Briefly, the RNA of the post-challenge swabs was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX, USA). Pre-challenge swabs were extracted without the use to Trizol. Quantitative real time RT-PCR (qRT-PCR) targeting the NDV M gene was performed using AgPath-ID one-step RT-PCR Kit (Ambion, Austin, TX, USA) and the

ABI 7500 Fast Real-Time PCR system. The calculated qRT-PCR lower detection limit for NDV was between $10^{1.5}$ and $10^{2.3}$ $EID_{50}$/mL.

*5.3.15. Hemagglutination inhibition (HI) assay*: HIs were performed as previously described [40, 41]. Briefly, 25 µl of PBS was placed into each well of a standard round bottom microtiter plate. Twenty-five microliters of bird serum were placed in the first well of a row or column and serially diluted. Twenty-five microliters of diluted antigen, containing 4 HA units/25µl, was placed into each well, then agitated on a microshaker for 1 min and incubated at 23 ± 3 °C for 30 min. Fifty microliters of 0.5% RBC's was added to each well, agitated for 1 min and incubated at 23 ± 3 °C for 45 min. The HI titer is the inverse of the last dilution that completely inhibited hemagglutination.

*5.3.16. Histopathology, immunohistochemistry, and in situ hybridization:*

Samples were collected immediately after euthanasia into 10% neutral buffered formalin and fixed for 54 h, then removed to 70% ethanol, and batch processed to paraffin. Sections were cut at 3µm for HE staining. Partner sections were placed on charged slides for immunohistochemistry procedures. Sections were heated at 70 °C to melt paraffin, then dewaxed in sequential changes of Hemo-De. Antigen retrieval consisted of microwaving in Antigen Unmasking solution, then the primary antibody was added for 4°C for overnight incubation. Signal enhancement was done with NovoLink Max Polymer Detection System (Leica Biosystems) according to manufacturers' instructions. Substrate was diaminobenzidine (DAB). Slides were counterstained lightly with hematoxylin and coverslipped for a permanent record.

For *in situ* hybridization, tissue sections were probed with riboprobes corresponding to NDV or the appropriate cytokine. The protocol is as described previously [46]. Briefly, tissue sections was deparaffinized, rehydrated, and digested with either 30

µg/ mL or 100 µg/ mL ProteinaseK at 37 ºC for 15 min. Hybridization was conducted overnight at 42 ºC with approximately 20 ng of probe in a prehybridization solution. After stringent washes, antidigoxigenin alkaline phosphatase was added to the sections. Development was done with chromogen/substrate nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolylphosphate. Tissues were counterstained lightly with hematoxylin and coverslipped for a permanent record.

*5.3.17. Statistical analysis:* Hemagglutination-Inhibition (HI) antibody titers and virus titers were expressed as arithmetic means plus or minus the standard error of the mean for each vaccine group. Group means were analyzed by ANOVA and Tukey's test for multiple comparisons when appropriate, and using Student's *t*-test when comparing only two groups at a time. The survival curves were analyzed using the Log-rank test. The level of significance used to determine statistical differences among groups was 5% ($\rho < 0.05$). The data was analyzed using Prism software version 6.0.

*5.3.18. Animal use and care:* All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee and were conducted with appropriate measures to maintain biosecurity and biosafety. General care of chickens was provided in accordance with the procedures reviewed and approved by the SEPRL Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

## 5.4. Results

### *5.4.1. Mean death time, intracerebral pathogenicity index and growth characteristics of viral strains*

The velogenic ZJ1 strain used for challenge had MDT and ICPI values characteristic of those of a velogenic NDV strain, 54.5 h and a 1.83, respectively. The NDT and ICPI levels of all vaccine strains were characteristic of lentogenic strains, with all MDTs longer than 100 h and all ICPI values at 0.3 or below [31, 36, 37] (Table 5.1). No significant differences in growth kinetics or viral yields were observed between the ZJ1-L-IL4R, ZJ1-L, LS-IL4R, and LS-RFP stains (Figure 5.1).

### *5.4.2. Effect of a primary NDV infection on lymphocyte proliferation and cytokine production*

A viral MOI of 0.1 induced low, non-significant levels of IL10 ranging between 0 and 10 pg/ml in lymphocytes isolated from adult SPF white leghorn chickens infected with ZJ1-L-IL4R or ZJ1-L in the presence or absence of ConA (data not shown). These levels were at or below the minimum detectable dose for the kit (9.375pg/mL). At MOI of 1.0, induced levels of IL10 were below the minimum detectable dose in lymphocytes inoculated with media, ConA alone, ZJ1-L-IL4R, and ZJ1-L, while the ConA + ZJ1-L-IL4R and ConA + ZJ1-L treatment groups had average IL10 levels of 24 and 20 pg/ mL, respectively. These levels were non-significant between groups (data not shown).

A viral MOI of 0.1 induced non-significant levels of IL4 ranging between 25.0 and 32.0 pg/ml. A viral MOI of 1.0 induced non-significant levels of IL4 ranging between 37.2 and 84.6 pg/ml (data not shown).

5.4.2.1. T Cell Proliferation levels. No significant differences in proliferation were observed between cells treated with ZJ1-L-IL4R, and ZJ1-L at either MOI in the presence or absence

of ConA (Figure 5.2). At an MOI of 0.1, ConA and ConA + ZJ1-L-IL4R – treated cells had significantly higher proliferative responses, compared to ZJ1-L-IL4R, and ZJ1-L treated cells (Figure 5.2a). At an MOI of 1.0, ConA, ConA + ZJ1-L and ConA + ZJ1-L-IL4R treated cells had average Δ means that were significantly higher compared to ZJ1-L-IL4R, and ZJ1-L (Figure 5.2b).

5.4.2.2. IFNγ levels. No significant differences in levels of IFNγ production between media, ZJ1-L-IL4R, and ZJ1-L inoculated cells were observed at MOI 0.1 and 1.0 (Figure 5.3a-b). Media controls had average IFNγ production levels of 17.4 ± 1.5 pg/ mL, while ConA controls had average IFNγ production levels of 1,505 ± 461 pg/ mL.

To account for the great variability observed in IFNγ production between lymphocytes from individual birds inoculated with ConA, ConA + ZJ1-L-IL4R and Con A + ZJ1-L, the ConA positive control for each bird was used to normalize the data. Lymphocytes inoculated with 0.1 MOI Con A + ZJ1-L produced significantly higher levels of IFNγ, compared to the ConA control and to cells inoculated with 0.1 MOI Con A + ZJ1-L-IL4R (Figure 5.3c). Lymphocytes inoculated with 1.0 MOI Con A + ZJ1-L produced significantly higher levels of IFNγ, compared to the ConA control and to cells inoculated with 1.0 MOI Con A + ZJ1-L-IL4R (Figure 5.3d). Lymphocytes inoculated with 1.0 MOI Con A + ZJ1-L-IL4R produced significantly higher levels of IFNγ compared to the ConA control.

5.4.3. *Vaccine experiment 1: CMI recall response of chickens vaccinated at 19 DOE with $10^{3.5}$ of Various NDV Vaccines.*

The survival of birds vaccinated with the ZJ1-L (71.1%) and LS-RFP (62.2%) vaccines was significantly lower than the survival of sham vaccinated birds (86.6%) (Figure 5.4a). LS vaccinated bird survival (26.7%) was significantly lower than that of all other groups.

Bird survival between 7 and 21 DPH was between 96.3 and 79.4% for all groups and was not significantly different between groups (Figure 5.4b). The birds vaccinated with LS that survived to 7 DPH were all challenged and therefore not included in the 7 to 21 DPH bird survival data.

5.4.3.1. Average Body Weights

At 14 DPH, ZJ1-L and LS-RFP vaccinated birds had significantly lower average body weights compared to both the mock and ZJ1-L-IL4R vaccinated groups (Table 5.2). At 21 DPH, ZJ1-L and LS-RFP birds had significantly lower weights compared to the mock vaccinated group. Since the surviving LS birds were challenged at 7 DPH, non-challenged bird weights were not measured at 14 and 21 DPH for this group.

5.4.3.2. Vaccine Shedding

Birds vaccinated with ZJ1-L and LS had average viral shed titers which were significantly higher compared to the ZJ1-L-IL4R and LS-RFP vaccinated birds (Figure 5.5).

5.4.3.3. HI Titers

At 23 DPH, birds vaccinated with LS-RFP had average HI which were significantly lower than the HI titers of ZJ1-L-IL4R and ZJ1-L vaccinated birds (Figure 5.6a). Post challenge, the HI titers of the vaccinated groups were not significantly different from one another (Figure 5.6b). HI titers of rZJ1-L-IL4R and ZJ1-L vaccinated birds were not significantly different from age matched birds challenged 16 days before. However, LS-RFP vaccinated birds challenged had significantly higher HI titers ($2^{8} \pm 2^{0.8}$) compared to the HI titers of age matched non-challenged vaccinated birds ($2^{5.5} \pm 2^{0.7}$).

5.4.3.4. Body Weights Post Challenge

At 7 DPC, mean body weights of ZJ1-L-IL4R vaccinated birds were significantly higher than those of ZJ1-L vaccinated birds (Table 5.3). At 14 DPC, average body weights of ZJ1-L-IL4R, LS-RFP and LS vaccinated birds were significantly higher than the ZJ1-L birds.

5.4.3.5. Viral Shedding Post Challenge

Birds vaccinated with ZJ1-L had significantly higher viral shed titers through the CL route at 2 DPC compared to all other groups (Figure 5.7b). At 4 DPC, mock vaccinated birds had viral shed titers of $10^{5.0}$ $EID_{50}$/ mL via the OP route and $10^{5.75}$ $EID_{50}$/ mL via the CL route (Figure 5.7c-d). Birds vaccinated with ZJ1-L-IL4R, LS-RFP and LS had significantly lower viral shed titers through the OP route at 4 DPC compared to the mock vaccinated birds (Figure 5.7c). Birds vaccinated with ZJ1-L-IL4R were shedding significantly lower viral shed titers through the OP route at 4 DPC compared to birds vaccinated with ZJ1-L. Birds vaccinated with LS-RFP were shedding significantly higher viral shed titers through the OP route at 4 DPC compared to birds vaccinated with ZJ1-L. Birds vaccinated with ZJ1-L-IL4R had lower viral shed titers compared to the mock vaccinated bird at 4 DPC through the CL route (Figure 5.7d).

5.4.3.6. Survival Post Challenge

After challenge with vNDV, mock vaccinated bird survival reached 0% at 5 days post challenge (Figure 5.8). The survival post challenge of birds vaccinated with ZJ1-L-IL4R was 100% survival, which was significantly higher than that of birds vaccinated with LS-RFP (58%) and the mock vaccinated birds. Birds vaccinated with ZJ1-L and LS, each had 3 birds succumb to challenge, resulting in 75% survival post challenge.

*5.4.3.7. CMI recall response*

Lymphocytes isolated from birds vaccinated with ZJ1-L-IL4R and stimulated using a BPL inactivated NDV antigen had significantly higher spontaneous proliferation (Δ means = 2394 ±310 RFU) compared with lymphocytes isolated from birds vaccinated with ZJ1-L (Δ means =1317 ±277 RFU) (Figure 5.9). The Δ means of lymphocytes isolated from birds vaccinated with ZJ1-L-IL4R, ZJ1-L, LS-RFP and stimulated with ConA were 7722 ±393 RFU, 7939 ±395 RFU and 7934 ±268 RFU, respectively (Figure 5.9). They were significantly higher than the Δ means of lymphocytes isolated from unvaccinated birds and stimulated with ConA, 6070 ±428 RFU.

5.4.3.8. Lymphocyte subpopulations. No significant differences in the splenic T cell and B cell subset expression were observed (Table 5.4). Birds vaccinated with ZJ1-L-IL4R had a lower numeric percentage of CD4+CD8- T cells compared to the BHI, ZJ1-L and LS-RFP vaccine groups and both ZJ1-L-IL4R and ZJ1-L led to higher percentages of CD4-CD8+ cells, compared to the mock and LS-RFP vaccine groups. The ratio of CD4-CD8+ to CD4+CD8- T cells was 1.8 ± 0.2 in the ZJ1-L-IL4R vaccine group, compared to 1.5-1.6 in all other vaccine groups. In fact, the CD4-CD8+ to CD4+CD8- ratio for 4 out the 5 birds in this group was between 1.8 and 2.2 (data not shown).

The average number of B cells IgM-Bu1+ cells remained between 1.1 and 1.4% for all groups. The proportion of IgM+Bu1+ in the ZJ1-L vaccine group was lower compared to that of the BHI, ZJ1-L-IL4R and LS-RFP vaccine groups. All vaccine groups had a decrease in the percentage of IgM+Bu1- cells compared to the BHI mock vaccine group.

5.4.4. Vaccine Experiment 2: *In ovo* vaccination and challenge. No significant differences in body weights were observed at 0, 1 and 2 DPH (Table 5.5). At 7 and 14 DPH, LS

vaccinated birds had average body weights which were significantly lower compared to mock, ZJ1-L-IL4R and LS-RFP vaccinated birds. At 23 and 30 DPH, the LS-RFP birds had significantly lower body weights than those of the ZJ1-L-IL4R vaccinated birds.

5.4.4.1. Vaccine viral shedding. At 0 DPH, birds vaccinated with LS were shedding significantly higher vaccine titers through the OP and CL route compared to ZJ1-L-IL4R vaccinated birds (Figure 5.10a-b). AT 1 DPH, the vaccine shed titers of birds vaccinated with ZJ1-L-IL4R were significantly lower through the OP route compared to the ZJ1-L, LS-RFP and LS vaccinated birds (Figure 5.10c). The vaccine shed titers of LS vaccinated birds were significantly higher than those of birds vaccinated with ZJ1-L and LS-RFP through the OP route at 1 DPH. The vaccine shed titers of LS vaccinated birds were significantly higher than those of all other vaccine groups through the CL route at 1 DPH (Figure 5.10d). At 2 DPH, LS vaccinated birds had significantly higher vaccine shed titers compared to all other groups (Figure 5.10e). No differences in vaccine shed titers were observed through the CL route at 2 DPH or through the OP or CL route at 7DPH (Figures 5.10f-h).

5.4.4.2. Serum HI Titers. At 8 DPH, birds vaccinated with LS had significantly lower serum HI titers compared to birds vaccinated with ZJ1-L-IL4R and LS-RFP (Figure 5.11a). No significant differences in serum HI titers were observed at 14 DPH (Figure 5.11b). At 30 DPH, birds vaccinated with LS-RFP had significantly lower serum HI titers compared to birds vaccinated with ZJ1-L-IL4R and ZJ1-L (Figure 5.11b).

5.4.4.3 Serum HI Titers post vNDV challenge. At 23 DPH, no significant differences in serum HI titers of all surviving birds challenged 7 DPH with vNDV were observed (Figures 5.12a). At 30 DPH, no significant differences in serum HI titers of all surviving birds

challenged 14 DPH with vNDV were observed. (Figures 5.12b). The serum HI titers of 30 DPH birds either challenged (Figures 5.12b) or not challenged (Figures 5.11c) at 14 DPH were compared. Serum HI titers were not significantly different for ZJ1-L vaccinated birds at 30 DPH, compared to 30 DPH birds that had been challenged at 14 DPH. Serum HI titers were significantly lower in LS-RFP vaccinated birds at 30 DPH, compared to 30 DPH birds that had been challenged at 14 DPH. Serum HI titers were significantly higher in ZJ1-L-IL4R vaccinated birds at 30 DPH, compared to 30 DPH birds that had been challenged at 14 DPH.

5.4.4.4. Body Weights Post Challenge. Birds vaccinated with ZJ1-L-IL4R had significantly higher body weights at 3 DPH (16 DPC) compared to birds vaccinated with ZJ1-L, LS-RFP or LS (Table 5.6).

