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Induction of Antiviral Response Against Avian Infectious Laryngotracheitis Virus Infection

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Induction of Antiviral Response Against Avian Infectious Laryngotracheitis Virus Infection

by

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ABSTRACT

Toll-like receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs). The PAMPs that act as ligands for TLRs prompt downstream signalling leading to antimicrobial effects. However, the details of antiviral responses of lipotechoic acid (LTA) and CpG DNA, which act as ligands for TLR-2 and -21 respectively, elicited against avian viruses are scarce. We investigated whether *in ovo* delivery of LTA and CpG DNA induces antiviral responses against infectious laryngotracheitis virus (ILTV) infection in chickens. We found that *in ovo* delivery of these two ligands reduces ILTV infections in lungs pre- and post-hatch. However, only CpG DNA could reduce mortality and morbidity due to ILTV infection encountered post-hatch. The expression of IL-1β mRNA and increase of macrophage numbers in lungs were found to be correlates of observed antiviral responses. Thus, LTA and CpG DNA can be candidate TLR ligands worthy of further investigation for the control of ILTV infection in chickens.

Keywords: *In ovo*, toll-like receptor-2 and -21, LTA, CpG-motif containing synthetic DNA, infectious laryngotracheitis virus, lung, chicken

PREFACE

The studies described in this thesis were performed at the Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada. The work described in this thesis was carried out by myself, Simrika Thapa, from September 2013 to May 2015 under the joined supervision of Drs. Faizal Careem and Markus Czub. The technical and analytical supports in real-time PCR optimization and standard curve generation were obtained from Amber M. Kameka and Jasmine Hui. Mohamed Sarjoon Abdul Cader supported me in tissue sectioning and immunohistochemistry staining and Kalamathy Murugananthan supported me in extracting nucleic acid from tissues. The thesis contains the materials already published elsewhere, which are listed below.

1. **Thapa, S.**, Abdul Cader, M.C., Murugananthan, K., Nagy, E., Sharif, S., Czub, M. and Abdul-Careem, M.F. 2015. *In ovo* delivery of CpG DNA reduces avian infectious laryngotracheitis virus induced mortality and morbidity. Viruses 7 (4):1832-52

2. **Thapa, S.**, Nagy, E and Abdul-Careem, M.F. 2015. *In ovo* delivery of toll-like receptor 2 ligand, lipoteichoic acid induces pro-inflammatory mediators reducing post-hatch infectious laryngotracheitis virus infection. Vet. Immunol. Immunopathol. 164 (3-4):170-8

3. Haddadi, H., **Thapa, S.**, Kameka, A.M., Hui, J., Czub, M., Nagy, E., Abdul Careem, M.F. 2015. Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection *in vitro* and *in vivo*. Dev Comp Immunol. 48(1):22-32

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DEDICATION

I would like to dedicate this thesis work to my beloved Mom and Dad

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LIST OF ABBREVIATIONS

ABC	Avidin–biotin–peroxidase complex
AD	Anderson-Darling test
AIV	Avian influenza virus
AP	Activator protein
APC	Antigen presenting cells
ATCC	American Type Culture Collection
BF	Bursa of Fabricius
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
cDNA	Complementary DNA
CEK	Chicken embryonic kidney cells
CEL	Chicken embryonic liver cells
CEO	Chicken embryo origin
CFIA	Canadian Food Inspection Agency
CK	Chicken kidney cells
CMV	Human cytomegalovirus
CpG DNA	Deoxycytidyl-deoxyguanosine dinucleotides
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

Dpi	Days post-infection
dsRNA	Double-stranded RNA
ED	Embryo day
EDTA	Ethylenediaminetetraacetic acid
EID50	50% Embryos infective dose
FACS	Fluorescence-activated cell sorting
FARM	Free avian respiratory macrophages
FBS	Fetal bovine serum
FPV	Fowlpoxvirus vectored vaccines
G	Glycoprotein
H & E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HBV	Hepatitis B virus
HRIC	Health Research Innovation Center
HSV-1	Herpes simplex virus-1
HVT	Turkey herpesvirus vectored vaccines
IBV	Infectious bronchitis virus
ICP	Infected cell protein
IFNs	Interferons
IKK	IκB kinase
IL	Interleukin
ILT	Infectious larygotracheitis

ILTV	Infectious larygotracheitis virus
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
Kbp	Kilo base pairs
LCMV	Lymphocytic choriomeningitis virus
LMH	Leghorn male hepatocarcinoma cell line
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LTA	Lipoteichoic acid
MD	Marek's disease
MDV	Marek's disease virus
MHC	Major histocompatibility complex
MQ-NCSU	Macrophage-North Carolina State University
mRNA	Messenger RNA
miRNA	Micro RNA
MyD88	Myeloid differentiation primary response factor 88
NF	Nuclear factor
NK	Natural killer
NLR	NOD-like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OCT	Optimal cutting temperature
OD	Optical density

ORFs	Open reading frames
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFU	Plaque forming unit
PK	Protein kinase
PRR	Pattern recognition receptors
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SPF	Specific Pathogen Free
ssRNA	Single-stranded RNA
TAK-1	Transforming growth factor-β-activated protein kinase 1
TCO	Tissue-culture origin
T_h1	Type 1 helper T-cells
TK	Thymidine kinase
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter inducing interferon- β
UCVM	University of Calgary Faculty of Veterinary Medicine
U_L	Long unique regions

U_S	Short unique regions
VSRS	Veterinary Science Research Station

CHAPTER ONE: INTRODUCTION

1. General introduction

Innate host responses as first line of defense are initiated after recognition of pathogen-associated molecular patterns (PAMPs) by receptors that belong to the innate immune system. The molecular components of innate immune system are notably conserved among plants, insects, birds and mammals. The innate responses are potent, quick and non-specific conferring broad-spectrum of protection. The plants and invertebrates such as insects and worms are solely dependent on this arm of the immune system to overcome the challenges of pathogens. In higher organism, antigen specific adaptive immune responses play a critical role in long-term protection against encountered pathogens which relies on the active role of innate immune system. As such, targeting the innate immune system as prophylactic and therapeutic means for the control of pathogens is an emerging field. In the innate immune system, toll-like receptors (TLRs) present on immune cells which recognize the PAMPs are gaining increasing attention in disease control. The binding of TLRs with their ligands (i.e. PAMPs) transiently activates the innate immune system [1]. In fact, the activation of innate immune system leads to the production of pro-inflammatory cytokines such as interleukin (IL)-1β and antiviral cytokines such as type I interferons (IFNs) and expansion of local populations of innate immune cells such as heterophils/neutrophils, dendritic cells (DCs), macrophages and natural killer (NK) cells. Furthermore, the activated innate immune cells, particularly DCs and macrophages, can initiate antigen presentation in addition to their participation in clearance of pathogens. The function of innate immune cells in antigen presentation suggests a role of TLRs as linking molecules operating between innate and adaptive immune systems [2]. Altogether, initiation of innate immune responses through activation of TLR signalling can be developed as a promising mean of viral disease control.

A range of poultry viruses uses respiratory mucosa for host entry. Thus, the induction of innate immune responses at the respiratory mucosa is decisive for the control of these viral infections which could be done by TLR activation. One way of activating TLR signalling at the respiratory mucosa in chickens is delivery of TLR ligands *in ovo* during embryo development. Due to the economic importance and also due to the limitations in current vaccine mediated control measures, infectious larygotracheitis virus (ILTV) infection control is receiving attention. This thesis work focussed on investigating the antiviral effects of *in ovo* delivery of TLR-2 ligand, lipoteichoic acid (LTA) and TLR-21 ligand, unmethylated deoxycytidyl-deoxyguanosine dinucleotides containing deoxyribonucleic acid (CpG DNA) against ILTV infection in chickens.

2. ILTV

2.1. Taxonomy of ILTV

ILTV is an avian respiratory virus and renamed as *Gallid* herpesvirus-1 recently. Since this virus is better known with old nomenclature, ILTV is used throughout the thesis. ILTV belongs to the genus *Iltovirus*, subfamily *Alphaherpesvirinae* and the family *Herpesviridae* [3].

2.2. Structure of ILTV

ILTV is an enveloped virus with linear double stranded DNA (dsDNA) genome and has an icosahedral symmetry [4]. The ILTV genome is approximately 150 kilo base pairs (kbp) in size consisting of 77 open reading frames (ORFs) with long (U_L) and short (U_S) unique regions and inverted repeats bordering the U_S region [5]. As illustrated in Figure 1.1, the viral genome is surrounded immediately by a capsid, which is further encircled by a tegument consisting of proteins and outer lipid bilayer known as envelope. Dwelling between envelope and nucleocapsid,

are the tegument proteins of the virus that play a significant role in transportation of capsid into cytoplasm and then to the nucleus [6]. The surface of the envelope contains the spikes of viral glycoproteins, namely glycoprotein (g) B, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM [7] and are responsible for virus entry and transporting nucleocapsid for initiating viral replication. In addition, glycoproteins are important as major antigens inducing antibody- and cell-mediated immune responses in the host [8].

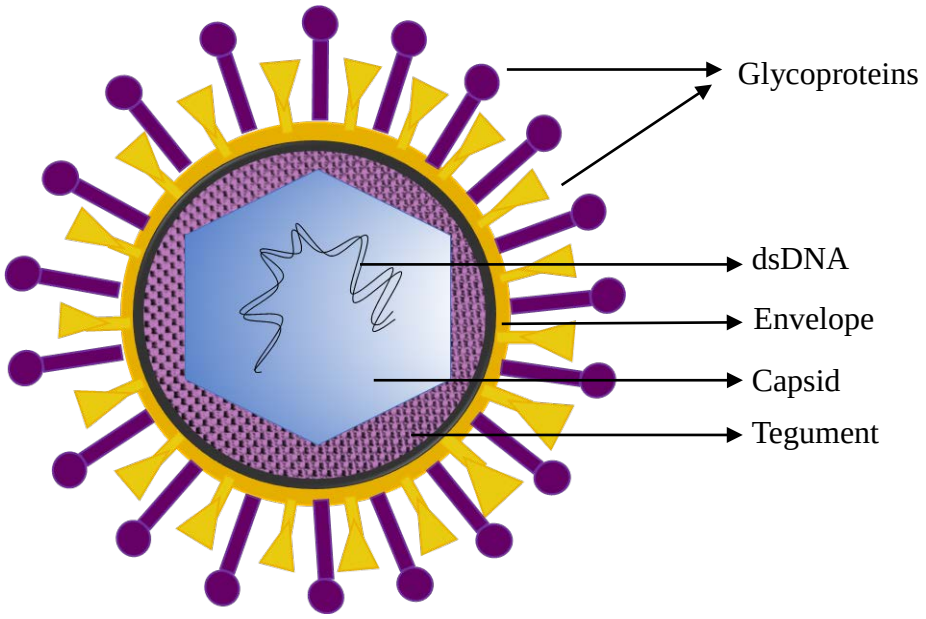


Figure 1.1. Structural components of an ILTV viral particle. ILTV consists of glycoproteins embedded in the envelope which surrounds the capsid. The dsDNA is contained inside the capsid. There is a protein layer called tegument existing between envelope and capsid [4, 9].

2.3. Epidemiology of ILTV infection

2.3.1. Susceptible hosts

ILTV primarily infects chickens of all ages [10, 11]. Other hosts of ILTV include pheasants, peafowl [12] and turkeys [13, 14]. Some other avian species such as sparrows, crows, pigeons and ducks have been reported to be ILTV resistant [12, 15].

2.3.2. Distribution of ILTV infection

Although infectious laryngotracheitis (ILT), the disease caused by ILTV, was first reported in Canada in 1925 [16], it is now a threat with significant economic losses to poultry industries globally [17, 18]. It has been reported that the outbreaks of ILT occurring in different regions of the world are mainly related to live attenuated ILTV vaccine strains rather than wild-type viral strains. In North America, the isolated field strains of ILTV have been described to be genetically close to vaccine strains [19-21]. Similarly, studies in Australia [22-24], Europe [25], Asia [26, 27] and South America [28] suggested the potential displacement of wild-type strains with vaccine variants.