5.4.4.5. Viral shedding post challenge in birds challenged with vNDV at 7 or 14 DPH. At 2 DPC (9 DPH), no differences were observed in viral shed titers between vaccinated groups through the OP or CL route (Figure 5.13a-b). At 4 DPC (11 DPH), average viral shed titers of mock vaccinated birds through the OP and CL route were significantly higher compared to all vaccine groups (Figure 5.13c-d). No differences were observed in viral shed titers between vaccinated groups through the OP route (Figure 5.13c). Birds vaccinated with LS-RFP were shedding significantly higher vaccine shed titers through the CL route at 4 DPC (Figure 5.13d).

At 2 and 4 days post 14 DPH challenge no significant differences were observed in the viral shed titers across all vaccinated groups through OP and CL route (Figure 5.14a-d). Mock vaccinated birds shed significantly higher viral titers compared to all vaccinated group at 2 DPC through the OP route and at 4 DPC through both the OP and CL route.

5.4.4.6. Survival Post Challenge. Mortality of mock vaccinated birds challenged with vNDV at 7 and 14 DPH was 100%, which was significantly higher than the mortality observed in all vaccinated groups (Table 5.7). After challenge at 7 DPH, the survival of ZJ1-L-IL4R (90%), ZJ1-L (80%) and LS (70%) vaccinated birds was not significantly different. The survival of LS-RFP (30%) vaccinated birds was significantly lower than the survival of ZJ1-L-IL4R vaccinated birds, but not the survival of ZJ1-L and LS vaccinated birds. The mortality observed in the ZJ1-L-IL4R, ZJ1-L and LS vaccine groups occurred within 2 DPC, compared to the mortality observed in the BHI mock and LS-RFP vaccinated groups, which occurred at 4 DPC. After challenge at 14 DPH all vaccinated groups had 100% survival, except for the mock vaccine group, which had 100% mortality between 4 and 5 DPC.

5.4.4.7. Lymphocyte Subpopulations. At 14 DPH, the birds in the LS vaccine group had a significantly lower percentage of CD4-CD8+ T cells compared to the mock vaccine group. Statistically, no significant differences in the splenic T cell and B cell subset expression were observed at 7, 14 or 30 DPH between any other groups (Tables 5.8 and 5.9).

The proportion of CD4+CD8- T cells was higher in the ZJ1-L-IL4R and LS vaccine groups compared to mock vaccinated birds at 7 DPH (Table 5.8). At 7 DPH, the proportion of CD4-CD8+ T cells was higher in the LS-RFP vaccine group compared to the mock vaccine group. While the CD4:CD8 ratio was 1.4 ±0.2 for the mock vaccinated birds, it was 3.1 ±1.0 and 2.1 ±0.6 for the LS-RFP and LS vaccine groups, respectively.

At 14 DPH, an increase in the proportion of CD4+CD8- cells was observed in the ZJ1-L-IL4R, ZJ1-L and LS vaccine groups compared to the mock vaccine group (Table 5.8). This was paired with a decrease in the proportion of CD4-CD8+ cells from 40.3 ±4.2 % in the mock vaccine group, compared to 32.1 ±1.7, 33.2 ±1.0, 27.0 ±2.7 % in the ZJ1-L-IL4R, ZI1-L and LS vaccine groups, respectively. The ratio of CD4-CD8+ to

CD4+CD8- cells at 14 DPH decreased by 0.6 or more in ZJ1-L-IL4R, ZI1-L, LS-RFP and LS vaccinated birds, compared to mock vaccinated groups.

The ZJ1-L vaccine group had an elevated population of IgM- Bu1+ and IgM+ Bu1+ cells compared to the mock vaccine group (Table 5.9). The LS-RFP vaccine group had an elevated population of IgM+ Bu1+ cells at 14 DPH compared to the mock vaccine group. At 30 DPH no great differences were observed in the proportions of T and B cells between vaccine groups.

Overall, there was a lymphocyte sub-population shift from 7 DPH to 30 DPH. At 7 DPH, CD4+CD8- cells were present in proportions above 30% compared to CD4-CD8+ cells, which are below 25% (Table 5.8). By 30 DPH however there is a reversal in these proportions, with the percentage of CD4+CD8- above 20% and CD4-CD8+ cells between 40.4 and 43.4%. In B lymphocyte subpopulations, the most noteworthy shift is seen in IgM+Bu1- cells, which are present in less than 2.2% at 7 DPH and increase above 30% by 30 DPH (Table 5.9).

5.4.4.8. Immunohistochemistry. Minimal viral replication was observed in most tissues that had IHC signal, with the exception of the larynx. IHC positive cells had a score of 0.4 in spleens collected from birds vaccinated with LS at 0 DPH, and a score of 0.2 in spleens from the ZJ1-L vaccine group at 1 DPH, and LS vaccine group at 1 DPH (Table 5.10). At 0 DPH, bursal IHC scores were between 0.2 and 0.4 in bursas from ZJ1-L, LS-RFP and LS vaccine groups (Table 5.10). At 1 DPH, the IHC score of the bursa of ZJ1L-L vaccinated birds was 1.2 compared to 0.6 in the ZJ1-L-IL4R vaccine groups and 0 in the LS-RFP and LS vaccine groups. The highest bursal IHC scores were observed at 2 DPH; between 1.2 and 1.6 for the ZJ1-L-IL4R, ZJ1-L and LS vaccine groups and 0.5 for the LS-RFP vaccine group. No signal was seen in the bursas of any group at 7 DPH.

While signal on the thymus was observed at 0 DPH in ZJ1-L-IL4R and LS-RFP vaccinated birds, the scores were between 0.2 and 0.4 (Table 5.10). At 1 DPH all vaccine groups except LS had signal in the thymus, and signals were between 0.8 and 1.2. At 2 DPH, all groups except ZJ1-L-IL4R had positive signal, with the scores ranging between 0.2 and 0.75. No signal was observed in the thymus at 7 DPH.

Though intestine was collected from each bird at each timepoint, no signal was observed and therefore, the data was not included in table (Table 5.10). The pharynx was largely positive especially for the LS vaccine group, which had the highest IHC scores at 0, 1 and 7 DPH. The LS vaccine group had an average IHC score of 2.2 at 0 DPH, compared to a score of 0.6 in the LS-RFP vaccine group and 0 in the ZJ1-L-IL4R and ZJ1-L vaccine groups. The ZJ1-L-IL4 and ZJ1-L vaccinated birds had IHC signal at 1 DPH, scores were between 0.5 and 0.6 compared to the LS vaccinated birds, which had IHC scores of 1.2. Positive IHC signal was observed in all vaccine groups at 2 DPH and 7 DPH. At 2 DPH, ZJ1-L birds had the highest average score, and at 7 DPH, LS vaccinated birds had the highest score.

5.4.4.9. Histopathology. The only noteworthy histologic finding was at 7 DPH, where the bursa and thymus were depleted in several birds. This depletion was greater in the parental strains LS and ZJ1-L, with average scores of 2.7 and 2.5 out of 4 in the thymus and 2 and 3 in the bursa (Figure 5.15). In contrast, LS-RFP and ZJ1-L-IL4R vaccinated birds had average scores of 0.8 and 1.8 in the thymus and 1.0 for both in the bursa, respectively. No lesions were observed in mock vaccinated birds.

5.4.5. Vaccine experiment 3: *In ovo* vaccination with antisense chIL4 using two different backbones

Birds vaccinated with LS-RFP had survival rates which were significantly lower compared to those of ZJ1-L-IL4R, ZJ1-L and BHI mock vaccinated birds (Figure 5.16). Birds vaccinated with LS had survival rates which were significantly lower compared to all other groups.

5.4.5.1. Average Body Weights.

Birds vaccinated with ZJ1-L and LS had significantly lower body weights at 7 DPH compared to ZJ1-L-IL4R, LS-IL4R, LS-RFP and mock vaccinated birds (Table 5.11). At 14 DPH, the body weights of birds vaccinated with ZJ1-L and LS were significantly lower compared to the body weight of LS-IL4R, LS-RFP and mock vaccinated birds.

5.4.5.2. HI Titers. At 14 DPH, the serum HI titers of birds vaccinated with ZJ1-L-IL4R vaccine group were significantly lower than those of birds vaccinated with ZJ1-L (Figure 5.17a). Birds vaccinated with LS had serum HI titers that were significantly higher than those of birds vaccinated with LS-RFP and LS-IL4R. Birds vaccinated with LS-RFP had serum HI titers that were significantly higher than those of birds vaccinated with LS-IL4R. At 28 DPH, the serum HI titers of birds vaccinated with LS-RFP and LS-IL4R were significantly lower than those of birds vaccinated with LS (Figure 5.17b).

5.4.5.3. Vaccine viral shed titers. Birds vaccinated with LS-IL4R and LS-RFP vaccinated birds were significantly lower viral shed titers through the OP route at 2 and 4 DPC compared to the vaccine shed titers birds vaccinated with of LS, ZJ1-L-IL4R and ZJ1-L (Figure 5.18a, b). Birds vaccinated with ZJ1-L and ZJ1-L-IL4R were not shedding significantly different vaccine shed titers at any timepoints through the OP route (Figure

5.18a-f). Birds vaccinated with LS, LS-RFP and LS-IL4R were not shedding significantly different vaccine shed titers after 7 DPH through the OP route (Figure 5.18c-f).

Birds vaccinated with ZJ1-L-IL4R were shedding significantly lower vaccine shed titers at 2 and 14 DPH through the CL route compared to birds vaccinated with ZJ1-L (Figure 5.19c, f). Birds vaccinated with LS-RFP and LS-IL4R were shedding significantly lower vaccine shed titers at 7 through the CL route compared to LS vaccinated birds (Figure 5.19c).

5.4.5.4. *CMI recall response.* At 72 h post stimulation, lymphocytes isolated from the spleens of LS-IL4R vaccinated birds at 35 DPH had significantly higher proliferation responses when stimulated in culture with an inactivated NDV antigen (Figure 5.20, table 5.12). No other proliferative differences were noted between any other vaccine groups stimulated with NDV or with ConA.

5.4.5.5. *CMI recall response and cytokine production.* Analysis of the IFNγ levels secreted in the supernatants at 24 h from enriched lymphocytes from ZJ1-L-IL4R vaccinated birds yielded significantly higher levels of IFNγ compared to enriched lymphocytes from mock, LS-RFP and LS vaccinated birds (Table 5.12). No differences in IFNγ production were observed from enriched lymphocytes stimulated with ConA for all vaccine groups. At 72 h post stimulation, the enriched lymphocytes from LS-IL4R vaccinated birds cultured in media alone had significantly higher IFNγ levels compared to all other treatment groups (Table 5.12). While no statistical differences were observed in the levels of IFNγ produced by lymphocytes stimulated with NDV for 72 h, numerically, the ZJ1-L-IL4R vaccinated birds had higher levels of IFNγ than enriched lymphocytes from birds vaccinated with ZJ1-L. Similarly, enriched lymphocytes from LS-IL4R vaccinated birds and stimulated with inactivated NDV had

higher levels of IFNγ compared to LS-RFP and LS. ConA-stimulated enriched lymphocytes from LS-IL4R vaccinated birds had higher levels of IFNγ compared to lymphocytes isolated from mock vaccinated birds (Table 5.12). Numerically, enriched lymphocytes from ZJ1-L-IL4R, ZJL, LS-RFP and LS birds had higher IFNγ levels in media alone compared to mock vaccinated birds.

## 5.5. Discussion

All vaccine viral strains had ICPI and MDT characteristics of lentogenic strains, however the ZJ1-L and LS backbones were from different genotypes. The ZJ1-L strain is an attenuated version of the velogenic strain ZJ1, a genotype VIId virus, whereas the LS strain belongs to the genotype II [47, 48]. The only difference between ZJ1-L and vZJ1 is the fusion cleavage site, as it was attenuated through site directed mutagenesis [31].

No differences in survival between the ZJ1-L-IL4R and mock vaccinated groups were observed. In this study, the survival of ZJ1-L vaccinated birds was not significantly different than that of mock or ZJ1-L-IL4R vaccinated birds, whereas in previous experiments done in our laboratory it was numerically lower, and often significantly lower compared to either BHI or ZJ1-L-IL4R. Vaccination with the LS strain and its recombinants led to great mortality, but this mortality was decreased by the addition of the RFP and IL4R inserts to the LS genome.

Consistent with our previous work, bird body weights between groups vaccinated with ZJ1-L-IL4R and the mock vaccine were not significantly different, but birds vaccinated with ZJ1-L and LS had significantly lower body weights, which if used would be costly for poultry producers. The weights of LS-RFP vaccinated birds on the other hand, were inconsistent, being significantly lower than that of mock vaccinated birds in previous experiments but not in the present study. In this and previous experiments, the recombinant ZJ1-L-IL4R, LS-RFP and LS-IL4R vaccines shed significantly lower viral

titers compared to their parental strains ZJ1-L and LS. The HI serum titers of birds vaccinated with ZJ1-L-IL4 and ZJ1-L were able to obtain protective levels. However, they have only been significant in one of four studies. This contrasts with LS-RFP and LS-IL4R vaccinated birds, which yielded significantly lower serum HI titers than LS-vaccinated birds. The SPF flock at SEPRL are composed of outbred birds, which may contribute to the variability observed in HI titers and weights across the groups. Another possible explanation is that the vaccine dose of $10^{3.5}$ $EID_{50}$ was too low of an infectious dose of LS-RFP, and some birds may not have become infected *in ovo*. These birds would become infected after hatch through aerosolization of the virus during the hatch process in the incubator.

Bird survival after challenge at 7 DPH was also consistent with previous work done in our laboratory, with 90-100% survival in birds vaccinated with ZJ1-L-IL4R, compared to 70-80% survival in birds vaccinated with ZJ1-L and LS, and between 30- 58% survival in birds vaccinated with LS-RFP. While mortality was observed in the ZJ1-L and LS vaccinated birds, this mortality had an earlier onset of 1-2 days before the sham vaccinated group. Thus, this early mortality was attributed to the vaccine, which can replicate for up to two weeks in chickens, instead of the challenge virus. This was supported by the survival post hatch observed in previous experiments done in our laboratory, as mortality due to the vaccine in unchallenged birds was observed to continue past 7 DPH.

No noteworthy differences in IHC signal were observed between groups. However, IHC is limited to the section of tissue selected and it is possible that we would have found greater differences if we had used a greater number of birds and/ or several sections per bird. The greatest replication was observed in the pharynx, and the IHC scores correlated with the OP shedding observed, with parental strains ZJ1-L and LS shedding greater amounts of virus compared to the recombinant strains ZJ1-L-IL4R and LS-RFP.

Thymic and bursal depletion observed at 2 DPH was greater in the parental strains ZJ1-L and LS compared to the recombinant strains ZJ1-L-IL4R and LS-RFP. The depletion observed appears to be associated with days of highest viral shed titers, with a similar vaccine shed titers observed between 2 and 7 DPH in this experiment, and at 2 and 3 DPH in previous experiments. However, this thymic and bursal depletion did not correlate with lymphocyte phenotype, or seem to permanently affect antibody production observed at 14 and 28 DPH. This transient depletion was not observed in tissue collected at 7 DPH. It is possible that this is a direct or indirect effect of vaccination through infection of cells at the thymus and bursa or progenitor cells at the bone marrow. We did not observe any apoptotic bodies in our H&E or intense IHC signal indicating viral replication at those sites. In addition, none of our vaccines have demonstrated suppressed proliferative capabilities, with antibody production and T cell proliferation assays plus protection after challenge. It is possible that the depletion is an indirect effect of infection in other tissues, that it is due to infection of progenitor cells in the bone marrow, or that these lymphocytes are maturing and being recruited for infection. Virulent strains of NDV has been shown to cause lymphocytic depletion in the spleen, bursa, thymus and bone marrow of chickens, and is paired with strong NDV IHC signal, [49]. In our study however, while we observed strong IHC signal in the bursa, this was not observed in the thymus and the levels did not correlate with the levels of depletion.