2.3.3. Transmission

Being a highly contagious respiratory infection, the main mode of transmission of ILTV is horizontal by direct or indirect contact and there is no evidence of vertical transmission via eggs [29].

2.3.4. Survival of ILTV in the environment

Being an enveloped virus, ILTV is inactivated following contact with chemicals that targets lipid envelop such as ether, chloroform, cresol and hydrogen peroxide (H_2O_2) [30, 31]. In the poultry barn environments, the most commonly used inactivation method for ILTV is the use of 5% H_2O_2 [31]. Although the virus is strongly cell associated, ILTV can survive and maintain its infectivity for days to months in respiratory secretions and chicken carcasses at a temperature range of 13-23°C. ILTV also survives for months to years at -20 to -60°C [17]. The sensitivity of ILTV to heat varies greatly among strains and certain strains are resistant than others when heated at 56°C for 1 hour [30].

2.4. Viral replication

Although ILTV replication cycle has not been studied in detail, it is expected that the ILTV replication cycle is similar to the replication cycle of its herpesvirus prototype, herpes simplex virus-1 (HSV-1) [7]. ILTV glycoproteins such as gB, gC, gD, gH and gL are assumed to be involved in the process of attachment to host cell receptor [5, 32]. However, only gC is found to be involved in the entry of ILTV into the host cell [33]. Unlike HSV-1, ILTV entry is thought to be heparin sulphate independent [32, 33]. After attachment, the envelope fuses with the host cell membrane allowing the tegument and nucleocapsid to get released into the cytoplasm. Once in the cytoplasm, the transportation of nucleocapsid towards the nuclear membrane occurs followed by the release of viral DNA into the nucleus [4]. Inside the nucleus the transcription and replication of viral DNA take place exploiting the host cell machinery. The viral replication process is highly regulated and there are three major categories of genes (α, β and γ) expressed at different periods of viral transcription and translation. The expression of α genes is self-regulated and occurs at the

beginning of ILTV infection, the protein products of which are non-structural and critical for expression of β genes between 4 to 16 hours post-infection [34]. The proteins of β genes are essential for viral replication and regulation of production of viral structural proteins encoded by late γ genes [35]. These proteins are not completely studied in case of ILTV, however, some information regarding function has been adapted from other alphaherpesviruses using sequence homology [5]. During the viral replication process in the nucleus, viral capsids are formed and DNA is packaged. The nucleocapsid, which is assembled in the nucleus is then translocated to the vacuoles in the cytoplasm [36]. The assembly of tegument proteins occurs in the trans-Golgi region and secondary envelope is formed during budding from trans-Golgi membrane which gets released from the host cell via exocytosis [7]. Besides, the formation of tubular structures and large cytoplasmic vacuoles with many virions are most often seen in the ILTV infected cells [36]. An *in vitro* infection study has reported that the ILTV replication starts with the formation of infectious progeny viral particles after 8 to 12 hours following infection and the maximum viral replication is detected within 24 to 30 hours post-infection [34].

2.5. ILTV pathogenesis

Naturally, ILTV infects upper respiratory tract, eye mucosa, lungs and air sacs [29] and the infection can be systemic where ILTV can be spread to liver, caecal tonsils and cloaca [29, 37]. Additionally, the ability of the ILTV to infect leukocytes [38] and macrophages *in vitro* [39] has been shown and this may be the possible explanation for the establishment of systemic infection by ILTV. Once in the tissues, ILTV starts the lytic phase of replication leading to clinical manifestations. The lytic infection is followed by the establishment of latency of ILTV in trigeminal ganglion [40] coinciding with the induction of effective adaptive immune responses.

The breakdown in the immunity due to various factors such as stress due to introduction of birds or onset of laying reactivates latent ILTV [41, 42]. This leads into lytic replication of ILTV in the epithelium and shedding of the virus through the respiratory route [29, 40]. Thymidine kinase (TK) gene in ILTV is essential for virulence and reactivation of virus from latency, but is not needed for virus replication [43, 44]. In addition to TK gene, it has been shown that putative latency associated transcripts that lies within infected cell protein (ICP) 4 gene whose expression is regulated by two micro ribonucleic acids (RNA) (miRNA) [45, 46]. These miRNAs may be involved in the regulation between lytic and latent phases of virus *in vivo* [47].

2.6. Clinical signs

Lytic replication of ILTV during primary infection or following reactivation from latency may lead to clinical manifestations in susceptible birds. Clinical signs can be seen 6-12 days post-infection (dpi) after natural infection whereas in experimental situation it can be recorded at 2-4dpi [17]. ILTV results in mild to severe respiratory manifestations. The severe form of ILTV infection leads to dyspnea and discharge of bloody respiratory mucus leading to mortality rate up to 70%. The mild form of ILTV infection is characterized by depression, low egg production and loss of body weights [17].

2.7. Laboratory host systems

For viral propagation, chicken eggs in incubation are widely used and ILTV can lead to plaque formation on chorioallantoic membrane (CAM) following CAM infection [48]. In *in vitro* conditions, ILTV can be propagated in chicken embryonic kidney (CEK) cells, chicken embryonic liver (CEL) cells and chicken kidney (CK) cells [48, 49]. For experimental purpose, leghorn

chicken hepatocarcinoma cell line (LMH) can be used for ILTV propagation once the virus is adapted to cell culture [50, 51].

2.8. Host responses against ILTV infection

2.8.1. Innate immune responses

The mucous lining in the respiratory tract and the ciliated epithelium provides a physical barrier for the viral infection and alters the viral particle movement [52, 53]. The particles, including microorganisms trapped in sticky mucous secreted by goblet cells are carried away by muco-ciliary escalator mechanisms resulting from constant beating of cilia towards the pharynx. Following viral infection of respiratory epithelium, the damage to the epithelium occurs leading to the disruption of the physical barrier as well as the muco-ciliary escalator mechanism leading to retention of microorganisms and subsequent secondary infections. The infected cells are able to produce chemokines and cytokines causing inflammation as part of innate immune responses [52]. Mechanisms of innate responses generated against ILTV infection have been poorly investigated. A mononuclear cell infiltration in response to ILTV infection has been observed in lamina propria and towards the epithelium in the respiratory tract, where migration of macrophages, lymphocytes and plasma cells takes place [54]. A microarray analysis suggested that in the chicken embryonic lung tissue infected with ILTV, there is an up regulation of messenger RNA (mRNA) expression of many genes related to cellular growth and proliferation, apoptosis, cell signalling and inflammation. For example, the expression of genes of cytokines and chemokines such as, IL-6, IL-8, IL-15, CXC K60, CCL17 and CCL20 have been shown to increase following ILTV infection [55] suggesting the role of inflammation during the early phase of infection. Nevertheless, the

susceptibility to ILTV infection and induction of protective responses depend on the genetic makeup of the chickens as well as the age [10, 56].

2.8.2. Adaptive immune responses

Additional to the innate host responses, the activation of adaptive immune responses leads to the antigen specific effector cells and molecules such as T-cells and antibodies. Due to ILTV replication in host cells, the recruitment of inflammatory cells occurs at the site of infection which may lead to the development of adaptive responses [57]. It has been shown that T-cells are distributed throughout and B-cells appear as a cluster in the respiratory mucosa following ILTV infection [57]. Passive immunity is not effective in providing protection to the young birds, although maternal antibodies against ILTV are transferred through eggs [58]. After ILTV infection, virus-neutralizing antibodies are found in the serum within 5-7dpi, reach a peak level at 21dpi and can remain for about a year [59, 60]. While the ILTV-specific total antibodies were noticed in tracheal swabs from 5dpi, IgA and neutralizing antibodies became detectable only at 6dpi and 14dpi respectively. Moreover, IgA and IgG secreting plasma cells started to increase in numbers between 3 to 7dpi [61]. Although it is known that the IgG and IgA protects the mucosal surfaces of respiratory tract from infections [62], systemic and local antibody responses are not protective against ILTV, rather it is cell-mediated immune responses play a critical role in the protection against ILTV [10, 63, 64]. An experiment to observe the efficacy of cytokines as adjuvants for recombinant gB based DNA vaccine revealed that ILTV drives type 1 helper T-cells (T_h1)-type responses [65, 66]. However, limited investigations have been conducted to study cell-mediated immune responses generated against ILTV infection.

2.9. Immunomodulation by ILTV

Most often, the viruses with larger genome size, such as herpes and pox viruses, have the ability to modulate host immune responses so that they can evade the host's immune system increasing their replication and survival. Similar to other herpesviruses, ILTV encodes proteins involved in blocking of complement system, cytokine signalling and antibody- and cell-mediated immune responses. A complex protein of gE and gI can bind to the Fc receptors, thus creating a interference for the binding of immunoglobulin, IgG. Similarly, gC binds to C3 in complement system, thus inhibiting the complement activation [67]. Moreover, it has been shown that alphaherpesviruses are capable of inhibiting apoptosis of infected cells and altering the NK cell- and T-cell-dependent host response pathways [68-72]. Similarly, ILTV also has the ability to inhibit apoptosis of infected cells, yet inducing apoptosis in the neighbouring uninfected cells *in vitro* [73-75].

2.10. Control measures

In regions where intensive poultry production systems are followed, the combination of biosecurity measures and immunization with live-attenuated vaccines are adopted to control ILT in both broiler and layer operations.

2.10.1. Biosecurity

ILTV could initiate an outbreak of ILT in commercial layer or broiler operations in number of ways. Firstly, ILTV could persist in chicken barn environment for months under favourable temperature conditions. The regular monitoring of site for hygiene is necessary to prevent the fomite transmission of ILTV through contaminated equipment, personnel, feed, waste and carcasses to newly stocked birds [17]. Secondly, since ILTV infection in birds is a lifelong

infection, it is suggested that the backyard flocks provides a constant source of ILTV. ILTV originated from backyard flocks may reach the commercial poultry flocks via fomites if tight biosecurity measures are not employed. Finally, the vaccination with live-attenuated vaccines can lead to latently infected carrier birds and the mixing of vaccinated and susceptible birds should be completely avoided to prevent transmission of the virus. Current ILTV vaccines induce latently infected carrier birds by two ways: 1. The live attenuated vaccine can be virulent itself and later undergo latency [76] and 2. ILTV vaccine virus itself can establish the latency and gain virulence when reactivated and passaged from bird-bird [77]. A recent study reported that strict biosecurity measures and prolonged downtime of flocks following ILT outbreaks may lead to the significant decrease in ILT reoccurrence [78].

2.10.2. Vaccination against ILT and its limitations

Vaccines are intended to stimulate the adaptive arm of the immune system of the host leading to antibody- and cell-mediated immune responses. Vaccinating the bursectomized chickens lacking antibody-mediated immune responses but not cell-mediated immune responses have been shown to be protective when challenged with virulent ILTV [10]. This fact confirmed the indispensable role of cell-mediated immune responses over antibody-mediated immune response in vaccine mediated protection against ILTV infection. Moreover, the presence of anti-ILTV antibodies locally in trachea has also been ruled out as protective for ILTV challenge [63].

Vaccines against ILT have been developed in the early 1930s utilizing the CAM virus propagation, in which the field isolates became weaker in virulence following several passages and these attenuated strains were used as vaccines. Still today this CAM attenuation method is used for development of ILTV vaccines commonly known as chicken embryo origin (CEO) vaccines. Other

widely used type of vaccine is tissue-culture origin (TCO) and generated by serial passage of virulent strains of ILTV in cell cultures [79]. Both CEO and TCO vaccines have been demonstrated to be effective in providing protection in chickens against ILTV challenge encountered up to 10 weeks post-infection. Still, in older chickens over 20 weeks of age, CEO vaccines appears to be superior in terms of providing better protection when compared to TCO vaccines [80].