Lymphocytic depletion after vaccination or exposure of chickens to NDV, Marek's disease virus (MDV), chicken anemia virus (CAV), infectious bursal disease virus (IBDV) and *E. coli* has been previously observed [50-55]. Infection of 1 day old chickens with CAV caused thymic depletion by 14 days after infection by apoptosis [50]. Lymphocytes repopulated the thymus by 20 days after infection. As CAV infects the bone marrow, bursa and thymus, this lymphocytic depletion may be due to infection of precursors in the bone marrow, to infection of T and B cells in the thymus and bursa respectively, or a combination

thereof. Lymphocytic depletion due to apoptosis in the presence of viral protein has also been observed in ECEs infected at 18 DOE with classical virulent IBDV, infection with an attenuated counterpart showed minimal changes [53]. Lymphocytic depletion after *in ovo* inoculation of MDV has been observed in the absence of viral genome and has been speculated to be due to indirect effects by infected cells such as cytokine production that may cause damage to resident cells [55].

ZJ1-L-IL4R remains the best candidate for *in ovo* vaccination. The ZJ1-L-IL4R recombinant strain successfully protected chicks against challenge at 7 DPH, producing protective levels of antibody titers, without compromising weight gain or survival post vaccination. To explore the possibility of immune modulation by our antisense vaccines, we measured its effects on lymphocyte proliferation and cytokine production following a primary challenge and recall response. Antisense vaccines have the potential to modulate the immune system within specific cells, limiting their impact [56, 57]. While cytokines such as IFNγ may have systemic impacts, a live attenuated NDV vaccine containing an antisense insert may have a more subtle effect, as it is limited to the cell types infected with the virus, without systemic effects that may have unintended harmful effects. While there is no current literature on the use of a recombinant antisense vaccine to protect against avian pathogens, antisense technology has proven successful in tumor and viral vaccines. Intracranial delivery of tumor vaccine and antisense TGF-β2 oligonucleotides to mice with brain tumors significantly enhanced their survival [57]. In human patients with non-small cell lung cancer, intradermal immunization with an TGF-β2 antisense tumor cell vaccine improved patient outcome [56]. A vaccine for foot and mouth disease containing a sense protein of the virus in conjunction with an antisense sequence targeting the 5' untranslated region of the virus successfully protected cells against foot and mouth disease infection and improved survival of mice challenged against the disease [58]. These studies successfully used antisense technology to improve disease outcome to

tumors and infection. This avenue should be further explored to improve vaccine and challenge outcomes against NDV.

The primary immune focus of this study was on T cells, and to a lesser degree on B cells, as they are major players in adaptive immunity. T cells can be further divided into CD8+ cytotoxic T cells and CD4+ helper T cells, which recognize antigen in the context of MHC class I and II, respectively [59]. Class I molecules play a crucial role in infection with intracellular pathogens such as viruses while class II molecules are expressed by APCs and lead to activation of helper and regulatory T cells [60]. CD4+ T lymphocytes can further differentiate into at least two functionally distinct helper subsets (Th1 and Th2). Th1-biased immune responses are most efficient at fighting invasion by intracellular organisms such as viruses, while Th2-biased responses are commonly associated with helminth infections and allergic responses [60, 61]. IFNγ, IL-2, and/or IL-12 cytokines are secreted by Th1 cells, while IL-4, IL-5, IL-6, and/or IL-10 are secreted by Th2 cells [62-66]. During NDV infection, the host's humoral immunity (B cells and Th2 cells) can protect the host across all age groups. Protective antibodies secreted by B cells are directed against either the HN or F glycopolypeptides and block binding of the Hn protein to the receptor on the cell membrane or fusion of the viral and host cell membrane, respectively [1]. These antibodies are detectable within 6-10 days post infection and are produced by differentiated B-lymphocytes known as plasma cells [2, 67]. Cell-mediated immunity (CD8+ and Th1 cells) on the other hand, is detectable as early as 2-3 days post infection, although it may or may not protect against NDV challenge by itself [68]. Some studies have deduced that NDV- specific CMI is unable to provide protection against NDV challenge alone [12], whereas other studies have found some protection [69].

We measured IL4, IL10 and IFNγ as our immune modulation indicators. Infection of lymphocytes with ZJ1-L-IL4R or ZJ1-L at different MOIs did not lead to significant differences in IL4 or IL10 production or T cell proliferation between the viral vaccines.

Previous work looking at changes in gene expression in chicken splenocytes infected with NDV did not reveal a significant increase in IL4 expression [70]. Mild changes in IL10 expression and several fold changes in IFNγ upon infection with NDV have been previously demonstrated with NDV infection [70, 71]. Previous work with NDV also did not detect an increase in IL10 production until 72 h post stimulation, and even then, the increase in IL10 production was variable between strains, with one strain displaying an increase of 1.83 fold and the other a 3.92 fold increase in IL10 production at 72 h post stimulation [71]. We expected an increase due to the addition of ConA, a lymphocyte mitogen previously shown to increase cytokine production through lymphocyte stimulation [72, 73]. Previous work in mice demonstrated that ConA stimulation can lead to an increase in IL10 production within 8 h post stimulation [72]. In the present study, an increase in the production of IL10 was observed in cells stimulated with a viral MOI of 1.0 in addition to ConA however, the increase was small (20- 24 pg/ mL) and was not significantly different between the ZJ1-L-IL4R and ZJ1-L inoculated birds.

Differences in IFNγ production were observed in lymphocytes infected with either MOIs of ZJ1-L-IL4R or ZJ1-L. While the differences were not significantly different in cells infected with virus alone, the addition of ConA demonstrated a significant lack of increase in IFNγ production in lymphocytes inoculated with ZJ1-L-IL4R compared to the ZJ1-L parental strain. Previous work in our laboratory explored differences in cytokine expression in the spleens of naïve chickens infected with the vZJ1 strain 2 and 3 DPC [74]. At 2 and 3 DPC they observed a 14.3 and a 74.5-fold increase in IFNγ production. The 74.5-fold increase observed at 3 DPC was the highest seen of the 8 mesogenic and velogenic strains used in the study. The second highest increase in IFNγ production was observed by the CA02 strain, which had an 18.8-fold increase, demonstrating a great capability of ZJ1 to induce IFNγ compared to other NDV strains [74]. In our study, we normalized the IFNγ protein production data to the ConA control and observed that at 0.1

and 1.0 MOIs the ZJ1-L strain induced a 25.4 and 25.7-fold increase in IFNγ protein production in the presence of ConA, demonstrating that it retains its ability to induce large quantities of IFNγ upon infection. In contrast, the ZJ1-L-IL4R strain displayed attenuation of this strain, inducing only a 12.7 and 8.8-fold increase in in IFNγ production at 0.1 and 1.0 MOIs, respectively.

In vaccine experiment 1, inbred birds obtained from Charles River Laboratories were vaccinated at 19 DOE, and their spleens were collected at 28 DPH. The lymphocytes isolated from the spleens of ZJ1-L-IL4R vaccinated birds displayed higher proliferation and were found to have higher proportions of CD4-CD8+ cytotoxic T cells compared to those of birds vaccinated with the ZJ1-L strain. The differences in T cell phenotyping were not consistent with those observed in vaccine experiments 2 and 3. Vaccine experiments 2 and 3 were performed using non-genetically similar birds from the SEPRL flock, and the inconsistencies may be due to the high genetic variability of the flock. However, at the time of experiment one, the SEPRL flocks were not producing enough eggs to carry out the experiment. The low stimulation seen in cells treated with the NDV inactivated antigen in vaccine experiment 3 are believed to be due to the stress the cells were under throughout the collection and isolation phase or to the short amount of time waited after the stimulation.

High IFNγ production in the splenocytes from vaccinated birds was observed at 24 and 72 h post stimulation with the inactivated NDV antigen. At both timepoints, IFNγ production was numerically higher in cultured cells from the recombinant vaccines compared to parental strains. However, IFNγ production was not significantly different between each parental strain and their respective recombinants. Direct infection of splenocytes with ZJ1-L induced high levels of IFNγ, while CMI recall response with inactivated NDV did not demonstrate a better recall response.

Previous work in our laboratory demonstrated that recombinant virulent NDV strains containing IFNγ can decrease viral load and decrease the pathogenicity of the virus in chickens [30]. However, in a separate study in our lab, delivery of IFNγ with inactivated, DNA or attenuated recombinant NDV did not improve disease outcome, having no effect on humoral response or survival post challenge [31]. This emphasizes the need to further study the role of IFNγ in disease outcome associated with NDV and strains with different pathogenicities. Overall, *in ovo* delivery of the ZJ1-L vaccine induced higher viral shed titers, lower body weights and lower survival rates post vaccination. *In vitro*, cells infected with ZJ1-L had high levels of IFNγ and lower proliferation profiles compared to the recombinant ZJ1-L-IL4R.

We hypothesized that the differences seen between the ZJ1-L-IL4R and ZJ1-L strains were due to some degree of immune modulation cause by the IL4R insert. However, the data in its current state does not support immune modulation due to the asIL4 insert. However, comparisons between the LS-IL4R and LS-RFP recombinant strains and the LS parent strain, we found no evidence of immune modulation specific to the chIL4R insert. The LS-RFP and LS-IL4R had comparable survival post vaccination, weight gain, HI titers, vaccine viral shed titers and IFNγ production rates. Differences were observed in T cell proliferations; however, these differences were accompanied by higher production of IFNγ in the media control at 72 h post infection, indicating that the cells had a higher baseline activation compared to those of the other groups and despite the treatment. It possible that the virulence and hence antigenicity of the LS and ZJ1-L backbones overshadows the insert, as IL4 is not produced in high quantities. It is also possible that differences were not observed due to the genetic variability of the SEPRL flock, as some differences were observed between the ZJ1-L-IL4R and ZJ1-L strains in experiments using genetically similar birds.

Our work shows a potential mechanism of action of ZJ1-L-IL4R, consisting of lower IFNγ induction post vaccination based on viral attenuation due to the insert, although not necessarily sequence specific. The LS-RFP and LS-IL4R strains also portray attenuation due to their respective inserts however, the LS backbone causes significantly higher mortality when applied *in ovo,* when compared to the ZJ1-L strain, making the ZJ1-L-IL4R recombinant a more suitable *in ovo* vaccine. While in some experiments the ZJ1-L strained performed well in terms of mortality, the birds were significantly lighter in weight, which would cause significant financial loss to the producer. Further studies to test this mechanism of action should involve genetically similar birds, a different non-sense insert in the ZJ1-L backbone, and trials in maternal antibody ECEs to ensure that this vaccine can successfully establish immunological memory in the presence of passively acquired maternal antibodies.

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**Table 5.1. Mean death time (MDT) and intracerebral pathogenicity index (ICPI) of the NDV vaccine strains.**

| | MDT[b] | ICPI[c] |
|---|---|---|
| vZJ1[a] | 54.5 | 1.83 |
| ZJ1-L-IL4R | >168 | 0.25 |
| LS-IL4R | >168 | 0.26 - 0.30 |
| ZJ1-L | >168 | 0.43 |
| LS-RFP | 127 | 0.00 |
| LS | 110-153.25 | 0.15 - 0.30 |

[a] Velogenic ZJ1L strain
[b] Mean death time
[c] Intracerebral pathogenicity index

**Figure 5.1. Growth Curves in ECEs and HD11 Cells.** The growth kinetics of ZJ1-L, ZJ1-L-IL4R, LS and LS-RFP were determined using SPF ECEs. Each ECE was inoculated with $10^{2.5}$ $EID_{50}$ per 0.1ml of virus in 0.1 ml of allantoic fluid. Allantoic fluid was collected at 1, 6, 12, 24, 36, 48, 72 and 96 h post infection and each time point was done in triplicate.

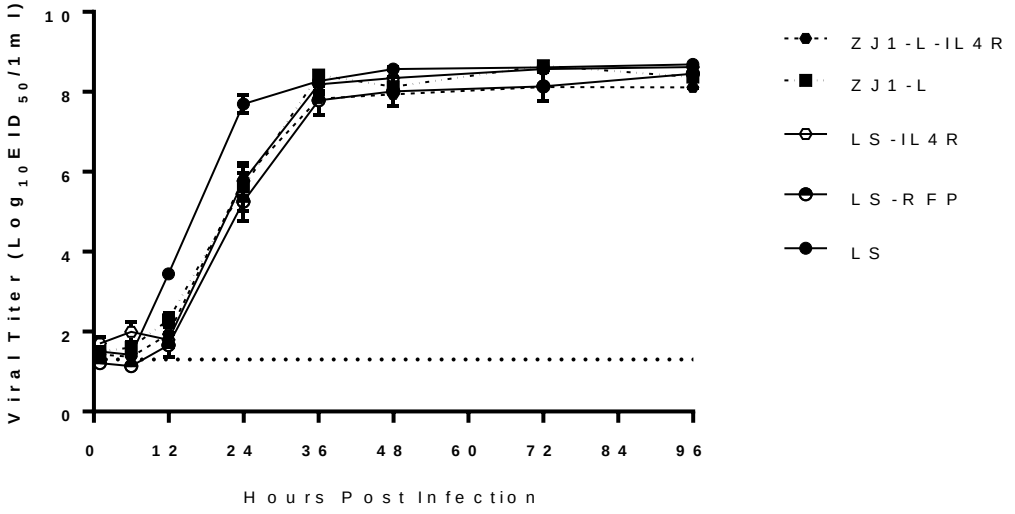



**Figure 5.2** ***Effect of primary infection with NDV on lymphocyte proliferation*****.** Splenocytes of adult SPF chickens were isolated and 5 x $10^5$ cells per well were plated. Cell were infected with ZJ1-L-IL4R or ZJ1-L at a multiplicity of infection (MOI) of 0.1 (a) or 1.0 (b) in the absence or presence of ConA. T cell proliferations were measured by adding 20 µL of AlamarBlue® at 48 h post stimulation and reading at 72 h post stimulation. Delta means were calculated by subtracting the average relative fluorescence units (RFU) of the media control. Differences were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test (*$p \leq 0.05$*). Significant differences are denoted by different letters. Groups sharing letters are not significantly different from one another.

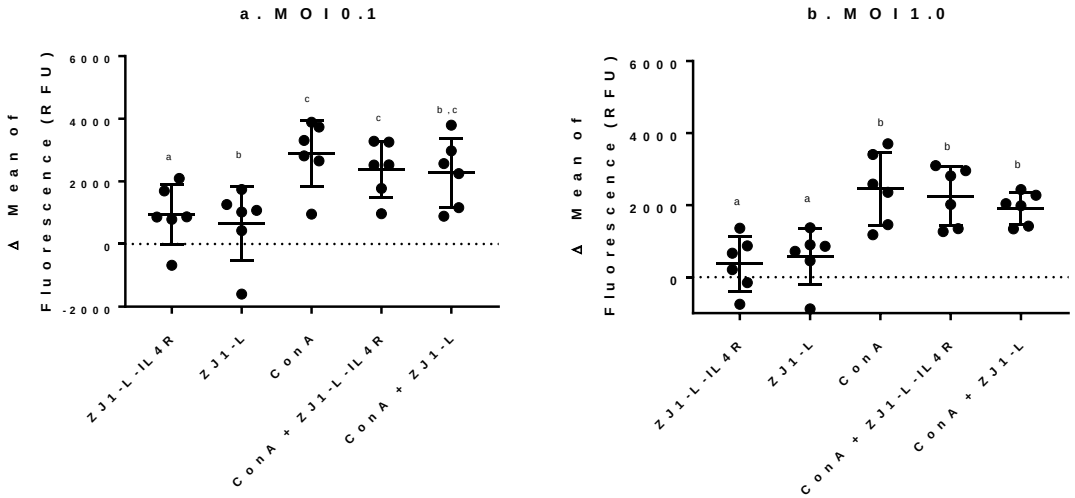


**Figure 5.3** ***Effect of primary infection with NDV on cytokine production*****.** Splenocytes of adult SPF chickens were isolated and 5 x $10^5$ cells per well were plated. Cell were infected with ZJ1-L-IL4R or ZJ1-L at a multiplicity of infection (MOI) of 0.1 (a,c) or 1.0 (b,d) in the absence (a,b) or presence (c,d) of ConA. The levels of IFNγ in supernatant at 24 h were detected by ELISA. Differences were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test ($p \leq 0.05$). Significant differences are denoted by different letters. Groups sharing letters are not significantly different from one another.

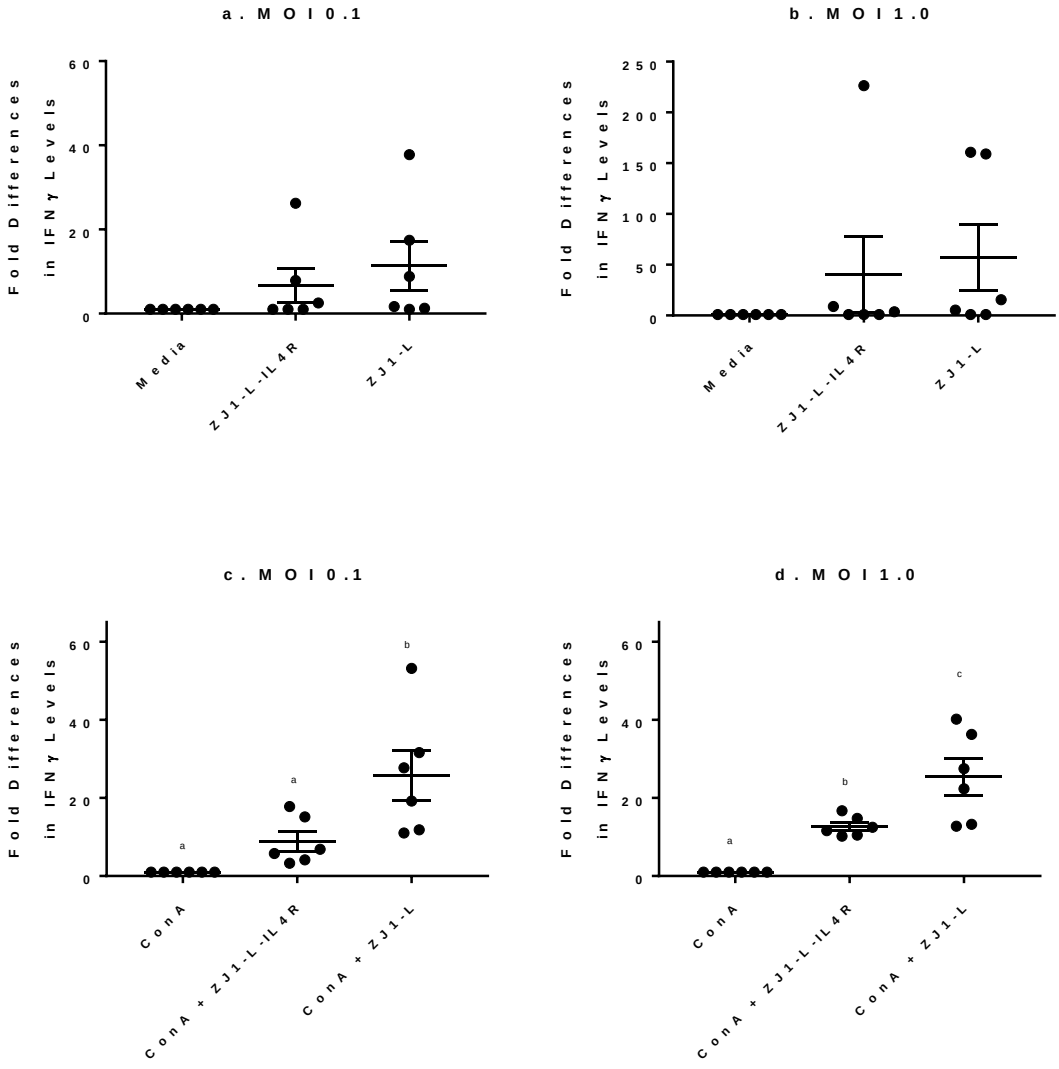
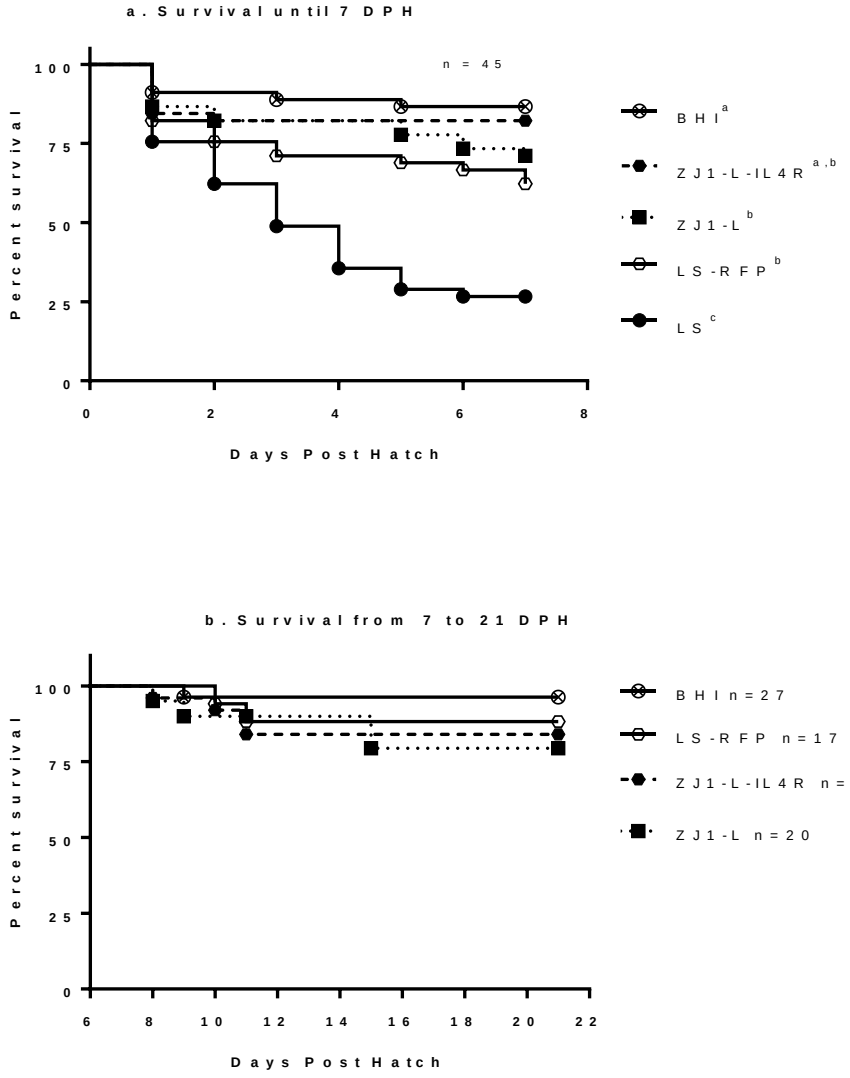


**Figure 5.4. Post hatch survival of 19 DOE NDV vaccinated birds.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-, LS-RFP, LS or brain heart infusion (BHI) control. Post hatch, birds were housed in negative pressure isolators and their survival was checked daily until 7 DPH (A). At 7 DPH, 12 chicks in each group were challenged with $10^5$ $EID_{50}$ vZJ1 by the oculo-nasal route. The survival of the non-challenged chicks was checked daily until 21 DPH (B). The survival curves were analyzed using the Long-rank test ($p \leq 0.05$).



| Table 5.2. DPH body weight of 19 DOE NDV vaccinated birds. | | | | | |
|---|---|---|---|---|---|
| **Day Post Hatch** | **BHI** (Mean ± SEM) | **ZJ1-L-IL4R** (Mean ± SEM) | **ZJ1-L** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
| **1** | 34.8 ± 0.5 | 33.8 ± 0.3 | 34.7 ± 0.3 | 33.2 ± 0.4 | 34.5 ± 0.6 |
| **7** | 62.9 ± 1.8 | 59.9 ± 2.0 | 54.0 ± 2.7 | 54.9 ± 2.8 | 54.2 ± 3.8 |
| **14** | 129.1 ± 1.4 [a] | 123.4 ± 4.5 [a] | 98.7 ± 7.1 [b] | 102.9 ± 7.2[b] | N/A |
| **22** | 228.6 ± 3.1 [a] | 220.1 ± 8.4 [a,b] | 182.7 ± 9.6 [c] | 188.7 ± 13.0 [b,c] | N/A |
| [#]body weight in grams, significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test, n = 20 at 1 DPH. n = 20 at 7 DPH except for LS group where n = 13. n at 14 DPH = 15-16 birds. N = 15 at 22 DPH. | | | | | |

**Figure 5.5. Viral shed titers of 19 DOE vaccinated 2-day-old birds.** At 19 DOE, SPF embryonated chicken eggs (ECEs) were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. Vaccine shed titers through the oropharyngeal (OP) route was measured at 2 DPH. $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test. Significant differences are denoted by different letters, n=10 birds/ vaccine group.

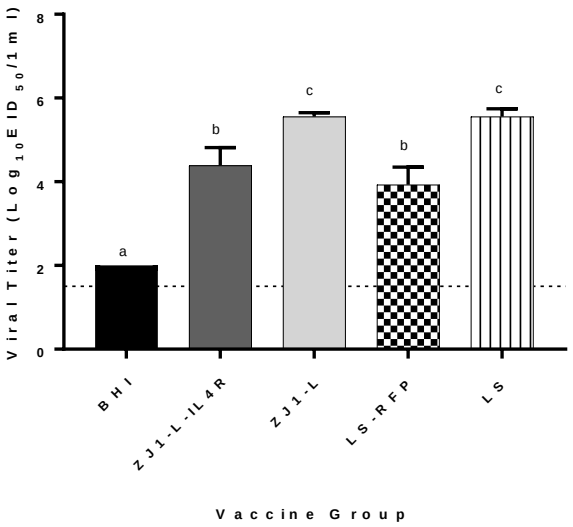
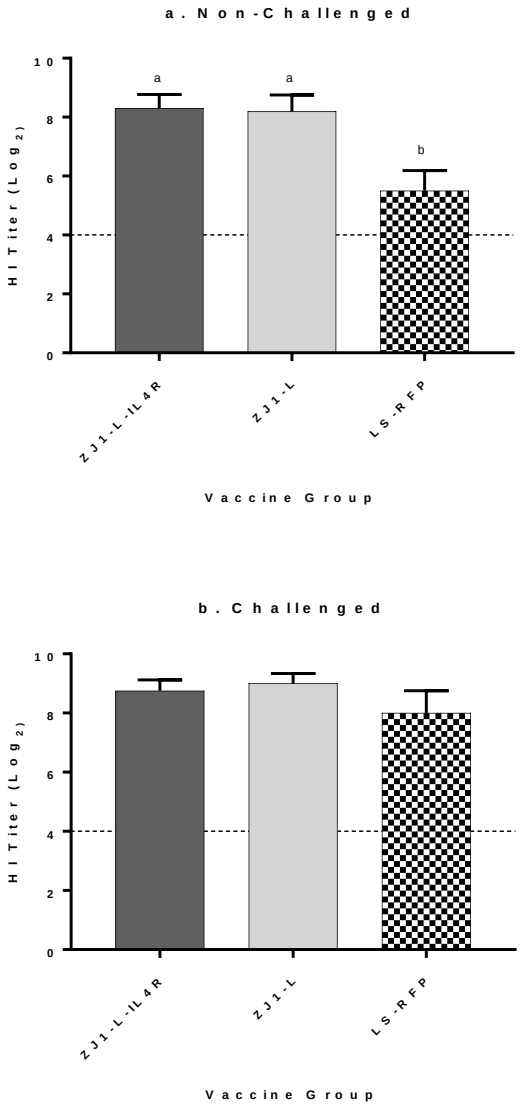


**Figure 5.6. vNDV challenged or non-challenged serum HI titers from 19 DOE NDV vaccinated 23-day-old birds.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. Serum was collected from 23-day-old vaccinated birds challenged with $10^5$ $EID_{50}$ vZJ1 at 7 DPH and age matched birds that had not been challenged (a). $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test. Significant differences are denoted by different letters, n=10 birds/ vaccine group.



| Table 5.3. DPC body weights of birds vaccinated at 19 DOE with NDV. | | | | |
|---|---|---|---|---|
| **Days Post Challenge** | **ZJ1-L-IL4R** (Mean ± SEM)[#] | **ZJ1-L** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
| **7** | 116.0 ± 3.7 [a] | 103.2 ± 4.6 [b] | 112.1 ± 4.5 [a,b] | 113.3 ± 2.8 [a,b] |
| **14** | 202.1 ± 7.3 [a] | 166.4 ± 7.8 [b] | 204.3 ± 8.1 [a] | 214.7 ± 6.3 [a] |
| [#] body weight in grams, Analysis performed by DPC, significant differences are denoted by different letters, $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test, n = 7-12 birds/ vaccine group. | | | | |

**Figure 5.7. Viral shed titers of 19 DOE NDV vaccinated birds vNDV challenged at 7 DPH.** At 19 DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 7 days DPH, 12 chicks from each group were challenged with $10^5$ $EID_{50}$ vZJ1 via the oculo-nasal route. Viral shed titers through the oropharyngeal (OP) (a, c) and cloacal (CL) (b, d) routes were measured at 2 (a-b), 4 (c-d), days post challenge (DPC). $p \leq 0.05$, One-way ANOVA followed by a multiple comparisons Tukey's test. Significant differences are denoted by different letters. n= 9-12 birds/vaccine group except for the BHI mock vaccine group at 4 DPC, in which only 1 birds remained alive.

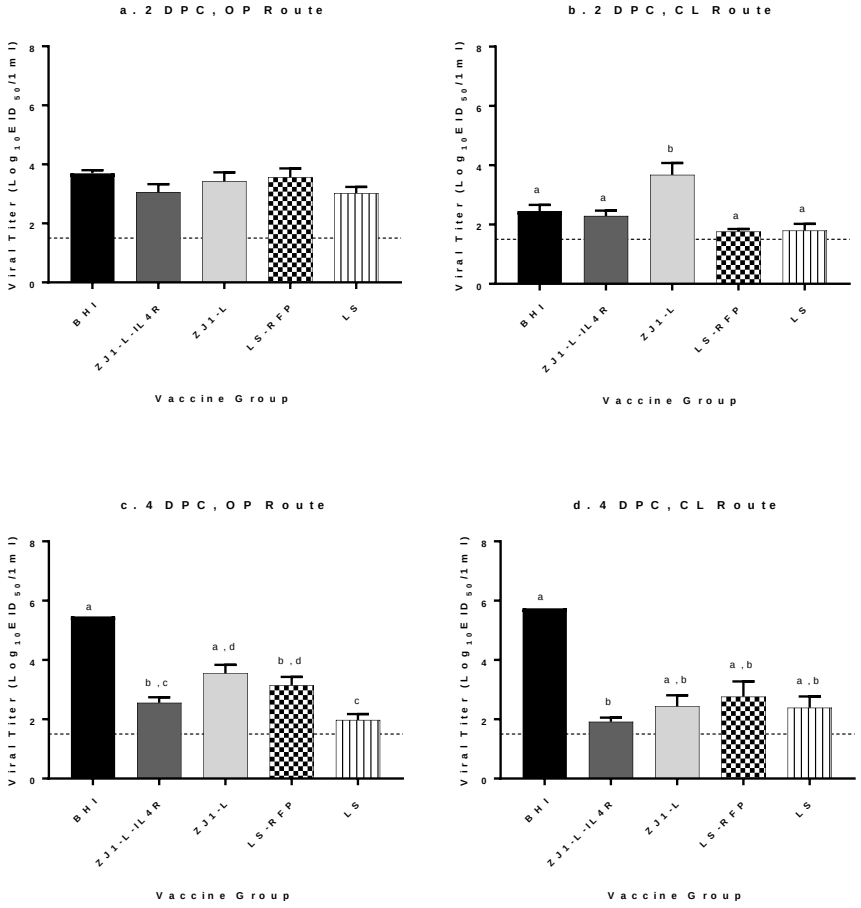


**Figure 5.8. Fourteen-day post challenge survival of NDV or mock *in ovo* vaccinated birds vNDV challenged at 7 DPH.** At 19 days DOE, SPF embryonated chicken eggs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 7 days post hatch (DPH)12 chicks from each group were challenged with $10^5$ $EID_{50}$ vZJ1 via the oculo-nasal route. Mortality was recorded daily until 14 DPH. Survival curves were analyzed using the Long-rank test ($p \leq 0.05$).