Mass vaccination of chickens with ILTV vaccines via drinking water and aerosol spray routes has been done commonly for labour saving purposes [17]. Consequently, vaccine uptake by the birds are not uniform, when vaccines are administered using these mass vaccination methods. The inherent property of commonly used ILTV vaccines, which is spread of vaccine virus from bird to bird may compensate for birds that did not take the adequate dose [81]. On the other hand, uptake of higher dose of the vaccine due to the mass vaccination methods may lead to adverse reactions in some birds [17].

Although currently available live-attenuated vaccines are effective in providing protection against ILT [59], limitations allow the use of these vaccines predominantly in ILT endemic areas. These limitations include virulence of vaccine strains leading to clinical manifestations and establishment of latency by vaccine strains, reactivation and transmission of vaccine virus from vaccinated to non-vaccinated birds and gaining virulence in the process [77, 82]. The vaccine-related ILT outbreaks have become an increasing concern and are most common in intensive poultry production areas globally [5, 19, 20, 24, 25]. Moreover, the ILTV vaccine use has been complicated by the recent record of the recombination events between circulating ILTV and vaccine strains in an area leading to novel virulent strains of ILTV [23].

Recombinant ILTV gene deletion mutants have been investigated as vaccine candidates [33, 44, 83-85]. A gG-deletion mutant vaccine has been found to be a potential candidate under

experimental situations [86]. However, further studies are needed to understand its efficacy in field conditions. Furthermore, viral-vectored vaccines with ILTV glycoproteins expressed in vectors such as herpesvirus of turkeys (HVT) and fowlpoxvirus (FPV) have been developed. Although these vaccines lacks the risk of gaining virulence like live attenuated vaccines [87], their use is limited in poultry operations due to partial protection [88]. Thus, development of novel strategies to overcome the limitations in existing ILT control measures are needed. One such option could be targeting the TLRs to activate and strengthen innate responses in newly hatched chickens which may in turn direct the adaptive immune system leading to long lasting protection.

3. Avian immune system

As in mammals, the avian immune system is classified into two categories; innate and adaptive immune systems [89]. The innate immune system provides a non-specific first line of defense against pathogens through anatomical (such as skin and mucus membrane), physiological (enzymes, fever and anti-microbial peptides) and cellular barriers (innate immune cells) [90]. The innate cells such as macrophages, heterophils, thrombocytes, DCs and NK cells are the active cellular components recruited during innate response [91, 92]. These cells can detect the PAMPs associated with broad range of microbes via pattern recognition receptors (PRR) and some innate cells such as macrophages, DCs and heterophils can engulf pathogens via phagocytosis [91, 92]. Both these events lead to the release of cytokines and chemokines by these cells that can attract more immune cells to the site of infection leading to inflammatory responses [93, 94]. The inflammatory response can be effective in combating infection in some cases [95-97].

The adaptive immune responses are comparatively slower to initiate, long-lasting and antigen-specific. One of the remarkable characteristic of adaptive immune system when compared to the

innate immune system is their capability to remember the encountered antigens eliciting quicker and stronger secondary responses up on reinfection [98]. The effector component of the adaptive immune system consists of B- and T- lymphocytes [99]. Unlike in mammals, B-cells in chickens are matured in a specialized organ called bursa of Fabricius (BF) whereas T-cells are developed in thymus in both avian and mammals [100, 101]. The specific recognition of antigens by T-cells takes place following antigen presentation by antigen presenting cells (APC) such as macrophages, DCs and B lymphocytes. In fact, macrophages and DCs characterized as innate immune cells recognize the pathogens via PRRs and present the proteins encoded by the pathogens as peptides on class I and II major histocompatibility complex (MHC) molecules for recognition by T-cells [102]. This suggests that generation of adaptive immune responses is highly dependent on pathogen recognition in the innate arm of the immune system.

3.1. Role of macrophages in avian respiratory viral infections

Macrophages are one of the most important cellular components of the innate immune system that exist in the avian respiratory system to counteract respiratory viral infections [103, 104]. In the mucosal surface of avian lungs, free avian respiratory macrophages (FARM) are found to be less frequent when compared to that of mammals according to respiratory lavage analysis [105]. However, external stimuli such as Freund's adjuvant introduced into the abdominal air sacs and bacterial infections may lead to recruitment of FARMs into both lungs and air sacs [106, 107]. The FARM are mainly present on the epithelial lining of atria and infundibulae of the lung. Moreover, macrophage are also found in the connective tissue below epithelium on the atrial floor and inter-atrial septa and surface of air sacs [108-111]. Unlike in mammals, the surface of the air capillaries in birds lacks macrophages [108]. During inflammatory response, macrophages can reach the site

of infection through chemotaxis [110]. Chemokines such as chicken restricted chemokine 9E3/CEF4 [112, 113] (similar to mammalian chemokine IL-8 [114]) and IL-1β [115] are responsible for attracting macrophages rapidly to the site of infection for phagocytosis. Besides functioning as a phagocytic cells, macrophages in chicken lungs have the ability to respond to various stimuli and upregulate the expression of MHC class II, cluster of differentiation (CD)40 and CD80 on the surface for antigen presentation and T-cell priming [116].

Avian macrophages are known to expand their population in the respiratory tract early following viral replication in these tissues. This has been shown in chickens following Marek's disease virus (MDV) infection [117]. Although MDV utilizes macrophages as a site of replication, their significance in innate immune responses following MDV infection suggest their involvement in the virus clearance and induction of adaptive immune responses [117]. Such responses are phagocytosis [118, 119], production of pro-inflammatory (IL-1β) [117, 120] and antiviral cytokines (interferon (IFN)-γ) [121] and nitric oxide (NO) through expression of inducible nitric oxide synthase (iNOS) [118, 122, 123]. Similarly, the significant increase in macrophages early in both lungs and trachea after subsequent avian infectious bronchitis virus (IBV) infection correlating with the mRNA expression of pro-inflammatory mediators has been reported [124]. It has also been shown that partial depletion of macrophages residing in chicken respiratory tract using clodronate encapsulated-liposomes increases IBV load in trachea (Kameka, A.M., unpublished results). These results overall suggest the antiviral response of avian respiratory macrophages against IBV infection. Furthermore, the antiviral role of avian macrophages against ILTV and avian influenza virus (AIV) *in vitro* has also been shown to be associated with the mRNA expression of IL-1β and IFN-γ [51, 125]. The IL-1β is known to attract macrophages and

other innate cells such as heterophils to the infection sites as described in case of AIV and MDV infections [115, 117, 126, 127].

During viral infection, cytoplasmic PRRs including endosomal TLRs, retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) are actively involved in recognition of viral double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG DNA [128, 129]. Among PRRs, TLRs are the most efficient early innate molecules involved in recognizing viral infections in chickens [130]. The interaction between PRRs and viral components leads to the signalling, culminating in the production of innate molecules such as pro-inflammatory cytokines, chemokines, type I and II IFNs acting as antivirals.

3.2. Toll-like receptors

TLRs were originally discovered as critical proteins involved in innate immunity against a fungal infection in the fruit fly, *Drosophila melanogaster* [131]. These receptors are also considered as linking molecules between innate and adaptive arms of the immune system [2]. Structurally, TLRs are transmembrane proteins containing an extracellular domain with leucine-rich repeats (LRRs) and a cytoplasmic tail with a conserved Toll/IL-1 receptor (TIR) domain. TLR are one of the PRRs, which recognize pathogen encoded PAMPs at mucosal surfaces. The interaction of TLRs and PAMPs leads to the induction of innate immune responses directed against microbial infections including viral infections [132, 133]. The PAMPs can be natural or synthetic in origin which include LTA, lipopolysaccharides (LPS), flagellin, dsRNA and CpG DNA [134, 135].

In mammals, the presence of the following TLRs has been recorded: TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, TLR-7, TLR-8, TLR-9, TLR-10, TLR-11, TLR-12 and TLR-13 [136]. TLRs

are generally conserved and less variable among different animal species [137-139]. TLR-15 and TLR-21 are unique for avian genome and TLR-21 is the functional homolog of mammalian TLR-9 [140, 141]. In addition, the orthologs of mammalian TLR-8 and TLR-13 are absent in avian species [142-145]. The TLRs are mostly found on the plasma membranes of the immune and epithelial cells of chickens with the exception of endosomal TLR-3, TLR-7 and TLR-21 [146]. The endosomal TLR-3, TLR-7 and TLR-21 recognise PAMPs encoded by intracellular pathogens such as dsRNA, ssRNA and CpG DNA motifs respectively. Unlike CpG DNA recognition in mammalian species, in avian species CpG DNA motifs are recognized by TLR-21 [135]. The different TLRs present in chickens, their locations, ligands and signalling pathways are depicted in Figure 1.2.

3.2.1. TLR signalling

Upon recognizing PAMPs or danger associated molecular patterns (DAMPs), the TLRs lead to the activation of signalling pathways resulting in innate immune responses [2]. There are two distinct signalling pathways down stream of TLRs, a myeloid differentiation primary response (MyD) 88-dependent pathway resulting in activation of transcription factor nuclear factor (NF)-κB and a MyD88-independent pathway utilizing TIR-domain-containing adapter inducing interferon- β (TRIF) resulting in production of type I IFNs [2, 147].

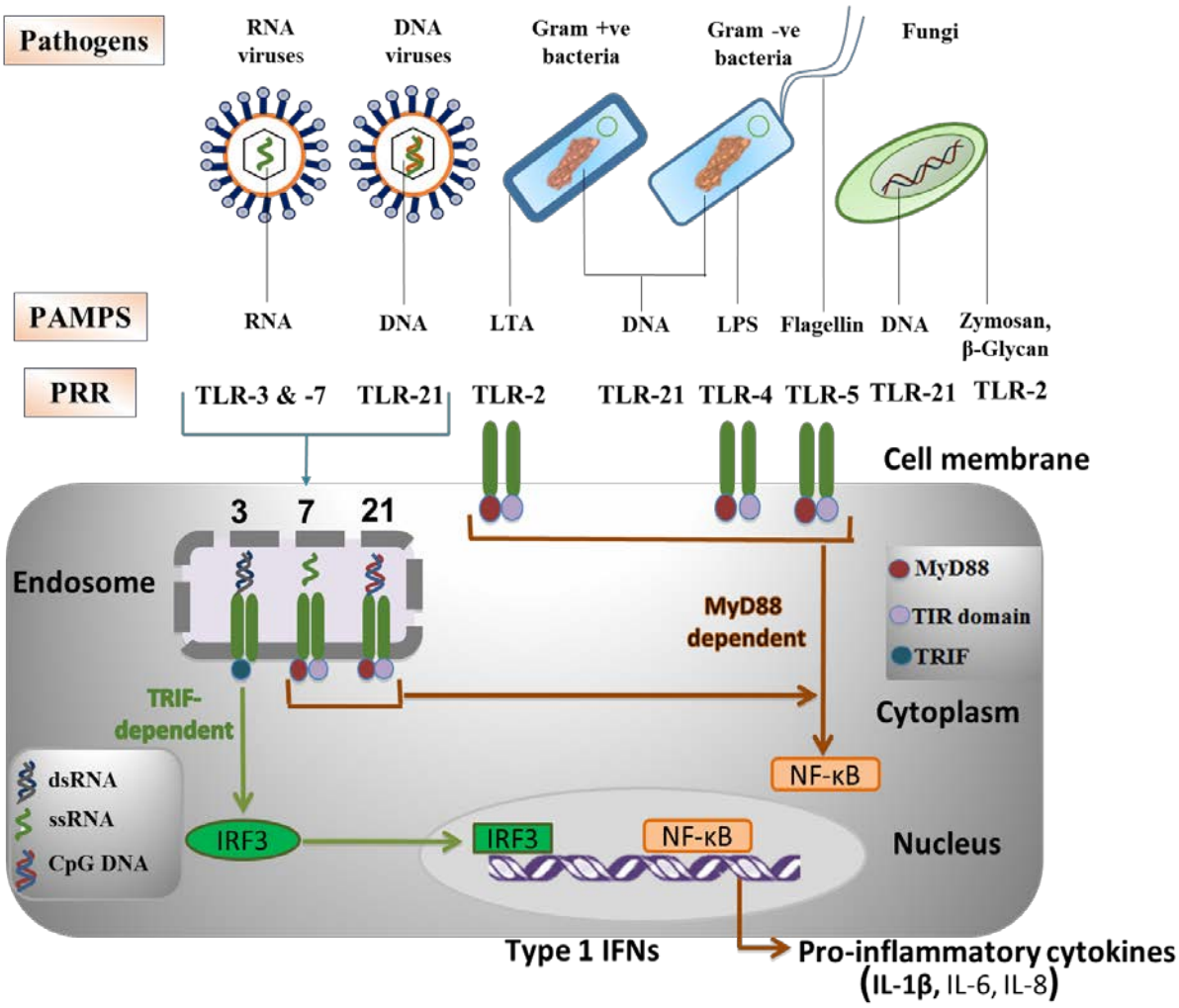


Figure 1.2. The TLRs present in chickens and their signaling pathways. Adapted from O'Neill and colleagues and St. Paul and colleagues [136, 148].