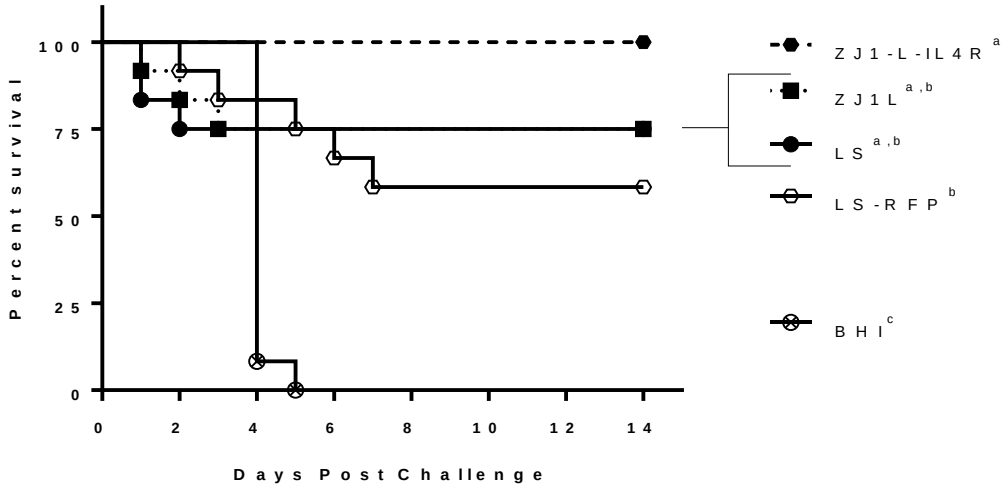
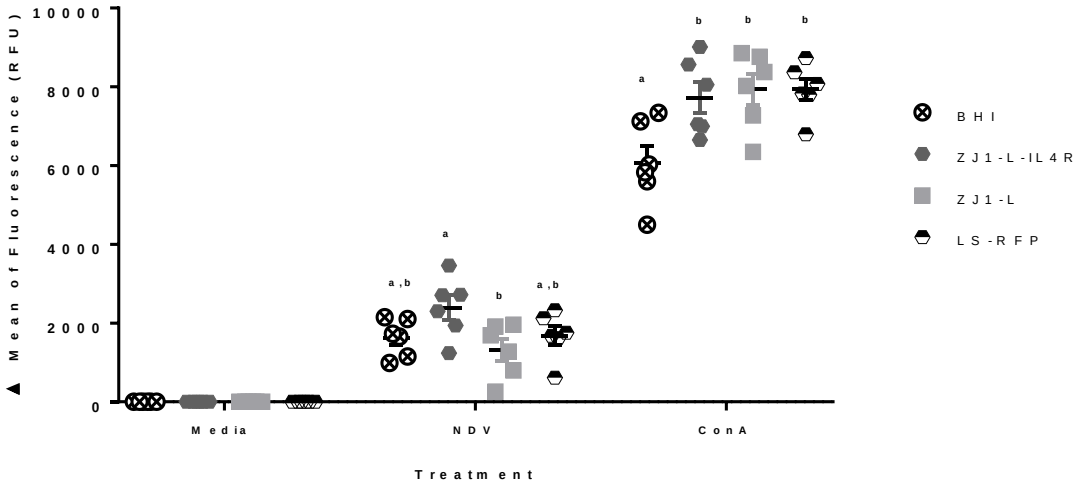



**Figure 5.9. Twenty-eight-day CMI recall response in 19 DOE NDV vaccinated chickens.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP or brain heart infusion (BHI) control. At 28 days post hatch (DPH), spleens were collected for lymphocyte proliferation response. Differences in T cell proliferation were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test (*$p \leq 0.05$*). Significant differences are denoted by different letters. The mean fluorescence for the media controls were 3085, 1958, 1938 and 4646 RFU for the ZJ1-L-IL4R, ZJ1-L, LS-RFP and BHI groups, respectively.



| Table 5.4. Lymphocyte subpopulations of 19 DOE vaccinated 4-week old birds. | | | | |
|---|---|---|---|---|
| | **BHI** (Average ± SEM) | **ZJ1-L-IL4R** (Average ± SEM) | **ZJ1-L** (Average ± SEM) | **LS-RFP** (Average ± SEM) |
| **CD4+ CD8-** | 30.1 ± 1.5 | 27.3 ± 2.2 | 30.9 ± 2.1 | 30.5 ± 1.9 |
| **CD4- CD8+** | 44.3 ± 1.8 | 48.5 ± 3.1 | 47.3 ± 2.2 | 44.7 ± 0.8 |
| **CD8:CD4** | 1.5 ± 0.1 | 1.8 ± 0.2 | 1.5 ± 0.1 | 1.6 ± 2 |
| **IgM- Bu1+** | 1.4 ± 0.3 | 1.2 ± 0.2 | 1.1 ± 0.5 | 1.4 ± 0.5 |
| **IgM+ Bu1+** | 5.4 ± 1.1 | 4.9 ± 1.1 | 3.1 ± 0.7 | 4.0 ± 0.8 |
| **IgM+ Bu1-** | 3.4 ± 1.0 | 2.1 ± 0.4 | 1.3 ± 0.2 | 1.3 ± 0.3 |
| * $p \leq 0.05$, One-way ANOVA and multiple comparisons Tukey's test. | | | | |

| Table 5.5. Days Post Hatch (DPH) body weights of 19 DOE vaccinated birds. | | | | | |
|---|---|---|---|---|---|
| **DPH** | **BHI** (Mean ± SEM)* | **ZJ1-L-IL4R** (Mean ± SEM) | **ZJ1-L** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
| **0** | 32.2 ± 1.6 | 32.5 ± 1.3 | 30.5 ± 0.6 | 31.5 ± 0.5 | 32.4 ± 1.3 |
| **1** | 29.1 ± 1.6 | 29.0 ± 0.7 | 29.2 ± 1.1 | 32.9 ± 2.6 | 29.4 ± 0.9 |
| **2** | 29.0 ± 1.7 | 31.3 ± 0.9 | 30.7 ± 0.9 | 30.9 ± 1.6 | 26.0 ± 1.0 |
| **7** | 53.0 ± 1.9[a] | 52.2 ± 2.7[a] | 47.4 ± 2.3[a,b] | 53.4 ± 3.1[a] | 41.4 ± 3.0[b] |
| **14** | 109.6 ± 5.1[a] | 104.2 ± 2.9[a] | 99.9 ± 2.4[a,b] | 99.1 ± 3.9 [a,b] | 87.6 ± 3.1[b] |
| **23** | 189.6 ± 6.5[a,b] | 191.1 ± 5.6[a] | 177.4 ± 8.8[a.b] | 159.6 ± 7.5[b] | Deceased |
| **30** | 281.3 ± 9.8 [a.b] | 281.3 ± 7.3[a] | 268.2 ± 12.7 [a.b] | 243.9 ± 10.8[b] | Deceased |
| *Average body weight in grams<br>[a-b] Weights at each time point were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test ($p \leq 0.05$). Significant differences are denoted by different letters (n = 5 at 0-2 DPH, n = 10 at 7 and 14 DPH, n = 6-18 at 23 and 30 DPH). | | | | | |

**Figure 5.10. Viral shed titers of 0, 1, 2 and 7-day-old 19 DOE NDV vaccinated birds.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. Vaccine shed titers through the oropharyngeal (OP) (a, c, e, g) and cloacal (CL) (b, d, f, h) routes were measured at 0 (a-b), 1 (c-d), 2 (e-f) and 7 (g-h) days post hatch (DPH). Significant differences are denoted by different letters ($p \leq 0.05$, one-way ANOVA followed by multiple comparisons Tukey's test). N = 3-5 birds.

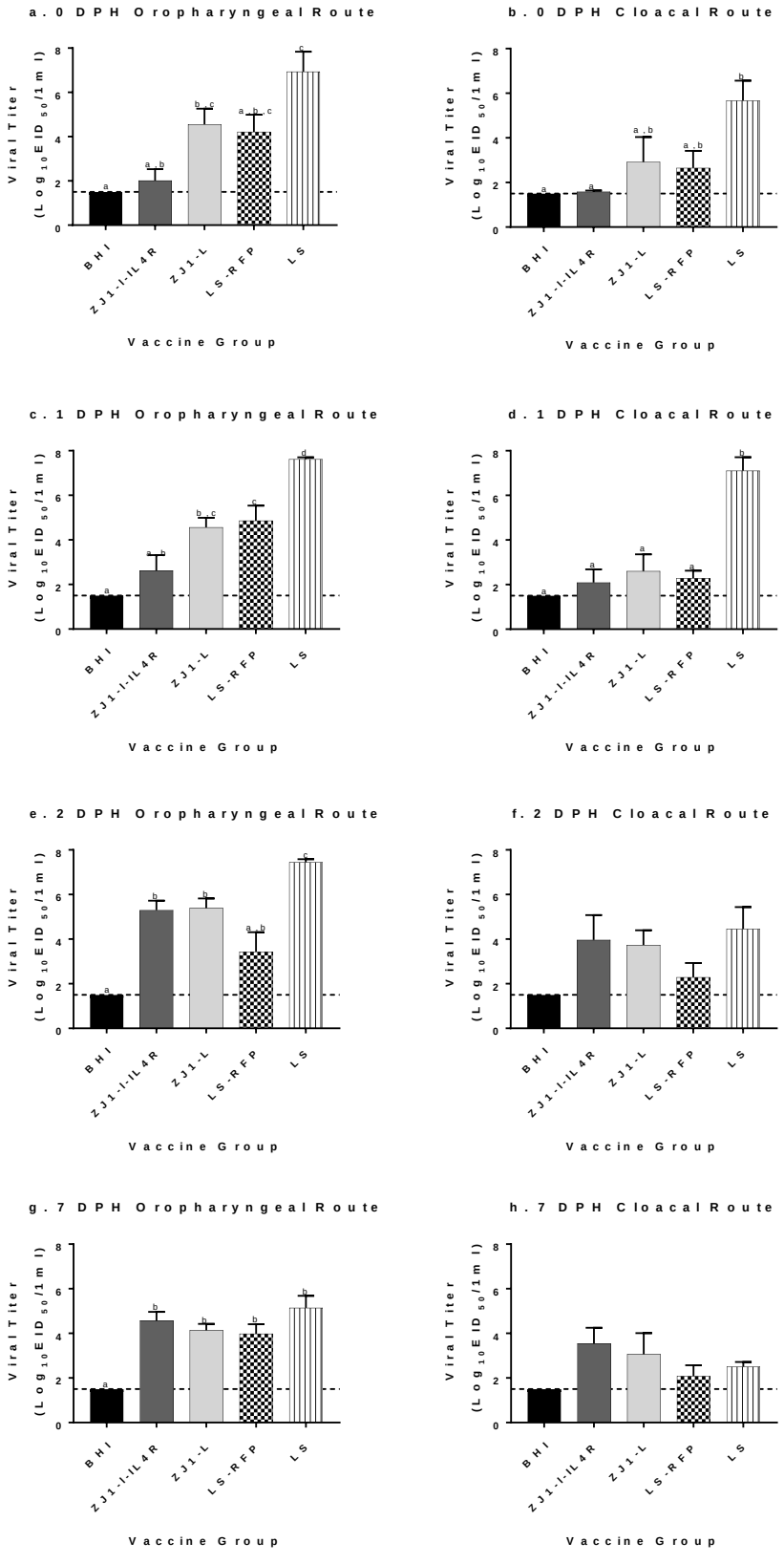

a. 0 DPH Oropharyngeal Route
b. 0 DPH Cloacal Route
c. 1 DPH Oropharyngeal Route
d. 1 DPH Cloacal Route
e. 2 DPH Oropharyngeal Route
f. 2 DPH Cloacal Route
g. 7 DPH Oropharyngeal Route
h. 7 DPH Cloacal Route
Viral Titer (Log10EID50/1ml)
BHI
ZJ1-I-IL4R
ZJ1-L
LS-RFP
LS
Vaccine Group

**Figure 5.11. HI titers of 19 DOE vaccinated SPF birds.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. Serum was collected at 8 (**A**), 14 (**B**) and 30 (**C**) days post hatch (DPH). Significant differences are denoted by different letters ($p \leq 0.05$, one-way ANOVA followed by multiple comparisons Tukey's test). At 8 DPH, n = 3 -5. At 14 DPH n = 10. At 30 DPH, n = 6 for the BHI groups and 12-17 for all other groups.

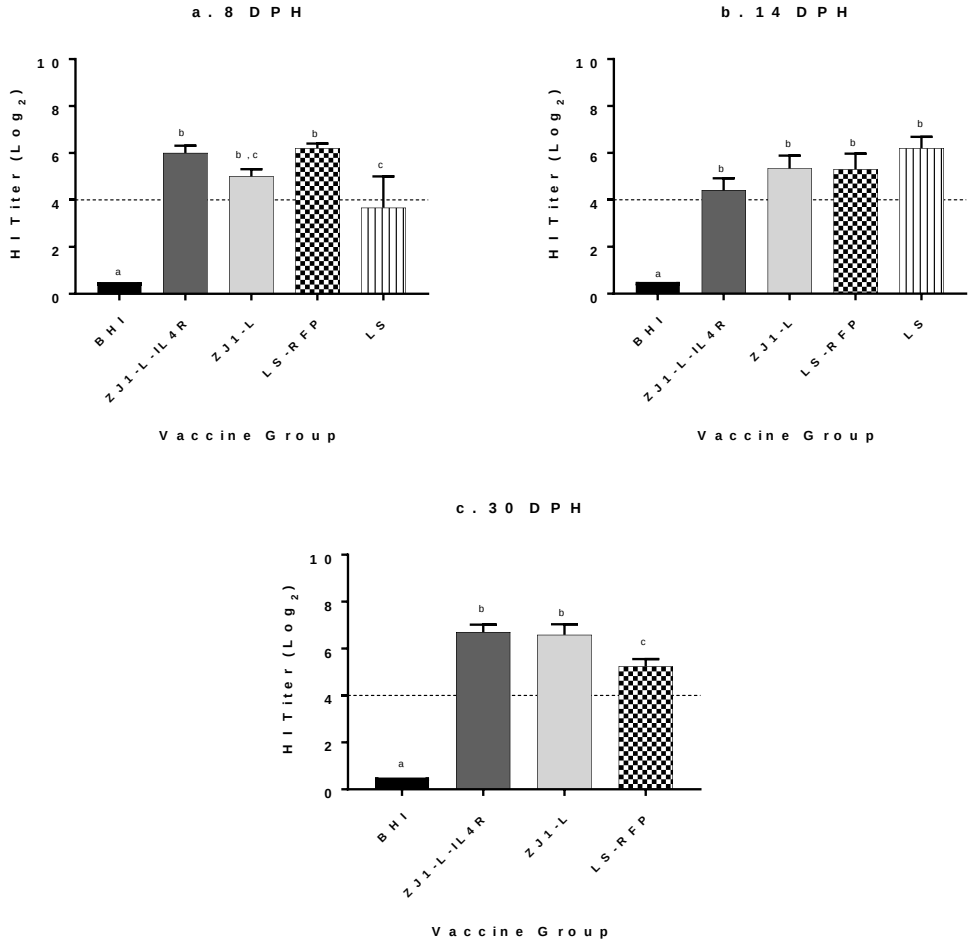
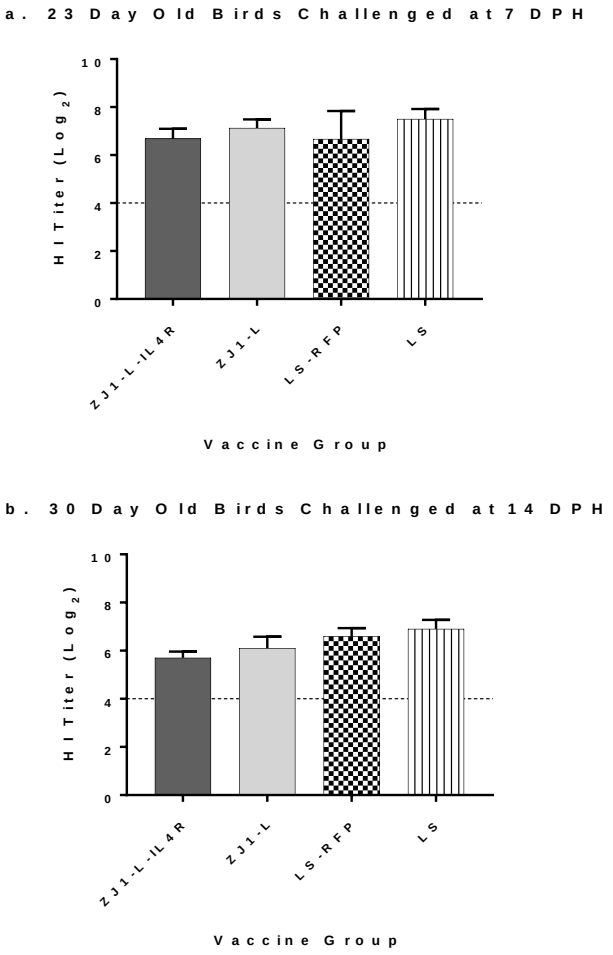


**Figure 5.12. HI titers of 23 and 30-day old birds vaccinated at 19 DOE and challenged at 7 or 14 DPH with vNDV (16 DPC).** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 7 and 14 days post hatch (DPH) 10 chicks in each group were individually bled and challenged with $10^5$ $EID_{50}$ vZJ1 via de oculo-nasal route. At 16 days after 7DPH (**A**) or 14 DPH (**B**) challenge, serum was collected. Significant differences are denoted by different letters ($p \leq 0.05$, one-way ANOVA followed by multiple comparisons Tukey's test). At 23 DPH, n = 3 -8. At 30 DPH n = 10.



| **Table 5.6. Average body weight of birds vaccinated at 19 DOE With $10^{3.5}$ $EID_{50}$ of Different NDVs and Challenged at 7 or 14 DPH respectively, with vNDV (16 DPC)** | | | | |
|---|---|---|---|---|
| | **ZJ1-L-IL4R** (Mean ± SEM)* | **ZJ1-L** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
| **23 DPH (16 DPC)** | 143.3 ± 6.5 | 141.4 ± 8.0 | 140.0 ± 15.3 | 128.8 ± 6.4 |
| **30 DPH (16 DPC)** | 253.0 ± 10.4[a] | 200.0 ± 6.5[b] | 219.0 ± 7.8[b] | 197.0 ± 6.7[b] |
| *Body weight in grams, Significant differences are denoted by different letters. ($p \leq 0.05$), One-way ANOVA followed by a multiple comparisons Tukey's test, n = 3-9 at 23 DPH. n = 10 at 30 DPH). | | | | |

**Figure 5.13. Viral titer of 19 DOE vaccinated SPF chickens VNDV challenged at 7 DPH.** At 19 DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 7 days post hatch (DPH) 10 chicks in each group were challenged with $10^5$ $EID_{50}$ vZJ1 by the oculo-nasal route. Viral shed titers through the oropharyngeal (OP) (a, c) and cloacal (CL) (b, d) routes were measured at 2 (a-b), 4 (c-d), days post challenge (DPC). Differences in viral shed titers were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test ($p \leq 0.05$). Significant differences are denoted by different letters. Groups sharing letters are not significantly different from one another. n= 9-10 birds/vaccine group except for the BHI mock vaccine group at 4 DPC, in which only 3 birds remained alive.

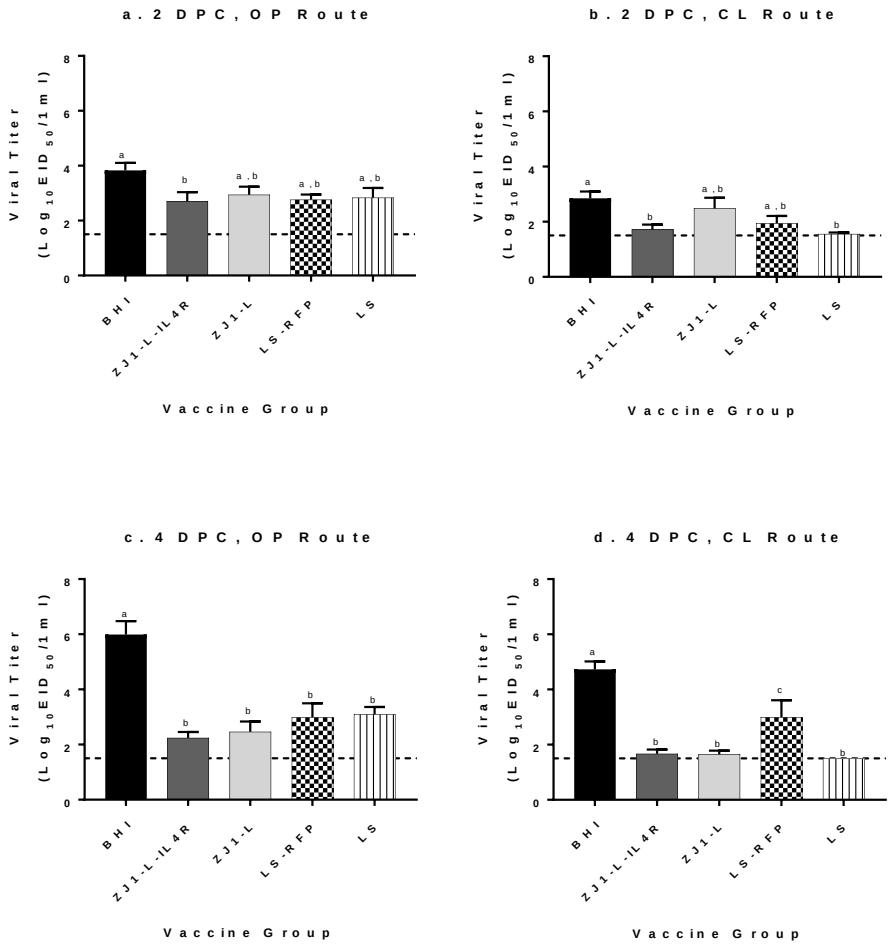
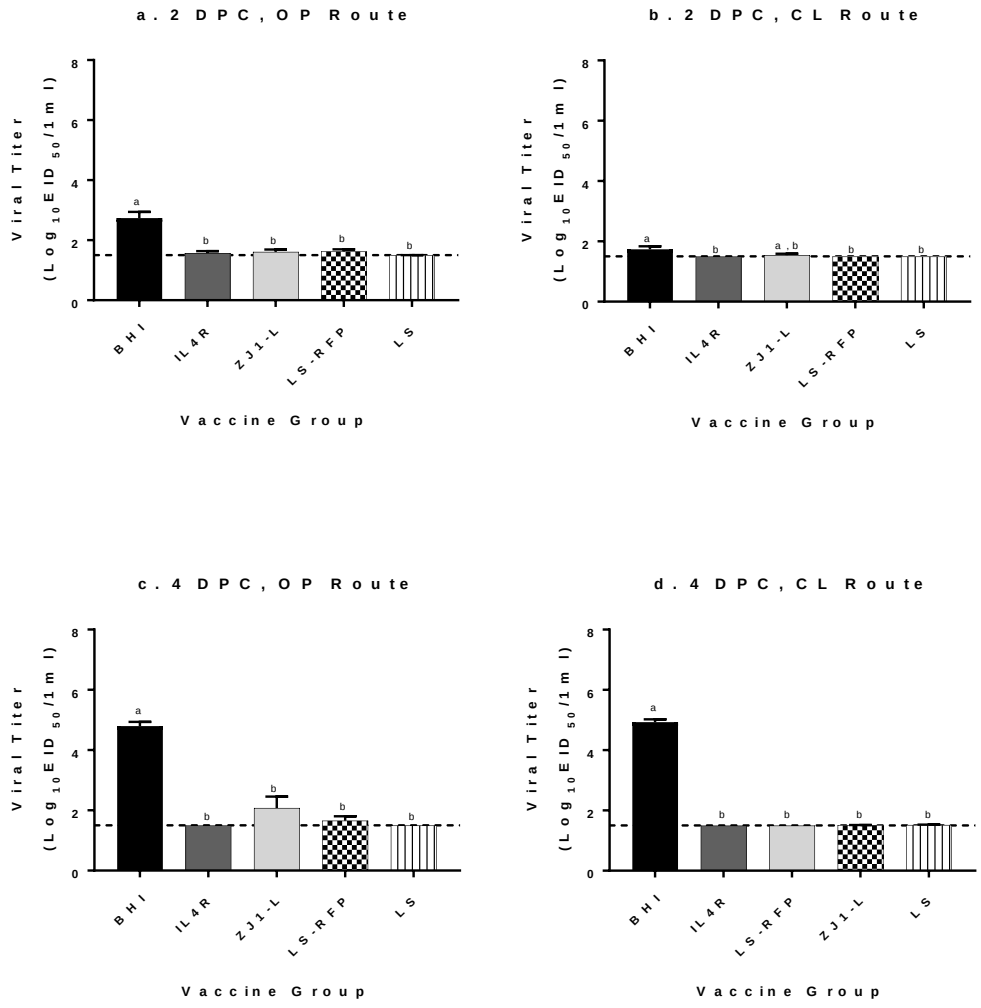


**Figure 5.14. Viral shed titers of 19 DOE NDV vaccinated 14DPH vNDV challenged chickens.** At 19 DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 14 days post hatch (DPH) 10 chicks in each group were challenged with $10^5$ $EID_{50}$ vZJ1 via the oculo-nasal route. Viral shed titers through the oropharyngeal (OP) (**A, C**) and cloacal (CL) (**B,D**) routes were measured at 2 (**A,B**), 4 (**C,D**), days post challenge (DPC). Significant differences are denoted by different letters ($p \leq 0.05$, one-way ANOVA followed by multiple comparisons Tukey's test). n= 10 birds/vaccine group except for the BHI mock vaccine group at 4 DPC, in which only 4 birds remained alive.



| Table 5.7 Percent Survival of Birds Vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$ of NDV or a mock and challenged at either 7 or 14 DPH with vNDV. | | |
|---|---|---|
| **Group** | **Percent Survival at 7 DPH** | **Percent Survival at 14 DPH** |
| BHI | 0[a] | 0 |
| ZJ1-L-IL4R | 90[b] | 100 |
| ZJ1-L | 80[b,c] | 100 |
| LS-RFP | 30[c] | 100 |
| LS | 70[b,c] | 100 |
| Groups with a different letter are significantly different from one another ($\rho$ = 0.05, Long Rank Test, n =10/ vaccine group). | | |

| Table 5.8. Splenic T lymphocyte subpopulations of birds vaccinated at 19 DOE with NDV. | | | | | |
|---|---|---|---|---|---|
| | **BHI** (Mean % ± SEM) | **ZJ1-IL4R** (Mean % ± SEM) | **ZJ1-L** (Mean % ±SEM) | **LS-RFP** (Mean % ± SEM) | **LS** (Mean % ± SEM) |
| **7 DPH[*] CD4+ CD8-** | 33.1 ±4.7** | 38.1 ±2.7 | 34.1 ±4.1 | 36.5 ±5.3 | 39.1±4.9 |
| **7 DPH CD4-CD8+** | 24.4 ±0.9 | 23.3 ±1.9 | 20.5 ±2.3 | 17.6 ±5.2 | 20.2±2.9 |
| **7 DPH CD4:CD8** | 1.4 ±0.2 | 1.7 ±0.2 | 1.7 ±0.3 | 3.1 ±1.0 | 2.1 ± 0.6 |
| **14 DPH CD4+ CD8-** | 20.9 ±3.0 | 26.2 ±2.2 | 26.1 ±1.9 | 22.7 ±2.7 | 33.0±5.9 |
| **14 DPH CD4-CD8+** | 40.3 ±4.2[a] | 32.1 ±1.7[a,b] | 33.2 ±1.0 [a,b] | 34.6 ±1.7[d,e] | 27.0±2.7[b] |
| **14 DPH CD8:CD4** | 2.1 ±0.5 | 1.3 ±0.2 | 1.3 ±0.1 | 1.6 ±0.2 | 0.9 ± 0.3 |
| **30 DPH CD4+ CD8-** | 21.4 ±0.9 | 22.4 ±1.8 | 21.8 ±1.5 | 22.8 ±2.4 | Deceased |
| **30 DPH CD4-CD8+** | 40.4 ±2.1 | 38.1 ±2.1 | 43.4 ±3.3 | 43.4 ±4.2 | Deceased |
| **30 DPH CD8:CD4** | 1.9 ±0.1 | 1.8 ±0.2 | 2.1 ±0.2 | 2.1 ±0.4 | Deceased |
| (*p ≤ 0.05*, One-way ANOVA and multiple comparisons Tukey's test. Significant differences are denoted by different letters across each time point.) [*] Days post hatch | | | | | |

| **Table 5.9. Splenic B lymphocyte subpopulations of birds vaccinated at 19 DOE with NDV.** | | | | | |
|---|---|---|---|---|---|
| | **BHI** (Mean % ± SEM) | **ZJ1-IL4R** (Mean % ± SEM) | **ZJ1-L** (Mean % ± SEM) | **LS-RFP** (Mean % ± SEM) | **LS** (Mean % ± SEM) |
| **7 DPH* IgM- Bu1+** | 3.8 ±1.4 | 5.4 ±0.7 | 7.2 ±0.9 | 5.1 ±1.9 | 4.7 ±0.4 |
| **7 DPH IgM+ Bu1+** | 6.0 ±1.6 | 6.6 ±0.6 | 9.2 ±3.6 | 5.0 ±1.0 | 6.3 ±1.9 |
| **7 DPH IgM+ Bu1-** | 0.1 ±0.0 | 1.8 ±1.1 | 0.8 ±0.3 | 1.2 ±0.7 | 2.1 ±1.0 |
| **14 DPH IgM- Bu1+** | 1.6 ±0.2 | 1.4 ±0.2 | 1.1 ±0.1 | 1.3 ±0.3 | 0.9 ± 0.4 |
| **14 DPH IgM+ Bu1+** | 7.2 ±2.9 | 9.4 ±1.3 | 9.2 ±1.6 | 12.9 ±2.2 | 5.4 ±1.3 |
| **14 DPH IgM+ Bu1-** | 3.8 ±1.2 | 3.7 ±0.4 | 3.7 ±0.8 | 3.3 ±0.3 | 2.2 ±0.2 |
| **30 DPH IgM- Bu1+** | 5.4 ±0.9 | 4.1 ±0.5 | 3.2 ±0.3 | 3.4 ±0.5 | Deceased |
| **30 DPH IgM+ Bu1+** | 10.5 ±1.3 | 6.4 ±0.8 | 10.3 ±1.2 | 6.4 ±1.1 | Deceased |
| **30 DPH IgM+ Bu1-** | 32.7 ±4.9 | 31.0 ±3.4 | 31.5 ±5.9 | 34.5 ±6.6 | Deceased |
| ($p \leq 0.05$, One-way ANOVA and multiple comparisons Tukey's test. Significant differences are denoted by different letters across each time point.) * Days post hatch | | | | | |

| Table 5.10. Anti NDV Immunohistochemical (IHC) findings in 0, 1, 2 and 7-day old SPF chickens vaccinated at 19 DOE with NDV *ovo* with $10^{3.5}$ $EID_{50}$ of NDV. | | | | | |
|---|---|---|---|---|---|
| | **Group** | **Spleen** | **Bursa** | **Thymus** | **Pharynx** |
| **0 DPH[a]** | ZJ1-L-IL4R | 0[b] | 0 | 0.2 | 0 |
| | ZJ1-L | 0 | 0.4 | 0 | 0 |
| | LS-RFP | 0 | 0.2 | 0.4 | 0.6 |
| | LS | 0.4 | 0.2 | 0 | 2.2 |
| **1 DPH** | ZJ1-L-IL4R | 0 | 0.6 | 0.8 | 0.5 |
| | ZJ1-L | 0.2 | 1.2 | 0.8 | 0.6 |
| | LS-RFP | 0 | 0 | 1.2 | 0 |
| | LS | 0.2 | 0 | 0 | 1.2 |
| **2 DPH** | ZJ1-L-IL4R | 0 | 1.3 | 0 | 1.3 |
| | ZJ1-L | 0 | 1.2 | 0.6 | 2.2 |
| | LS-RFP | 0 | 0.5 | 0.75 | 0.75 |
| | LS | 0 | 1.6 | 0.2 | 1.4 |
| **7 DPH** | ZJ1-L-IL4R | 0 | 0 | 0 | 1.0 |
| | ZJ1-L | 0 | 0 | 0 | 0.6 |
| | LS-RFP | 0 | 0 | 0 | 0.5 |
| | LS | 0 | 0 | 0 | 1.3 |

n =3-5 birds

[a] DPH = days post hatch

[b] Average score (0 = negative, 1 = rare cells with signal (1-5 cells), 2 = regular appearance of cells (5-20), 3 = numerous cells with signals (>20), 4 = too many to count)