Similar to mammals, avian TLRs except TLR-3 signals through MyD88 [149]. The recognition of PAMPs by TLRs activates MyD88 and IL-1 receptor-associated kinase (IRAK) family. The phosphorylation of IRAK4 and IRAK1 occurs resulting in their binding to tumor necrosis factor (TNF) receptor-associated factors (TRAF) 6 following the dissociation from receptor complex. TRAF6 together with ubiquitin-conjugating enzymes leads to the activation of transforming growth factor-β-activated protein kinase 1 (TAK1). This kinase later activates IκB kinase (IKK) complex and the mitogen-activated protein kinase pathway leading to activation of the transcription factors, NF-κB and activator protein (AP)-1 respectively. The outcome of NF-κB activation is the expression of pro-inflammatory cytokine genes, including IL-6 and IL-1β [150, 151].

MyD88-independent pathway, also termed as TRIF-dependent pathway, is mediated by TLR-3 and utilizes TRIF which recruits IKKε (also known as IKK-i) and TANK (TRAF family member-associated NF-κB activator)- binding kinase 1 (TBK1). The activation of TBK1 leads to phosphorylation of transcription factor, interferon regulatory factor (IRF) 3 resulting in activation of interferon-β (IFN- β) promoter. In mammals, TLR-4 signalling followed by binding to LPS can lead through TRIF/TRAM pathway for activation of IRF3 for IFN- β production. However, in chickens TLR-4 mediated TRIF-dependent pathway is not functional [152].

4. TLR-2 and -21 Ligands

Among TLRs, TLR-2 binds to peptidoglycan, LTA, and lipoproteins of Gram-positive bacteria and zymosan of yeast [153]. LTA is found in the surface of Gram-positive bacteria and is released after lyses of bacteria by lysozyme or antibiotics. LTA contains a hydrophobic region which is a glycolipid or a phosphatidyl glycolipid, whereas hydrophilic region is a 1-3-phosphodiesterase-

linked polymer with sugar or D-alanine substitution at C2 position of glycerol residues [154, 155]. It is the hydrophobic lipid moiety of LTA that allows the bacteria to bind to cell membranes and scavenger receptors, CD14 and TLR-2 in the cells, including macrophages [156].

CpG DNA is a TLR-21 ligand in chickens. CpG DNA motifs of microbial origin are known to be unmethylated and present in bacteria and viruses with DNA genome at much higher frequencies than in eukaryotes [157]. Synthetic CpG DNA shares similarity with CpG DNA originated from microbes. Depending on the structures and immune responses produced, synthetic CpG DNA has been divided into three major classes [158]. Class A CpG DNA contains a naturally present phosphodiester backbone with central poly CpG DNA motifs, the stimulation by which leads to type 1 IFN production [159, 160]. Class B CpG DNA has a phosphorothioate backbone throughout and it helps in B cell and monocytes stimulation [160, 161]. Class C CpG DNA has the properties of both class A and B stimulating type 1 IFN production and B-cell activation with one or two CpG DNA motif(s) within a phosphodiester backbone at the 5' end and a palindromic sequence on a phosphorothioate backbone at the 3' end [162].

5. Anti-microbial role of TLR-2 and -21 ligands

In avian species, the LTA recognition by the TLR-2 has been reported [163] where the activation of TLR-2 encoded on heterophils was shown to up regulate the expression of mRNA of the pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8 following LTA treatment [164]. Induction of TLR-2 signalling leading to antiviral responses against hepatitis B (HBV) virus [165] and human cytomegalovirus (CMV) [166, 167] infections *in vitro* has been shown. TLR-2- mediated antiviral activity was also shown against influenza virus induced pneumonia in mice [168] and

parainfluenza virus infection in guinea pigs [169]. The efficacy of LTA against an avian microbial infection is yet to be determined.

In mammals, the innate immunostimulatory properties of synthetic CpG DNA has been shown *in vitro* with production of cytokines such as IFN-α, IFN-γ, IL-6 and IL-12 by peripheral blood derived macrophages from porcine, bovine, ovine, rhesus monkey and murine origin [170-174]. It has been also shown that CpG DNA induces *in vivo* release of cytokines in mice, sheep and cattle [175-178]. In addition, CpG DNA has been found to have protective effect in mice against microbial infections such as *Leishmania major*, *Chlamydia trachomatis* and *Helicobacter pylori* [179-181], viral infections such lymphocytic choriomeningitis virus (LCMV), HBV, poxvirus and HSV-2 [182-185]. Similarly, in chickens, it has been reported that CpG DNA is a potential candidate in providing protection against bacteria such as *Escherichia coli* [186, 187], *Salmonella Typhimurium* [188] and *S. Enteritidis* [189] and viruses such as AIV [190]. *In ovo* administration of CpG DNA at embryo day (ED) 18 or 19 also has been shown to be protective against systemic *E. coli* and *S. Typhimurium* infections post-hatch [187, 188]. It has been indicated that CpG DNA induced protection against *E. coli* and *S. Enteritidis* appears to be associated with increased functions of heterophils [186, 191]. Nevertheless, the role of other innate immune cells such as macrophages and NK cells as well as other molecular events that contribute to the CpG DNA mediated antimicrobial effect has not been investigated. Moreover, the adjuvant property of CpG DNA in chickens has been shown with vaccination against AIV [192], Newcastle disease virus [193] and infectious bursal disease virus [194, 195]. Furthermore, recent studies showed that *in ovo* CpG DNA treatment at ED18 eggs induces antiviral effects against IBV reducing viral loads in chicken lungs, trachea, spleen and kidney tissues pre-hatch [196, 197]. However, the efficacy of *in ovo* delivered CpG DNA against ILTV infection is yet to be determined.

6. *In ovo* route of delivery

In ovo delivery of antigens is extensively investigated in poultry disease control since it is vital, due to the ubiquitous nature of pathogen distribution in poultry barn environments, to employ control methods pre-hatch. Induction of host responses pre-hatch empowers the bird's immune system at hatch and minimize the window of susceptibility. In fact, non-pathogenic avian herpes viruses and attenuated virulent viruses have been routinely used as *in ovo* vaccines most notably for the control of MDV associated lymphoma formation in chickens [198]. These *in ovo*-administered vaccines have proven to be efficacious in preventing morbidity and mortality induced by the MDV. The vaccines delivered *in ovo* reach the amniotic cavity as such the respiratory mucosa [199].

In order to obtain day old chickens, the fertile eggs are incubated at 37.2-37.6°C at 60-70% relative humidity for 21 days in which last 3 days, the eggs are kept in Hatcher compartment of the incubator to avoid turning. An embyonating egg has four basic compartments during the final stage of incubation; air cell, allantoic cavity, amniotic cavity and yolk sac. The air cell contains gas and is situated at the top of the broader side of egg between the shell and CAM. The allantoic cavity surrounds the amniotic cavity and contains fluid with waste. The amniotic cavity is the closest compartment to the developing embryo and consists of amniotic fluid. The yolk sac is located inside the amniotic cavity and is internalized by the time the egg is hatched. The yolk sac consists of reserved nutrients for the developing embryos and the hatched chickens rely on uptake of nutrients originated from internalized yolk sac for first 72 hours post-hatch.

In order to achieve maximal immune response by *in ovo* vaccination, it is very important to identify optimal time and most suitable embryo compartment for injection. Although the *in ovo* injection can be done between ED17.5 and ED19.2 of incubation to get maximum hatchability, for

vaccination to prevent Marek's disease (MD) ED18 is considered standard [200]. This might be due to the fact that at ED18 the eggs are transferred from Setter compartment to Hatcher compartment of the incubator and hence, it is convenient and less labor intensive to vaccinate during the transfer process. The studies done to evaluate the most suitable embryo compartment for *in ovo* MD vaccination to achieve maximum immune response suggested that 94% of birds originated from *in ovo* vaccinated eggs were protected after vaccine was delivered into the amniotic cavity. Moreover, inoculation of MD vaccine into the allantoic compartment resulted in 28.3% protection while into yolk sac provided no effective induction of protection against MD [201]. Thus, the current recommendation is that the *in ovo* injection should be done deep into the eggs through the air cell into the amniotic cavity in order to be effective.

STATEMENT OF RATIONALE

ILTV causes one of the most economically important respiratory diseases in chickens worldwide including North America. Being a herpesvirus, ILTV establishes latent infection leading to life-long infection in chickens. In North America, it has been suggested that backyard flocks infected such a way provide a source of virus for transmission. Due to the highly contagious nature of ILTV infection, the commercial poultry flocks are under constant threat of contracting ILTV from these sources. The control of ILT caused by ILTV is relied on the vaccination in addition to biosecurity measures. The current live attenuated vaccine use is confined to ILT endemic areas due to their limitations and recombinant vaccine option without these limitations has not been popular due to low efficacy. These limitations of the current ILT control methods in chickens could be addressed by developing novel strategies which may be used to substitute or add to the existing control measures.

One such option may be the stimulation of innate immune system through the activation of the TLR signalling pathways, thus strengthening the innate arm of the immune system. The stimulation of the TLR signalling pathways has been shown to increase innate host responses against viral diseases in mammals. The beneficial effects of activation of innate immune responses via TLR signalling in chickens for the control of AIV and MDV has also been shown. However, the efficacy of stimulation of TLR signalling pathways in chickens against ILTV infection is yet to be elucidated as has been the mechanisms involved.

HYPOTHESES

The hypotheses tested in the studies presented in this thesis are:

1. The TLR-2 ligand, LTA, when delivered *in ovo,* induces antiviral responses against ILTV infection encountered both pre- and post-hatch correlating with higher mRNA expression of pro-inflammatory mediators and expansion of macrophages.
2. The TLR-21 ligand, CpG DNA, when delivered *in ovo* induces antiviral responses against ILTV infection encountered both pre- and post-hatch correlating with higher mRNA expression of pro-inflammatory mediators and expansion of macrophages.

EXPERIMENTAL APPROACH

1. *In ovo* delivered LTA mediated antiviral responses against ILTV infection

Objective 1.1

To investigate whether *in ovo* LTA delivery can mediate antiviral responses against ILTV infection pre-hatch correlating with expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

Key steps involved:

- *In ovo* delivery of 50µg LTA in ED18 specific pathogen free (SPF) eggs with control group receiving PBS.
- Collection of lung tissues from a subset of LTA treated and control eggs at 24 hours post-treatment for quantification of relative mRNA expression of pro-inflammatory molecules using real-time PCR technique and staining of macrophages using immunohistochemistry technique.
- Infection of a subset of LTA treated and control eggs through same route at ED19 (24 hours post-treatment) with 1×10^5 PFU/egg of ILTV for the evaluation of the antiviral response.
- Collection of lung tissues 24 hours post-infection from embryos of LTA treated and control groups for DNA extraction and the ILTV genome load quantification.