**Figure 5.15. Bursal and Thymic Depletion Score.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-RFP, LS or brain heart infusion (BHI) control. At 2 days post hatch (DPH), between 2 and 5 birds were euthanized in each group, and the bursa and thymus were stained with H&E. Bursas (a) and thymii (b) were scored between 0 and 4 based on severity of lymphocyte depletion. Group averages and SEM are depicted.

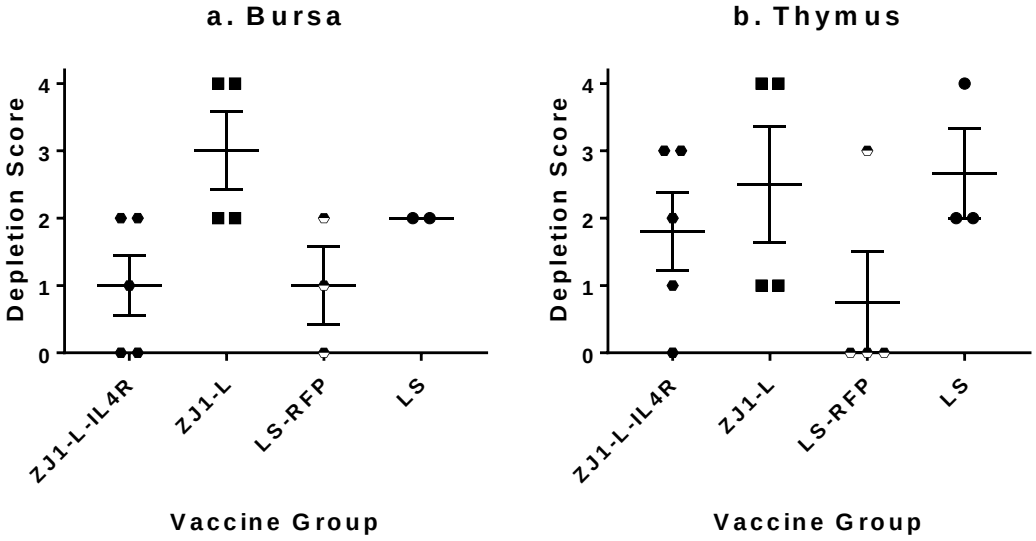
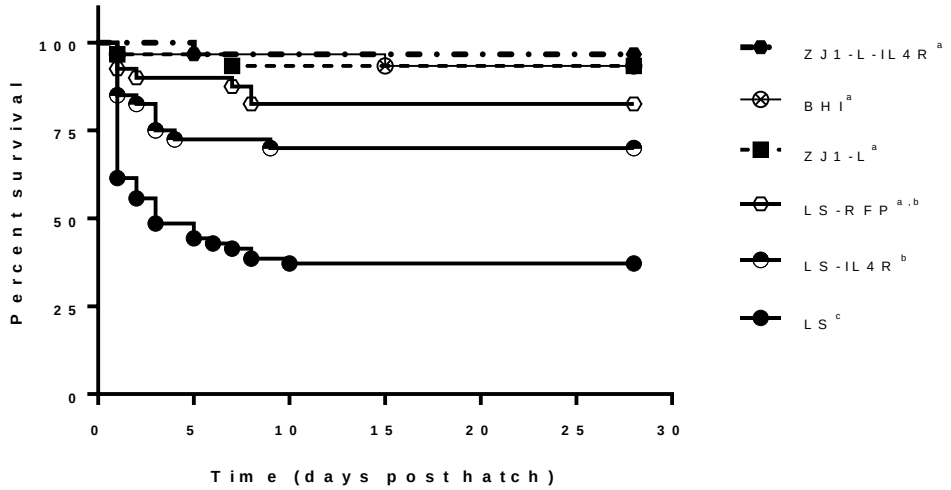



**Figure 5.16. Percent survival of 19 DOE NDV vaccinated SPF eggs.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R (n= 30), ZJ1-L(n= 30), LS-IL4R (n= 40), LS-RFP(n= 40), LS(n= 70) or brain heart infusion (BHI) control (n= 30). After hatch, birds were housed in negative pressure isolators and their survival was checked daily for four weeks. ($p \leq 0.05$, Long-rank test).



| **5.11. Body weights of 19 DOE NDV vaccinated birds at 7, 14 and 21 days post challenge with vNDV.** | | | | | | |
|---|---|---|---|---|---|---|
| | **BHI** (Mean ± SEM)* | **ZJ1-L-IL4R** (Mean ± SEM) | **ZJ1-L** (Mean ± SEM) | **LS-IL4R** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
| 7 DPH | 61.4 ± 1.4[a] | 60.2 ± 1.2[a] | 50.3 ± 2.4 [b] | 60.8 ± 2.1[a] | 63.1 ± 1.1[a] | 46.0 ± 2.4[b] |
| 14 DPH | 121.7 ± 2.2[a] | 116.2 ± 3.1[a,c] | 101.8 ± 4.5[b] | 121.4 ±3.0[a] | 127.3 ± 3.6[a] | 107.3 ± 3.5[b,c] |
| 21 DPH | 194.1 ± 4.9 | 197.8 ± 5.9 | 175.6 ± 7.5 | 197.6 ± 5.5 | 192.4 ± 5.9 | 179.4 ± 6.8 |
| *p ≤ 0.05*, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted by different letters. n = 20 birds at 7 and 14 DPH. n = 14 birds at 21 DPH. *grams, | | | | | | |

**Figure 5.17. Serum HI Titers of 14 and 28 day old 19 DOE vaccinated birds.** At 19 DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS or brain heart infusion (BHI) control. Serum from 14 birds per groups was collected at 14 (**A**) and 28 (**B**) days post hatch (DPH). (*p ≤ 0.05*, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted by different letters.) How many birds per group?

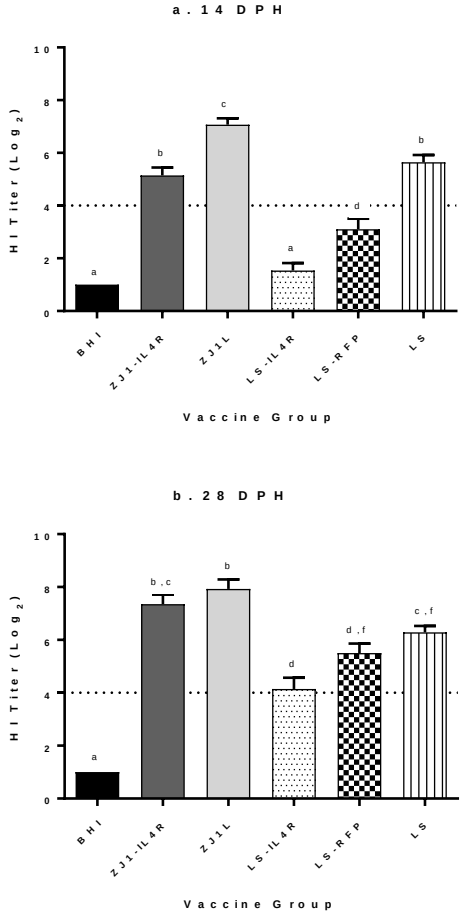


**Figure 5.18. Oropharyngeal viral titers of 2, 4, 7, 9, 11 and 14 days old 19 DOE NDV vaccinated chickens.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS or brain heart infusion (BHI) control. Vaccine shed titers through the oropharyngeal (OP) route was measured at 2 (**A**), 4 (**B**), 7 (**C**), 9 (**D**), 11 **(E**) and 14 (**F**) DPH from 10 birds in each group. ($p \leq 0.05$, one-way ANOVA, multiple comparisons Tukey's test. Significant differences among vaccine groups are denoted by different letters.)

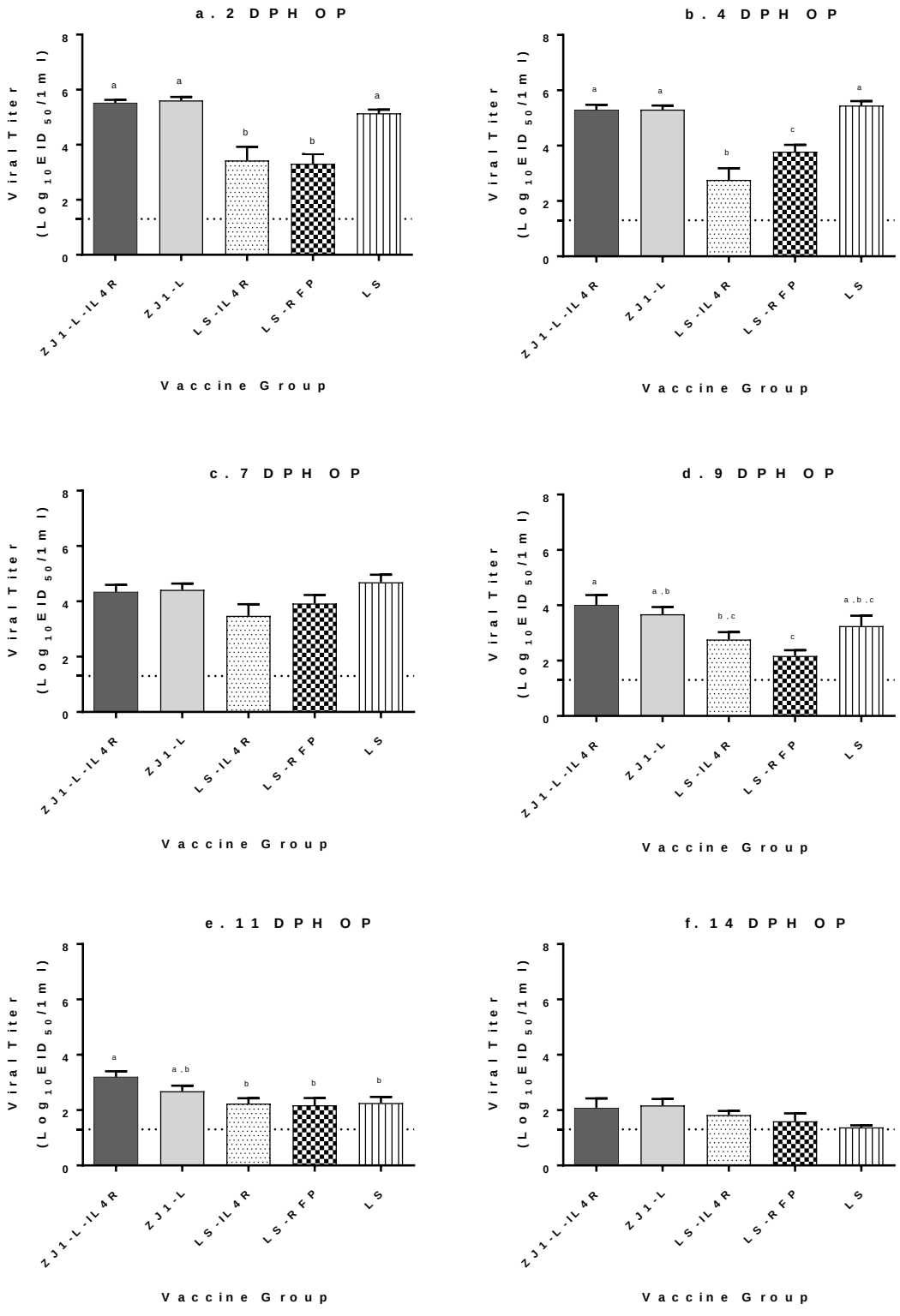

a. 2 DPH OP
b. 4 DPH OP
c. 7 DPH OP
d. 9 DPH OP
e. 11 DPH OP
f. 14 DPH OP
Viral Titer (Log10EID50/1ml)
0
2
4
6
8
a
a,b
b
b,c
c
a,b,c
ZJ1-L-IL4R
ZJ1-L
LS-IL4R
LS-RFP
LS
Vaccine Group

**Figure 5.19. Cloacal viral titers of 2, 4, 7, 9, 11 and 14 days old 19 DOE NDV vaccinated chickens.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS or brain heart infusion (BHI) control. Vaccine shed titers through the CL route was measured at 2 (**A**), 4 (**B**), 7 (**C**), 9 (**D**), 11 (**E**) and 14 (**F**) DPH from 10 birds in each group. ($p \leq 0.05$, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted by different letters.)