Objective 1.2

To investigate whether *in ovo* LTA delivery can mediate antiviral responses against ILTV infection post-hatch correlating with higher expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

Key steps involved:

- *In ovo* delivery of 50µg LTA in ED18 SPF eggs with control group receiving PBS.
- Collection of lung tissues from a subset of LTA treated and control eggs at 12, 24 and 48 hours post-treatment for quantification of relative mRNA expression of pro-inflammatory molecules using real-time PCR technique.
- Collection of lung tissues from a subset of hatched LTA treated and control chickens on the day of hatch for staining of macrophages using flow cytometry technique in addition to histological evaluation.
- Infection of a subset of hatched LTA treated and control chickens on the day of hatch through intra-tracheal route with 5×10^4 PFU/bird of ILTV for the evaluation of the antiviral response.
- Collection of lung tissues at 1, 3 and 5dpi for quantification of ILTV genome load.
- Stimulation of macrophages *in vitro* using LTA or PBS to identify the source of pro-inflammatory mediators.

2. *In ovo* delivered CpG DNA mediated antiviral responses against ILTV infection

Objective 2.1

To investigate whether *in ovo* CpG DNA delivery can mediate antiviral responses against ILTV infection pre-hatch correlating with expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

Key steps involved:

- *In ovo* delivery of 50µg CpG DNA in ED18 SPF eggs with control groups receiving 50 µg non-CpG DNA or PBS.
- Collection of lung tissues from a subset of CpG DNA treated and control eggs at 24 hours post-treatment for quantification of relative mRNA expression of pro-inflammatory molecules using real-time PCR technique and staining of macrophages using immunohistochemistry technique.
- Infection of a subset of CpG DNA treated and control eggs through same route at ED19 (24 hours post-treatment) with 1×10^5 PFU/egg of ILTV for the evaluation of the antiviral response.
- Collection of lung tissues 24 hours post-infection from embryos of CpG DNA treated and control groups for quantification of relative expression of ILTV protein kinase (PK) gene mRNA and genome loads.

Objective 2.2

To investigate whether CpG DNA when delivered *in ovo* can induce antiviral responses and protection against morbidity and mortality caused by ILTV infection post-hatch and is associated with expansion of macrophage population

Key steps involved:

- *In ovo* delivery of 50µg CpG DNA in ED18 SPF eggs with control groups receiving 50 µg non-CpG DNA or PBS.
- Collection of lung tissues from a subset of hatched chickens at day 1, 4 and 7 for the quantification of macrophages using flow cytometry techniques in addition to histological evaluation.
- Infection of a subset of hatched CpG DNA treated and control chickens on the day of hatch through intra-tracheal route with 5×10^4 PFU/bird of ILTV for the evaluation of the antiviral response and protection from the morbidity and mortality.
- Collection of tracheal swabs at 4 and 7dpi for quantification of relative expression of ILTV PK gene mRNA and genome loads.
- Stimulation of macrophages *in vitro* using CpG DNA or non-CpG DNA to identify the source of pro-inflammatory mediators.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Eggs and animals

SPF eggs were purchased from Canadian Food Inspection Agency (CFIA), Ottawa, ON, Canada. The eggs lack maternal antibodies which can be transferred from laying hens to chicks via yolk sac [202] and thus their interference with establishment of ILTV infection is avoided. The eggs were placed in the egg incubators (RcomPro 50 or 20 digital incubator, AutoElex Co., Ltd., GyeongNam, South Korea) for 21 days at the Veterinary Science Research Station (VSRS) or Health Research Innovation Center (HRIC), University of Calgary with the following setup for temperature and humidity. During ED1 to 18, the temperature was maintained at 37.6°C and relative humidity was maintained at 60% with turning of eggs at 90° angle every hour. During ED19 to 21, the temperature was maintained at 37.2°C and relative humidity was maintained at 70% with no turning of eggs. The eggs were allowed to hatch for post-hatch experiments and chickens were maintained in a high containment poultry isolators at VSRS or the Prion/Virology Animal Facility at the University of Calgary with *ad libitum* availability of food and water. All procedures requiring the use of eggs, embryos and live chickens have been approved by the University of Calgary's Veterinary Sciences Animal Care Committee and Health Sciences Animal Care Committee.

2.2. Cell cultures

An avian macrophage cell line, Muquarrab Qureshi-North Carolina State University (MQ-NCSU) cells [203] used in this study was provided by Dr. Shayan Sharif (University of Guelph, Canada). MQ-NCSU cells were cultured in LM HAHN medium which consisted of Leibovitz L-15 medium

(39.5 %), McCoy's 5A medium (39.5%), chicken serum (10 %), L-glutamine (1%), sodium pyruvate (1%), 100 units of penicillin and 100µg of streptomycin per ml, fungizone (250µg/ml), 2-mercaptoethanol (1.0mM) (Invitrogen, Burlington, ON, Canada), tryptose phosphate broth (1%) (Sigma-Aldrich, St. Louis, MO, USA), and fetal bovine serum (FBS) (8%) (Cellgro, Manassas, VA, USA) [204]. The maintenance of cells was done at 40°C at 5% carbon dioxide (CO_2) in an incubator (Binder GmBH, Tuttlingen, Germany).

Chicken blood derived macrophages were isolated from blood as has been described previously [205]. Briefly, for the isolation of chicken peripheral blood monocyte derived macrophages jugular blood was collected in heparinized tubes and mixed with an equal volume of phosphate-buffered saline (PBS). The mixture was subjected to Ficoll gradient density centrifugation using Ficoll-Paque^{TM} PLUS (GE Healthcare Bio-Sciences, Mississauga, ON, Canada) at 400×g for 40 minutes at 20°C. The cells at the interface were collected, discarding the upper plasma layer in the tube. The cells were washed in PBS and pelleted by centrifuging at 400×g for 5 min at 4°C. This washing step was repeated three times. Finally, the cells were resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium with 5% chicken serum (Invitrogen, Burlington, ON, Canada), 2% Hepes (Gibco Life Technologies, Burlington, ON, Canada), 100 units of penicillin and 100µg of streptomycin per ml and 100µM of L-glutamine and plated in T175 (175cm^2) flasks and incubated at 40°C for 48 hours.

LMH cells were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). 0.1% gelatine (Sigma-Aldrich, Oakville, ON, Canada) was used to coat the cell culture flasks or plates so that LMH cells get attached to the surface of coated flasks. LMH cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, ON, Canada) augmented with heat inactivated 10% FBS (Invitrogen, Burlington, ON, Canada) and 100 units of

penicillin and 100µg of streptomycin per ml (Invitrogen, Burlington, ON, Canada). The cells were incubated at 37°C.

2.3. ILTV virus titration

The ILTV (strain N-71851) was purchased from the ATCC (Manassas, Virginia, USA). The virus was adapted to the LMH cell culture system and the cell-adapted ILTV virus was titrated in the LMH cells using plaque assay. Briefly, LMH cells were cultured in 6-well plates with 5×10^{6} cells/well. The virus stock was 10-fold serially diluted from 10^{-1} to 10^{-6} dilution and cells in each well was infected with each dilution of virus inoculum. The experiment was done in triplicate. After 5dpi, the plates were stained with 1% crystal violet (Sigma-Aldrich, Oakville, ON, Canada). The number of plaques was counted in 10^{-4} well and virus titre was calculated by averaging the titre from three plates and expressed as plaque forming unit (PFU)/ml.

2.4. LTA and CpG DNA

Purified LTA of *Staphylococus aureus* were purchased from InvivoGen (San Diego, CA, USA) and CpG DNA 2007 and non-CpG DNA 2007 from Cedarlane (Burlington, ON, Canada).

2.5. Experimental Design: *In ovo* delivered LTA mediated antiviral responses against ILTV infection

2.5.1. Evaluation of antiviral effect of in ovo delivered LTA against pre-hatch ILTV infection

In ovo LTA delivery at ED18 was carried out manually as has been described previously [201, 206, 207]. Briefly, an 18-gauge needle was used to puncture the shell at the broader end of the egg. The delivery was done using a 23-gauge, 2.5cm long needle inserted in its entire length through the hole targeting the amniotic cavity. The ED18 embryos were injected with 50µg LTA in 200µl

PBS per egg (n = 3-5) *in ovo* and control ED18 eggs received 200µl PBS alone (n = 4-5). 24 hours post-treatment the embryonated eggs were infected with 1×10^5 PFU of ILTV per egg *in ovo*. 24 hours post-infection, the embryos were euthanized to collect lungs for DNA extraction. The experiment was repeated three times and the data represent results of three independent experiments pooled (total of n = 13 in PBS group and n = 14 in LTA group).

2.5.2. Evaluation of host responses pre-hatch following in ovo delivery of LTA or PBS

For the characterization of mRNA expression of downstream molecules activated in lungs pre-hatch following *in ovo* LTA delivery, ED18 eggs were injected with 50µg LTA in 200µl (n = 5 per time point) or 200µl of PBS (n = 5 per time point) per egg. The treated and control eggs were incubated and lungs of embryos were sampled at 12, 24 and 48 hours post-treatment for the extraction of RNA. The experiment was repeated with *in ovo* delivery of LTA (n = 3-4 per time point) and PBS (n = 3 per time point) and sampling of lungs at 12 and 24 hours post treatment for the extraction of RNA. The data were pooled for the purpose of analysis; LTA (12 hours, n = 9; 24 hours, n = 8; 48 hours, n = 5) and PBS (12 hours, n = 8, 12 hours, n = 8; 48 hours, n = 5).

In addition, samples of lungs from both LTA treated and control groups after 24 hours post-treatment *in ovo* were embedded in the optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetek USA, Inc., Torrance, CA, USA) separately before being frozen in dry ice. The sections were cut and cryosections were then evaluated using immunohistochemistry technique (explained in section 2.10) for macrophage quantification.

2.5.3. Evaluation of lung cellular infiltration post-hatch following in ovo LTA delivery

To study the LTA induced immune cell infiltration in lungs post-hatch, the lungs from one day old chickens that received *in ovo* LTA (n = 4) or PBS (n = 4) at ED18 were fixed in 10% neutral buffered formalin (VWR International, West Chester, PA, USA) for histological evaluation. The samples were submitted to the Histology Section of the University of Calgary Faculty of Veterinary Medicine (UCVM). The histological sections that were stained with haematoxylin and eosin (H & E) were examined and scored on a scale of 0-3 as has been indicated in the Appendix 1. As such, scoring was based on the extent of cellular infiltration (no cellular infiltration = 0, <25% lungs with cellular infiltration in the area of lung = 1, 25-50% lungs with cellular infiltration = 2 and >50% lungs with cellular infiltration = 3) [124].

2.5.4. Evaluation of lung macrophages in lungs post-hatch following in ovo LTA delivery

For the characterization of lung mononuclear cell populations such as macrophages following *in ovo* LTA delivery, ED18 eggs were injected with 50µg LTA in 200µl (n = 3-6) or PBS 200µl (n = 4-6) per egg. The eggs from both groups were allowed to hatch and, on the day of hatch, the chickens were euthanized to collect lungs for flow cytometry quantification of macrophages. The experiment to characterize macrophages was done three times independently and the data were pooled for the purpose of analysis; LTA (n = 15) and PBS (n = 15).

2.5.5. Evaluation of efficacy of LTA treatment in ovo against post-hatch ILTV infection

For the evaluation of efficacy of LTA treatment *in ovo* against post-hatch ILTV infection, ED18 eggs were injected as previously described with 50µg LTA in 200µl PBS per egg and control ED18 eggs received 200µl PBS alone. The eggs from both groups were allowed to hatch. After hatch,

day old chicks were challenged with 5×10^4 PFU of ILTV per bird intra-tracheally as shown in Figure 2.1. The birds were euthanized after 1, 3 and 5dpi to collect lungs for extraction of DNA to quantify ILTV genome loads in lungs. The experiment was repeated three times with 2-5 chickens per group per time point and the data were pooled for the purpose of analysis; 1 (n = 11-12 per group), 3 (n = 7 per group) and 5 (n = 11-13 per group) dpi.

To investigate whether LTA provides protection to chickens against ILTV infection, the subset of chickens from *in ovo* delivered LTA (n = 12) and PBS (n = 8) groups were allowed to hatch and infected with 5×10^4 PFU of ILTV per bird intra-tracheally on the day of hatch. The birds were monitored until 8dpi for development of clinical signs and determination of endpoints. We considered that the chicken reached the end point when the bird was assigned a cumulative score of 5 (ruffled feathers and huddling together =1, droopy wings =1, depression or head lowered with no movement = 1, mild increase in the respiratory rate =1, increased respiratory rate with constant beak opening =2, very severe increased respiratory rate as marked by gasping =3 and body weight loss rather than gain =1). The scoring criteria are summarized in Appendix 2.

2.5.6. Evaluation of embryo viability and hatchability of eggs following in ovo delivery of LTA

ED18 eggs were injected with either 50µg of LTA per egg in a volume of 200µl (n = 63) or PBS (n = 68) *in ovo*. The eggs were incubated and allowed to hatch for the calculation of percentage of hatchability in each group.

Figure 2.1. Intra-tracheal ILTV infection technique in chicken. The tongue of the chicken was pulled out using a forceps after being anesthetized with isoflurane. Then, the virus inoculum containing ILTV was delivered using a pipette into the glottis which opens into the trachea through larynx. Adapted from Kameka A.M., 2014 [208].

2.5.7. Stimulation of peripheral blood monocyte derived macrophages with LTA

5×10^5 cells per well were cultured in six-well plates and incubated for 24 hours. Then, the growth medium was removed and cells were washed with 1× Hanks balanced salt solution (HBSS) (Invitrogen, Burlington, ON, Canada). The cells were then treated with LTA (10µg/ml) prepared in phenol red free RPMI 1640 containing 10% FBS, 2.0mM L-Glutamine, and no antibiotics and untreated wells were given media alone to keep as controls. After 12 hours post-treatment supernatants were collected for NO assay using Griess reagent system (Promega, Madison, WI, USA) and cells were collected from the wells using cell scrapper in Trizol reagent for RNA

extraction. The quantification of mRNA expression of iNOS and IL-1β was done by real-time PCR technique. The experiment was done in triplicate.

2.6. Experimental Design: *In ovo* delivered CpG DNA mediated antiviral responses against ILTV infection

2.6.1. Evaluation of antiviral effect of in ovo delivered CpG DNA against pre-hatch ILTV infection

To determine the antiviral effect of *in ovo* delivered CpG DNA, a group of ED18 eggs (n =4-5) were injected with 50µg CpG DNA in 200µl PBS per egg and each egg in control group (n =4) were injected with 50µg non-CPG DNA in 200µl PBS to investigate the efficacy of *in ovo* delivered CpG DNA against pre-hatch ILTV infection. After 24 hours of treatment, each egg was infected with 1×10^5 PFU of ILTV per egg *in ovo*. The embryos were euthanized to collect lungs for RNA extraction 24 hours post-infection to evaluate ILTV replication. The experiment was done in duplicate (total of n = 9 in CpG DNA group and n = 8 in non-CpG DNA group).

2.6.2. Evaluation of host responses pre-hatch following in ovo delivery of CpG DNA, non-CpG DNA or PBS

In order to determine the mRNA expression of innate mediators (IL-1β and iNOS) that will be activated following *in ovo* delivery of CpG DNA (50µg per egg), 24 hours following treatment, the lungs of embryos from both CpG DNA treated group (n = 5) and non-CpG DNA treated group (n = 4) were collected for RNA extraction. The experiment was repeated with CpG DNA treated group having a total of n = 8 and non-CpG DNA treated group of n = 7.

In order to determine the macrophage populations that will be recruited in lungs following *in ovo* delivery of CpG DNA (50µg per egg), the lungs of embryos from both CpG DNA treated group (n = 9), non-CpG DNA treated group (n = 4) and PBS treated group (n = 6) were collected in OCT compound before being frozen in dry ice 24 hours following *in ovo* delivery. Cryosections were evaluated for macrophages using immunohistochemistry technique.

2.6.3. Evaluation of lung cellular infiltration post-hatch following in ovo CpG DNA delivery

The lungs collected from chickens on the day of hatch, 4 and 7 that received *in ovo* CpG DNA (n = 4 per time-point) or PBS (n = 4 per time-point) treatments at ED18 were fixed in 10% neutral buffered formalin (VWR International, West Chester, PA, USA) for histological evaluation. The samples were H & E stained at the Histology Section of UCVM, examined and scored as has been indicated in the Appendix 1.

2.6.4. Evaluation of lung macrophages in lungs post-hatch following in ovo CpG DNA delivery

For the evaluation of expansion of lung macrophage populations following *in ovo* CpG DNA delivery, ED18 embryos were injected with 50µg CpG DNA in 200µl PBS (n = 5) or non-CpG DNA control in 200µl PBS (n = 4) per egg. The eggs from both groups were allowed to hatch. On the day of hatch, the chickens were euthanized to collect lungs for characterization of macrophages using flow cytometry technique.

2.6.5. Evaluation of in ovo delivery of CpG DNA mediated protection against ILTV caused morbidity and mortality in chickens

SPF eggs were injected with 200µl CpG DNA (50µg) or 200µl PBS *in ovo,* allowed to hatch and hatched chickens (CpG DNA, n = 9 and PBS, n = 8) were infected with 5×10^4 PFU of ILTV intra-tracheally. The infected chickens were observed daily for 12dpi for development of clinical signs and determination of the end points. We considered that the chicken was reached the end point when the bird was assigned a cumulative score of 5 (Appendix 2). The chickens were also weighed (4, 7 and 11dpi) and tracheal swabs were collected (4 and 7dpi) for quantification of ILTV PK gene mRNA expression and ILTV genome load using real-time RT-PCR and PCR techniques respectively. The experiment was repeated (total of n = 17 in CpG DNA and n = 15 in PBS treated groups).

2.6.6. Determination of safety of in ovo delivery of CpG DNA

ED18 eggs were treated *in ovo* with 50µg CpG DNA (n = 22) and allowed to hatch along with PBS (n = 68) treated eggs to evaluate the hatchability.

2.6.7. Stimulation of macrophages in vitro with CpG DNA to determine the expression of pro-inflammatory mediators

MQ-NCSU cells were propagated and cells were seeded in 6 well plates (2×10^6 cells/well). After culturing the cells overnight, growth medium was removed and washed with 1× HBSS (Invitrogen, Burlington, ON, Canada). CpG DNA stimulation was done at 10µM in phenol red free RPMI 1640 (Invitrogen, Burlington, ON, Canada) containing 10% FBS, 2.0mM L-Glutamine, and no antibiotics. The plates were incubated for 1, 3, 6 and 12 hours at 40°C under 5% CO_2 before

collection of culture supernatants for NO assay and cells for RNA extraction. Each treatment was done in triplicate in two separate experiments and the results were pooled before being analyzed. To confirm our major finding *in vitro*, we used chicken peripheral blood derived macrophages. After 24 hours of culture of chicken peripheral blood derived mononuclear cells, the media was changed and after 48 hours, the adhered cells were trypsinized using TrypLE^{TM} Express (Life Technologies, Burlington, ON, Canada) and counted using hemocytometer. 1.5×10^6 cells per well were cultured in 6-well plates and incubated for 24 hours. Then the cells were treated with CpG DNA (10µM) prepared in phenol red free RPMI 1640 containing 10% FBS, 2.0 mM L-Glutamine, and no antibiotics and untreated wells with media alone were kept as control. After 12 hours post-treatment, both supernatant and cells were collected from the wells using cell scrapper for RNA extraction. The experiment was done in triplicate.

2.7. DNA and RNA extraction

Total DNA and RNA were extracted from lungs using Trizol reagent as described by manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada) and as has been described previously for other tissues [209]. Briefly, homogenization of a small piece of lung was done in 1ml of Trizol using a Pro200 Power Homogenizer (Diamed, Mississauga, ON, Canada). After chloroform separation, RNA and DNA phases were collected separately. For RNA extraction, RNA was precipitated using isopropanol from the upper inorganic phase. Subsequent 75% ethanol wash of RNA pellet was performed. RNA pellet was finally resuspended in 20µl of RNAase-free water. For DNA extraction, bottom organic phase was precipitated in 100% ethanol. The pellet was washed with sodium citrate/ethanol solution and 75% ethanol before being resuspended in 400µl of ethylenediaminetetraacetic acid (EDTA). Finally the supernatant containing DNA was collected.

The concentration and quality of both RNA and DNA were measured using Nanodrop1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) with absorbance at 260/280nm wavelength.

For DNA and RNA extraction from tracheal swabs, the swabs were collected in 1ml of Trizol and then after brief mixing using a vortex the swab was discarded and nucleic acids were extracted as has been described for lung tissues. However, the final volume was adjusted as follows: 10µl for RNA and 100µl for DNA.

2.8. Reverse transcription

Complementary DNA (cDNA) was synthesized from extracted RNA (2000ng for lung RNA and 400ng for tracheal swabs) by reverse transcription using 10XRT random primers (High Capacity cDNA Reverse Transcription Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.9. Real-time PCR assay

The DNA and cDNA were used in real-time PCR technique to quantify ILTV genome load and the relative expression of genes respectively using Fast SYBR® Green Master Mix Top 10 kit (Invitrogen, Burlington, ON, Canada). The primers used are listed in Table 2.1. The housekeeping gene β-actin primers were used for later normalization of data. 96-well PCR plate (VWR, Edmonton, AB, Canada) was used to run all real-time PCR assays with a reaction volume of 20µl and a dilution series of the plasmids were used to generate a standard curve. The SYBR® Green dye acts as a DNA intercalating agent and was used to detect the amplification in a CFX96 Real-Time System C1000 Thermal Cycler (Bio-Rad Laboratories, Mississauga, ON, Canada). For 20ng

or 100ng of DNA or cDNA, 5µM of gene-specific primers were used per reaction. In a run, a positive control (plasmid) and negative control (RNAse-free water) were also incorporated. The thermal conditions used were denaturation at 95°C for 20 seconds, 40 cycles of amplification/extension at 95°C for 3 seconds, and 60°C for 30 seconds. In addition, melting curve analysis was performed at 95°C for 10 seconds, 65°C for 5 seconds and 9°C for 5 seconds.

Table 2.1. Primers used in real-time PCR and RT-PCR assays

Primer	Sequence (5'-3')	Fragment (bps)	Reference
ILTV PK	F – TACGATGAAGCGTTCGACTG R – AGGCGTGACAGTTCCAAAGT	189	[210]
β-actin	F-CAACACAGTGCTGTCTGGTGGTA R-ATCGTACTCCTGCTTGCTGATCC	205	[211]
IL-1β	F- GTGAGGCTCAACATTGCGCTGTA R- TGTCCAGGCGGTAGAAGATGAAG	214	[211]
iNOS	F- GGCAGCAGCGTCTCTATGACTTG R- GACTTTAGGCTGCCCAGGTTG	185	[212]
MyD88	F- AGCGTGGAGGAGGACTGCAAGAAG R- CCGATCAAACACACACAGCTTCAG	264	[211]

2.10. Immunohistochemistry technique

The immunohistochemistry technique was performed as has been described previously [117]. The lungs were collected 24 hours following *in ovo* delivery of LTA or CpG DNA or non-CpG DNA

or PBS in order to enumerate macrophages in lung sections and kept in the OCT compound. The 5µm sections were cut from the lung tissues using the Cryostat CM1850 (Leica Biosystems Nussloch GmbH, Heidelberg, Nussloch, Germany). The sections were transferred to the glass slide and were stored at -20°C for later use. After fixing the tissue using acetone (VWR International, West Chester, PA, USA), the quenching of endogenous peroxidase activity was done by adding 3% hydrogen peroxide (H_2O_2) (EMD Chemicals Inc., Gibbstown, NJ, USA) in 0.3% goat serum in the sections placed in the humidifying chamber. Blocking was done using 3% goat serum. Mouse anti-chicken KUL01 monoclonal antibody (Southern Biotech, Birmingham, AL, USA) at a 1:400 dilution in blocking solution was used as the primary antibody and incubated for 30 minutes. The biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody and samples were incubated in it for 30 minutes. The immunoperoxidase staining of tissue sections was done using Avidin–biotin–peroxidase complex (ABC) system (Vectastain® ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA) and primary antibody bound sites were located using 3,3-diaminobenzidine-H_2O_2 solution from DAB substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA). The counterstaining of slides was done using Gill II haematoxylin solution (Leica Biosystems Canada, Inc., Winnipeg, Manitoba, Canada) and mounted in VectaMount™ mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The expansion of macrophage numbers was assessed quantitatively in each section by counting KUL01 positive cells with clear outlines in five highly infiltrated fields at 40× magnification.