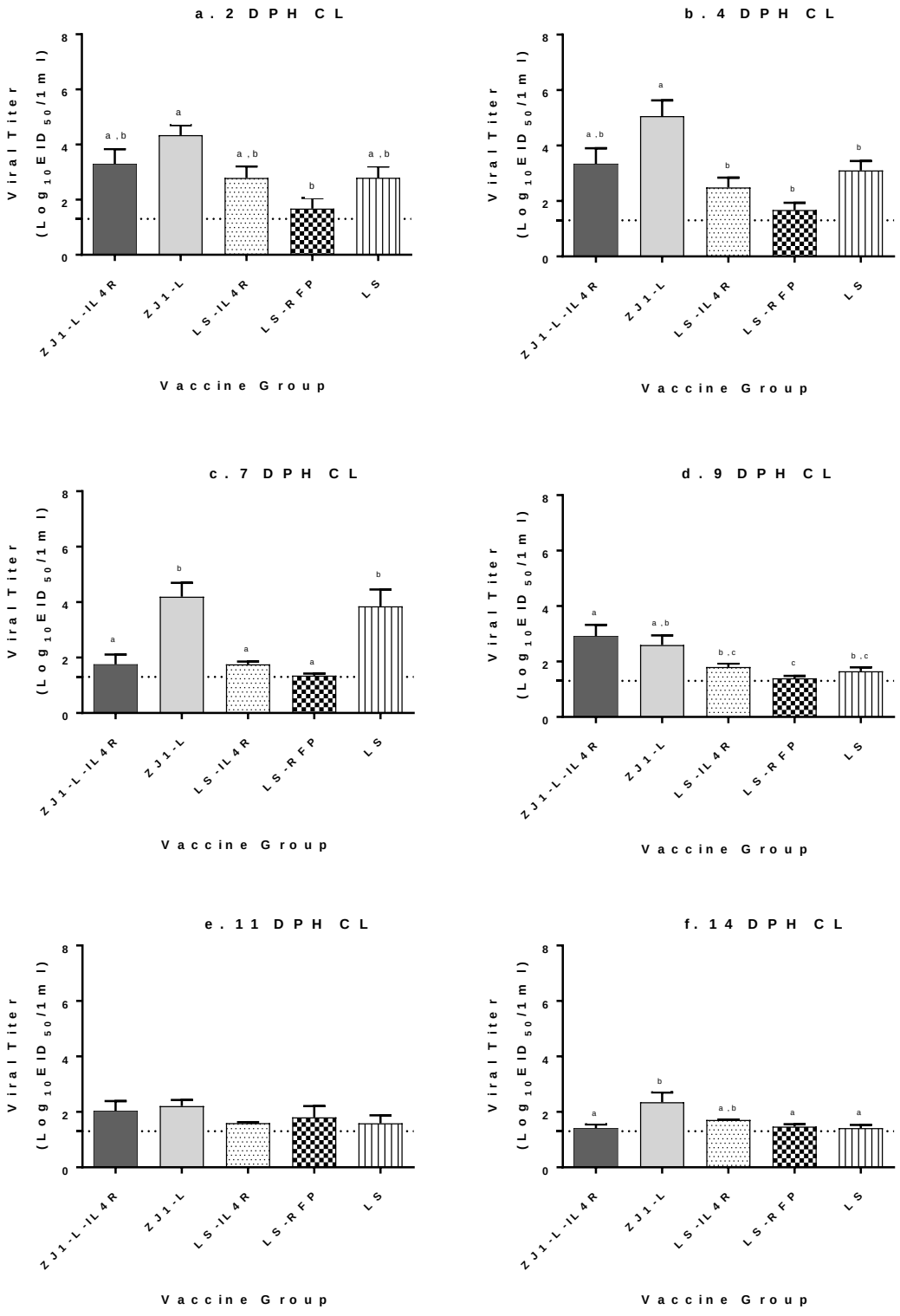

a. 2 DPH CL
b. 4 DPH CL
c. 7 DPH CL
d. 9 DPH CL
e. 11 DPH CL
f. 14 DPH CL
Viral Titer (Log10EID50/1ml)
0
2
4
6
8
a, b
a
b
ZJ1-L-IL4R
ZJ1-L
LS-IL4R
LS-RFP
LS
Vaccine Group

**Figure 5.20. CMI recall response of 19 DOE NDV chickens.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS or brain heart infusion (BHI) control. At 35 days post hatch (DPH), spleens were collected for T cell proliferation assay. (*p ≤ 0.05*, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted with*)

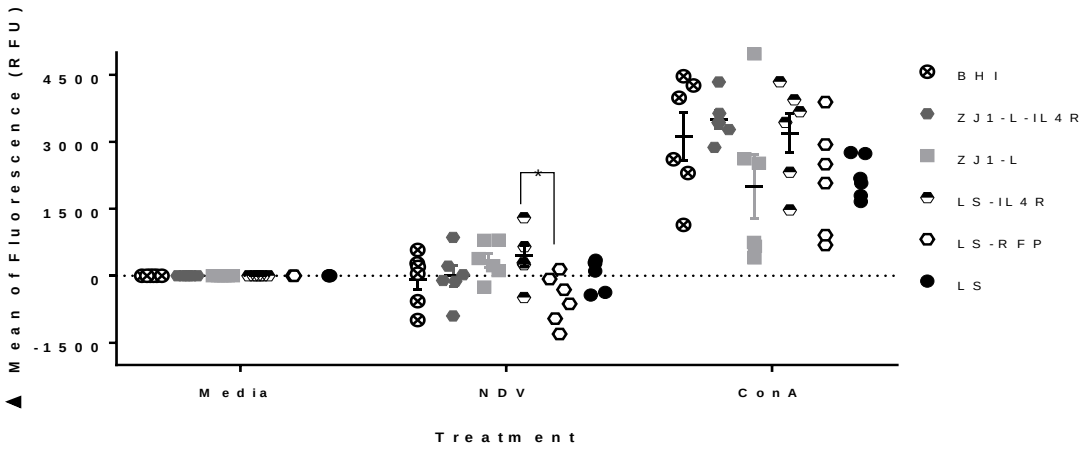
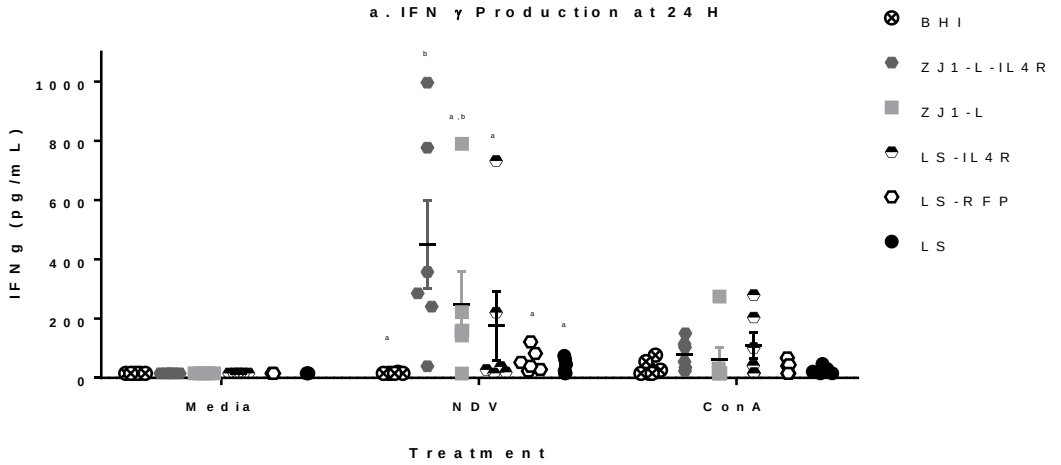


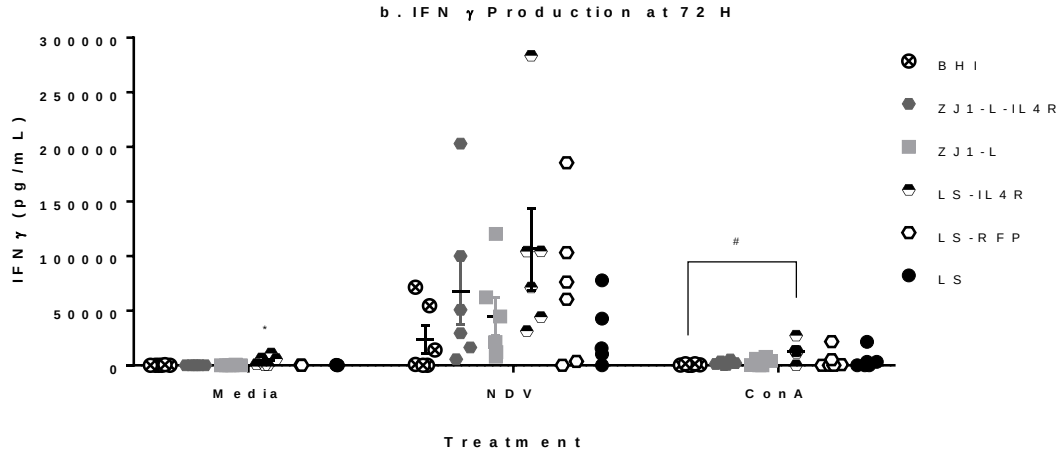

**Table 5.12. Chicken IFNγ production in splenocytes at 24 and 72 hours post stimulation.**

| | **BHI** (Mean ± SEM) | **ZJ1-L-IL4R** (Mean ± SEM) | **ZJ1-L** (Mean ± SEM) | **LS-IL4R** (Mean ± SEM) | **LS-RFP** (Mean ± SEM) | **LS** (Mean ± SEM) |
|---|---|---|---|---|---|---|
| 24 h, Media | 15.6 ± 0.0 | 15.6 ± 0.0 | 15.6 ± 0.0 | 15.6 ± 0.0 | 15.6 ± 0.0 | 15.6 ± 0.0 |
| 24 h, ZJ1-L | 16.2 ± 0.6[a] | 449.6 ± 147.6[b] | 247.9 ± 111.9[a,b] | 175.4 ± 115.9[a,b] | 58.0 ±15.4[a] | 39.8 ± 10.3[a] |
| 24 h, ConA | 34.2 ± 10.6 | 80.1 ± 20.5 | 61.5 ± 42.7 | 109.7 ± 44.8 | 33.1 ± 8.7 | 312.1 ± 286.9 |
| 72 h, Media | 125.7 ± 108.5 | 16.3 ± 0.7 | 151.3 ±116.8 | 4,027.9 ± 1,802.2 | 55.9 ± 40.2 | 111.0 ± 95.4 |
| 72h, ZJ1-L | 23,518.4 ± 12,877.4 | 67,578.3 ± 30,342.0 | 44,850.0 ± 17,330.5 | 106,525.7 ± 37,458.0 | 71,561.2 ± 28, 206.4 | 24,447.3 ± 12,444.4 |
| 72 h, ConA | 617.3 ± 277.8 | 2,178.3 ± 790.5 | 3,044.0 ± 1,395.7 | 13,057.8 ± 3,519.2 | 4,601.5 ± 3,532.5 | 4,733.3 ± 3,437.3 |

*p ≤ 0.05*, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted by different letters. n = 6.

**Figure 5.16. IFNγ production during recall response of 19 DOE NDV chickens.** At 19 days DOE, SPF ECEs were vaccinated *in ovo* with $10^{3.5}$ $EID_{50}$/ egg of ZJ1-L-IL4R, ZJ1-L, LS-IL4R, LS-RFP, LS or brain heart infusion (BHI) control. At 35 days post hatch (DPH), spleens were collected for lymphocyte stimulation and IFNγ levels were measured at 24 (a) or 72 (b) hours post stimulation ($p \leq 0.05$, one-way ANOVA, multiple comparisons Tukey's test. Significant differences are denoted by different letters.)



# CHAPTER 6

# CONCLUSIONS

Newcastle Disease (ND) causes devastating losses to the poultry industry worldwide despite heavy vaccination protocols and biosecurity measures. Vaccine failure may occur due to a variety of reasons, including, inappropriate application or storage of the vaccine, immunosuppression by other agents, interference by maternal antibodies in young birds, antigenic diversity and improper biosecurity measures. Vaccination *in ovo* can address some of these concerns by using an automated system that ensure vaccination of every embryonated chicken egg (ECE) with a uniform dose stored properly, without increased labor and vaccine costs. This delivery form will ensure the establishment of early immunity as long as the vaccine can avoid interference by maternal antibodies passively transferred from the hen to the chick in the egg yolk and white. In the present work, we evaluated recombinant ND virus (rNDV) vaccines as *in ovo* vaccine candidates to provide more efficient and early immunity against virulent NDV strains.

First, we evaluated the effect of dose and vaccination time on survival after vaccination, and protection after challenge. This was accomplished by vaccinating ECEs at different times *in ovo* with different doses of rNDV vaccines containing either an antisense chicken IL-4 insert (ZJ1-L-IL4R), an IL-2 expressing variant (-IL2), an IL-10 expressing variant (-IL-10) or an IFNγ expressing variant (-IFNγ) and testing them with challenge against homologous genotype VIId NDV (Chapter 3). In the second of experiments, we evaluated the effect of vaccination with rNDV an

IL4R on the development of acquired immunity by vaccinating embryonated chicken eggs with rNDV vaccines containing an IL4R (Chapter 4). To safely move our samples from our biosafety level 2 (BSL2) facility to a BSL1 facility for flow cytometric analysis, we first ensured that our protocol for fixing lymphocytes completely inactivated the virus (Chapter 5).

First, it was hypothesized that vaccination *in ovo* with rNDV would not negatively impact hatchability or survival after hatch. Results in Chapter 3 confirm our hypothesis, it is possible to develop an *in ovo* rNDV vaccine that does not negatively impact survival. Bird vaccinated with ZJ1-L-IL4R displayed no adverse effects and demonstrated robust weight gains. The other recombinants (ZJ1-L-IL2, ZJ1-L-IL10, and ZJ1-L- IFNγ) however, had high levels of mortality compared to the mock control group, making them unsuitable for *in ovo* vaccination.

Second, it was hypothesized that vaccination *in ovo* with rNDV would improve survival after vNDV challenge compared to non-vaccinated birds without negatively impacting survival compared to parental viral strains. Results shown in Chapters 3 and 4 demonstrate that the ZJ1-L-IL4R, ZJ1-L-IL10, and ZJ1-L- IFNγ recombinants provided 100% protective immunity against challenge with virulent NDV at 14 DPC. More importantly, vaccination with the ZJ1-L-IL4R recombinant allowed for 90-100% protection post challenge at 7 DPC. In contrast, the LS-RFP recombinant provided less than 60% protection.

Finally, it was hypothesized that the improvement in vaccine protection observed with the ZJ1-L-IL4R recombinant is due to the IL4R insert modulating adaptive immunity toward towards a predominantly cell-mediated response by

inhibiting IL-4 production. Unfortunately, we were unable to prove that this was the case. We measured IL4, IL10 and IFNγ production during primary infection *in vitro* and during recall CMI response. In addition, we looked at T cell proliferation and lymphocyte subpopulations in the spleen. No differences were observed in IL4 or IL10 production in cells infected with ZJ1-L-IL4R or ZJ1-L. A decrease in IFNγ production was observed in lymphocytes infected ZJ1-L-IL4R, compared to those infected with ZJ1-L, refuting our hypothesis that the IL4R insert modulates adaptive immunity toward towards a predominantly cell-mediated response during primary infection. No differences in proliferation during primary infection were observed.

The results from CMI recall response in Chapter 4 were inconsistent. In one experiment, birds vaccinated with ZJ1-L-IL4R had higher proliferation capabilities and proportions of CD4-CD8+ cytotoxic T cells compared to those of birds vaccinated with the ZJ1-L strain. In a different experiment however, no differences were seen in the T cell proliferation capabilities or the proportions of lymphocytes between birds vaccinated with ZJ1-L-IL4R and ZJ1-L. The birds from the first experiment were genetically similar birds obtained from Charles River Laboratories whereas those used in the later experiments were non-genetically similar birds from the SEPRL flock. It is possible that the inconsistencies may be due to the high genetic variability of the flock.

To test the ability of the IL4R insert to modulate immunity, we developed a new recombinant with a different backbone, LS, and compared it to a recombinant with a similar sized insert, LS-RFP and to the parental strain LS. While differences

were observed in the proliferation capabilities of lymphocytes collected with birds vaccinated with LS-IL4R, compared to those vaccinated with LS-RFP, neither were significantly different from lymphocytes vaccinated with a mock vaccine or with the parental strain. In addition, the LS-IL4R cells were producing high quantities of IFNγ regardless of whether they had been stimulated or not.

High IFNγ production in the splenocytes from vaccinated birds was observed at 24 and 72 hours post stimulation with the inactivated NDV antigen. At both timepoints, IFNγ production was higher in the recombinant vaccines compared to parental strains, but not significantly.

Our work exposes a viable vaccine candidate for *in ovo* vaccination against NDV. Further work needs to be done to explore the mechanism of action in genetically similar birds to reduce the variability observed in cytokine production and T cell proliferation. Future work should also test whether the vaccine is able to establish immunity in the presence of maternal antibodies, and whether lower doses of the vaccine applied at 19 DOE can provide similar protection.