2.11. Flow cytometry technique

Standardized protocol for flow cytometry was adopted for the experiments in this study. Briefly, the collected lungs were chopped into small pieces and treated with 400U/mL collagenase type I solution (Sigma-Aldrich, Oakville, ON, Canada) for 30 minutes at 37º C, and neutralized by HBSS. Using the broad end of syringe the digested lung tissue was smashed, diluted with HBSS and filtered to get the single cell suspension. The cell suspension was subjected to centrifugation and pellet was resuspended in 4ml of RPMI 1640 with 10% FBS. Then the cells were layered onto equal volume of Ficoll-PaqueTM PLUS (GE Healthcare Bio-Sciences, Mississauga, ON, Canada) and centrifuged for 40 minutes at 400×g at 20º C. The cloudy inter phase layer consisting of mononuclear cells was collected, washed with HBSS, pelleted and resuspended in complete RPMI-1640 medium for counting. Then the cells (1×10^6) were rinsed with 1% bovine serum albumin (BSA) fraction V (w/v; OmniPur, EMD, Darmstadt, Germany). After centrifugation for 10 minutes at 1500×g at 4ºC, Fc receptor blocking was performed by resuspending the cells in 100µl of 1:100 chicken serum (Invitrogen, Burlington, ON, Canada) in 1% BSA. Then, the cells were incubated in the dark on ice for 30 minutes with a final concentration of 0.5µg/ml mouse anti-chicken macrophage-monocyte conjugated with phycoerythrin (PE) (SouthernBiotech, Birmingham, Alabama, USA) monoclonal antibody. The respective isotype control or 1% BSA (unstained controls) were also included. Then, 1% BSA was used to wash the cells two times. The samples stained such a way were analyzed using a BD LSR II (BD Biosciences, Mississauga, ON, Canada).

2.12. Griess assay

The Griess assay was performed using the Griess reagent system (Promega, Madison, WI, Canada) using the manufacturer's protocol. Briefly, the standard curve (as shown in Appendix 3) was generated using serial dilution of different concentrations of nitrite. 1% sulfanilamide and 0.1% N-1-naftyletylendiamin dihydrochloride were used to obtain the colorimetric product following the reaction with nitrite. The optical density (OD) of the product was measured at 548nm in a SPECTRAMAX M2 microplate reader (Molecular devices, Sunnyvale, CA, USA). The generated standard curve was used for the quantification of nitrite (μM) from the OD values recorded for each sample (Appendix 3).

2.13. Data analyses

The ILTV genome load and mRNA expression were quantified based on the standard curve generated by the serial dilutions of the plasmid containing the ILTV PK gene. The amplification plots, dissociation curves, and standard curves generated for quantification of target gene genome load or mRNA expression are shown in Appendix 4. For the quantification of mRNA expression of ILTV PK gene and host target genes, the calculated copy numbers were normalized with β-actin copy numbers of the same sample and expressed as initial copy per 1×10^6 host cells [213]. The events collected during sample running in flow cytometer were further analysed using FlowJo version 7.6.4 (Ashland, OR, USA). Macrophage population (PE–KUL01 positive) was gated using the iso type control data, verified the absence of this population in unstained controls and the batch function was used to apply the gate to rest of the samples. The percentage of macrophages are illustrated graphically and group differences were identified using statistical analyses.

For the statistical analyses of the continuous data such as, mRNA expression, genome load and macrophage counts, the data were first analysed for their distribution before being analysed further using parametric or non-parametric tests. Anderson-Darling (AD) test from MINITAB® release 14 (Minitab Inc. State College, Pennsylvania, USA) was used to test the normal distribution of the data. The data were considered not normally distributed when the $P < 0.05$ following AD test, then, we used Mann-Whitney *U* test in GraphPad Prism 4 (GraphPad Prism Software, La Jolla, CA, USA) in order to identify differences between two groups, elsewhere we used Student's t test. Before being tested, each set of data was analyzed using the Grubbs' test (GraphPad software Inc., CA 92037, USA) to identify outliers which is the most deviated value from the sample mean. Difference in survival between two groups was analyzed by log-rank test using GraphPad Prism 4 (GraphPad Prism Software, La Jolla, CA, USA). The outliers shown in the box-plot for body weight generated using Statistical Package for the Social Sciences (SPSS) version 20 (IBM Corp., Armonk, NY, USA) were removed before being analyzed. For the analysis of categorical data such as histological scoring, Mann-Whitney *U* test was used to compare the two groups. Comparisons of the groups were considered significant at $P \leq 0.05$.

CHAPTER THREE: RESULTS

3.1. *In ovo* delivered LTA mediated antiviral responses against ILTV infection

3.1.1. Objective 1.1: To investigate whether *in ovo* LTA delivery can mediate antiviral responses against ILTV infection pre-hatch correlating with expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

3.1.1.1. Titre of the ILTV stock

ILTV virus titration in the LMH cells resulted in the visible and clear plaques after 5dpi. The titre of the ILTV stock was determined to be 2.35×10^7 PFU/ml. This batch of the ILTV stock was used in all the infection studies in the thesis.

3.1.1.2. In ovo delivery of LTA inhibits ILTV replication in the respiratory system of chicken embryos pre-hatch and that correlates with mRNA expression of MyD88 and IL-1β in lungs and the expansion of macrophage population

As TLR-2 mediated antiviral responses has been reported against influenza virus induced pneumonia in mice [168] and parainfluenza virus infection in guinea pigs [169], we hypothesized that LTA may induce antiviral responses against ILTV infection in chickens *in vivo*. The delivery of 50µg of LTA per egg *in ovo* at ED18 and infection of the embryos with ILTV at 24 hours post-treatment through the same route resulted in a significant decrease in the ILTV genome load in lungs ($P = 0.0078$, Figure 3.1) when compared to the controls. The amplification plot, dissociation curve, and standard curve used for ILTV genome quantification using plasmids for ILTV PK gene (A-C) are shown in Appendix 4.

In order to investigate correlates of LTA mediated antiviral response against ILTV infection, we quantified the mRNA expression of two downstream molecules of TLR-2 signaling, namely MyD88 and IL-1β in lungs at ED19 after *in ovo* LTA delivery into eggs at ED18 since [214]. The amplification plots, dissociation curves, and standard curves generated for relative quantification of IL-1β and MyD88 are shown in Appendix 4 (D-I). We found that IL-1β and MyD88 mRNA expressions in lungs were significantly increased following LTA treatment when compared to the controls (P = 0.0188 and 0.0001 respectively, as shown in Figure 3.2).

The lung macrophages were also quantified at 24 hours following *in ovo* LTA delivery, the time coinciding with ILTV infection *in ovo*. We found that macrophage numbers were significantly increased in lungs due to LTA treatment when compared to that in the controls (P = 0.0008, Figure 3.3 A-B).

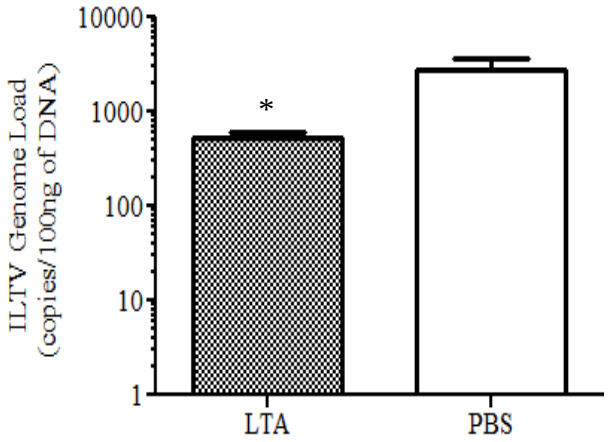


Figure 3.1. *In ovo* delivery of LTA decreases ILTV genome load in lungs pre-hatch. ED18 embryos were injected with 50µg LTA in 200µl of PBS (n = 3-5) *in ovo* and control ED18 eggs receiving 200µl PBS (n = 4-5) were kept as controls. 24 hours post-treatment the embryonated eggs were infected with 1×10^5 PFU of ILTV per egg *in ovo*. 24 hours post-infection, the embryos

were euthanized to collect lungs for DNA extraction. Real-time PCR assay was performed to assess ILTV genome load. The data represent results of three independent experiments pooled (n = 13 PBS group and n = 14 LTA group). Mann-Whitney *U* test was performed to elucidate the difference in ILTV genome load between the LTA treated and control groups. * indicates significance at $P \leq 0.05$.

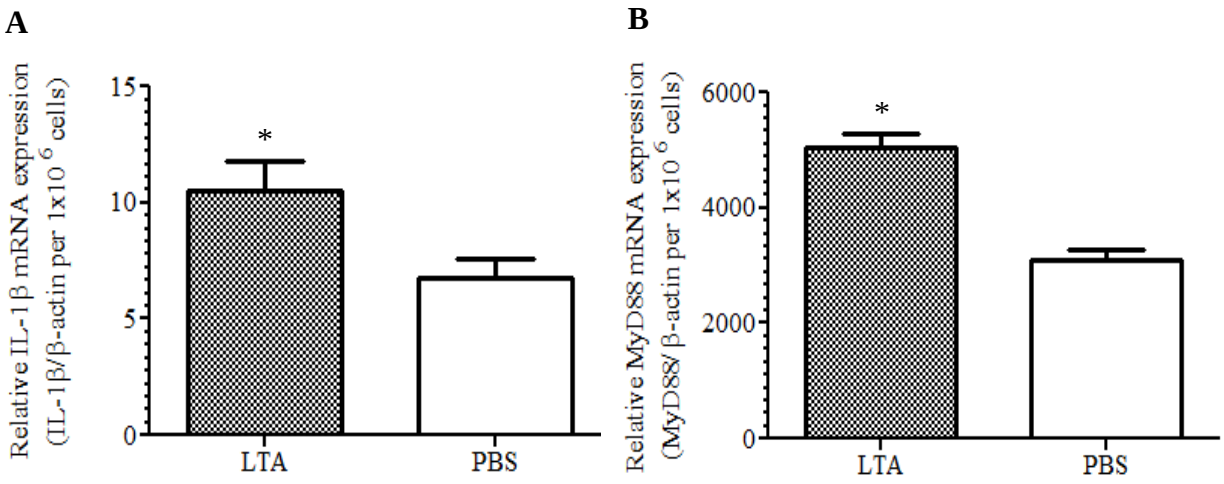


Figure 3.2. *In ovo* delivery of LTA upregulates mRNA expressions of IL-1β and MyD88 in lungs pre-hatch. ED18 embryos were treated with LTA along with a control group as has been described in the legend of Figure 3.1. LTA (n = 6) treated and PBS (n = 6) treated ED18 embryos were euthanized 24 hours post-treatment (ED19) to collect lungs for RNA extraction and real-time PCR assay. (A) and (B) represent mRNA expression of IL-1β and MyD88 respectively. Student's t-test was performed to elucidate the differences in mRNA expressions between the treatment and control groups. * indicates significance at $P \leq 0.05$ when LTA treated and PBS treated groups were compared.

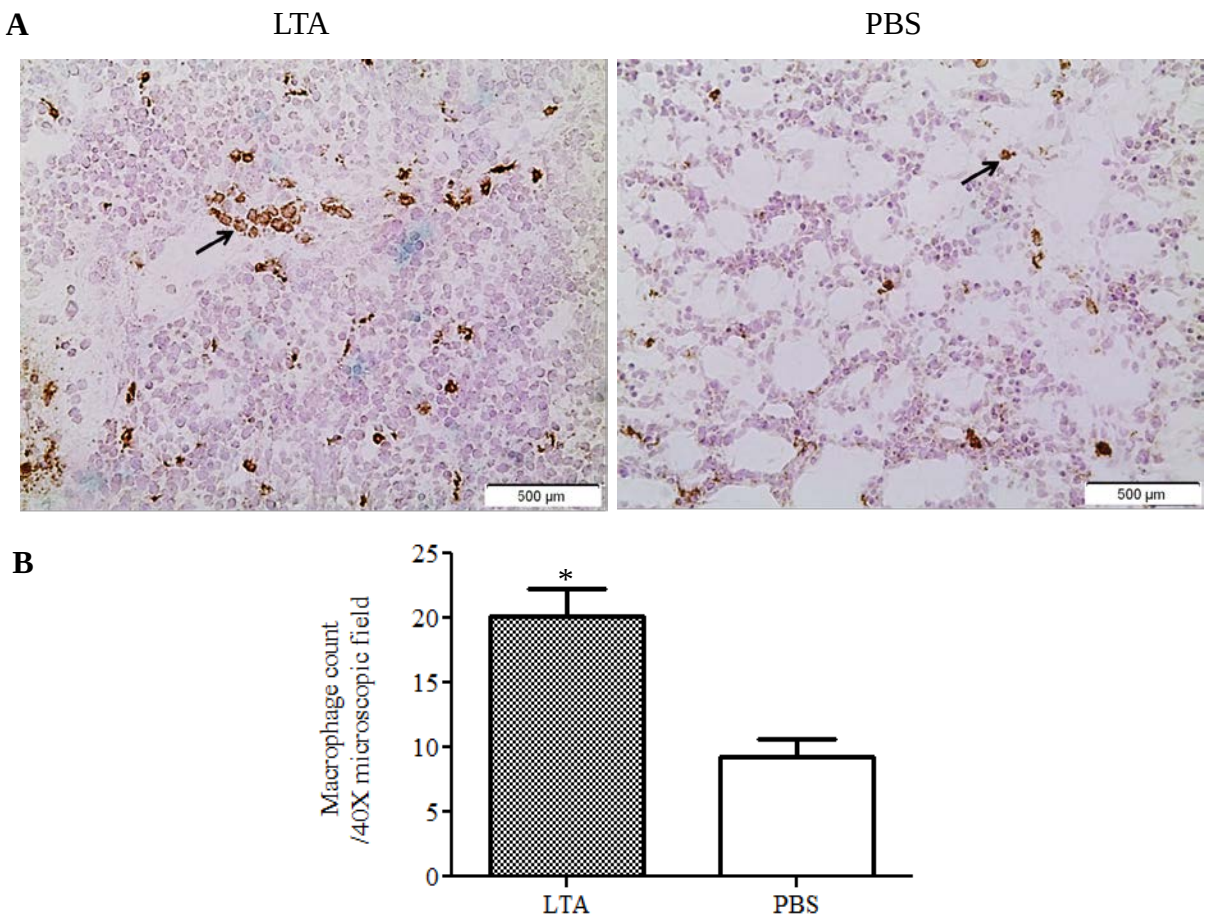


Figure 3.3. *In ovo* delivery of LTA expands macrophage numbers in lungs pre-hatch. ED18 embryos were treated with LTA along with a control group as has been described in the legend of Figure 3.2. LTA (n = 6) treated and PBS (n = 6) treated ED18 embryos were euthanized 24 hours post-treatment (ED19) to collect lungs for immunohistochemical staining of macrophages. The sections were stained for macrophages using mouse anti-chicken macrophage/monocyte KUL01 monoclonal antibody. Arrows show KUL01+ macrophage cells. (A) and (B) show representative sections of lungs originated from LTA and PBS treated embryos respectively. Student's t-test was performed to elucidate the difference between the treatment and control groups. * indicates the significance at $P \leq 0.05$ when LTA treated and PBS treated groups were compared. The scale bar of images is 500µm.

3.1.2. Objective 1.2: To investigate whether *in ovo* LTA delivery can mediate antiviral responses against ILTV infection post-hatch correlating with higher mRNA expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

3.1.2.1. In ovo delivery of LTA reduces ILTV infection encountered post-hatch in lungs of chicken

Since *in ovo* delivered LTA induced antiviral response in lungs pre-hatch, we examined the lungs at 1, 3 and 5dpi for ILTV genome load following *in ovo* delivery of LTA followed by infection on the day of hatch. We found a significant decrease in the ILTV genome load in the lung of LTA treated group at 1 (P = 0.0105) and 5 (P = 0.0264) dpi when compared to the PBS treated group as shown in Figure 3.4. At 3dpi, the ILTV genome load was not significantly different between two groups (P = 0.1914).

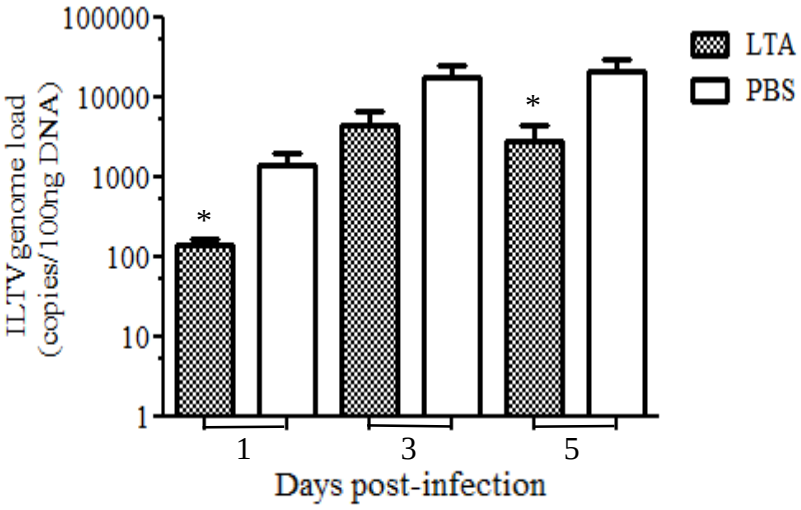
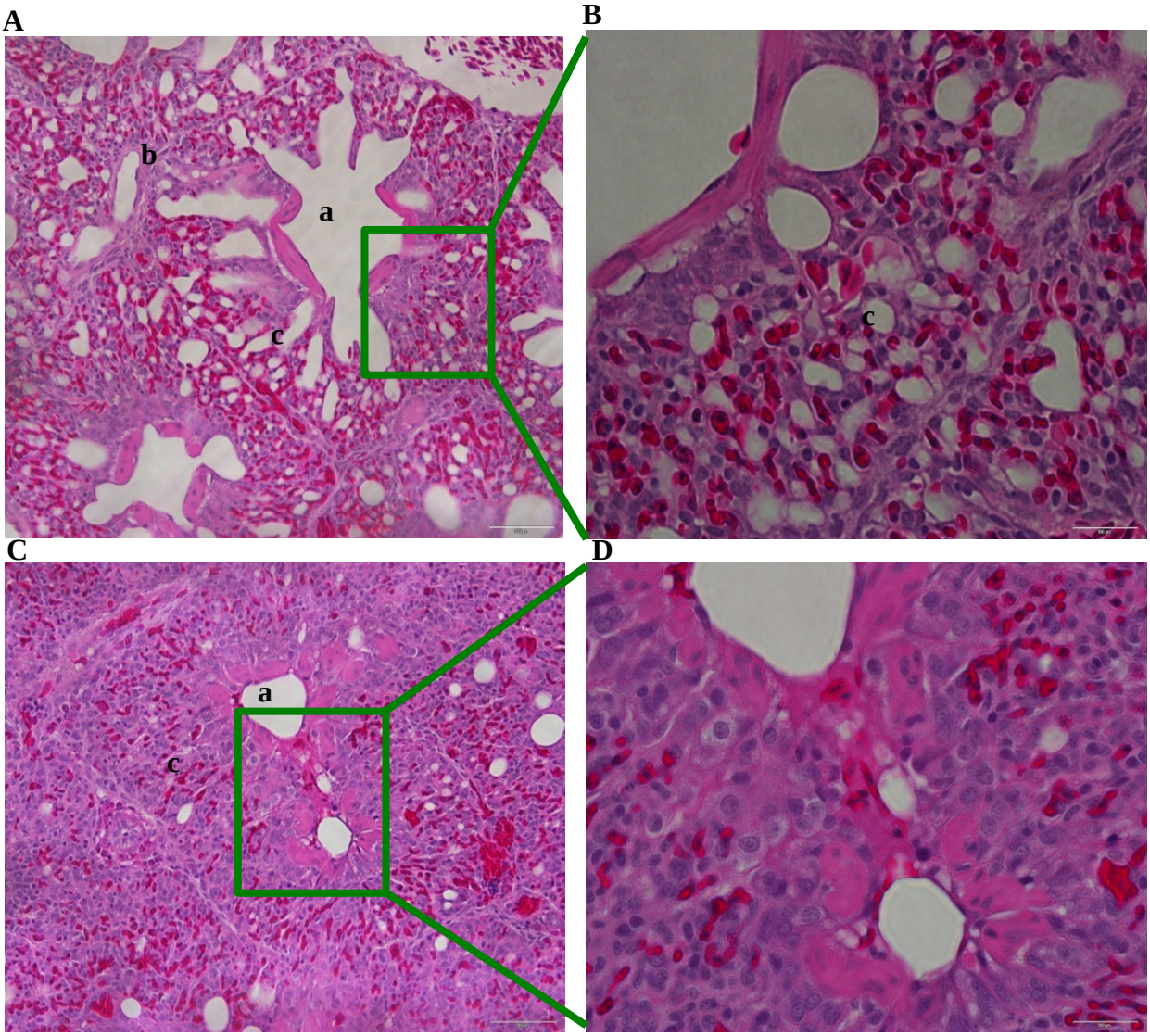


Figure 3.4. LTA delivered *in ovo* reduces ILTV infection encountered post-hatch in lungs of chicken. Eggs were injected with 50µg of LTA per egg (n = 12) in a volume of 200µl *in ovo* with control group receiving 200µl of PBS (n = 12). The eggs were allowed to hatch and chicks were infected with 5×10^4 PFU of ILTV per chicken intra-tracheally on the day of hatch. The chickens

were euthanized at 1, 3 and 5dpi respectively (n = 4 per group) from both LTA-treated and PBS-treated groups to collect lung for DNA extraction. Real-time PCR assay was done using DNA to investigate ILTV genome load in each group for each time point. The experiment was repeated three times with 2–5 chickens per group per time point and the data were pooled for the purpose of analysis; 1 (n = 11–12 per group), 3 (n = 7 per group) and 5 (n = 11–13 per group) dpi. Mann-Whitney U test was performed to analyze the differences between the groups at each time point post-infection. * represents significant differences in ILTV genome loads between treated group compared to control group at $P \leq 0.05$.

3.1.2.2. In ovo delivery of LTA recruits mononuclear cells into lungs with expansion of predominantly macrophage numbers post-hatch

In order to investigate correlates of antiviral response against ILTV infection encountered post-hatch, we examined the lung sections from *in ovo* LTA or PBS delivered day old chickens post-hatch using routine H & E staining. We observed that *in ovo* LTA delivery recruited significantly higher number of mononuclear cells into the lungs when compared to the PBS treated lungs (Figure 3.5 A-E, P = 0.0114). Moreover, in order to investigate whether macrophages are portion of recruited mononuclear cells into the lungs post-hatch following *in ovo* LTA delivery, we quantified the macrophages in lungs post-hatch using flow cytometry technique. We found that *in ovo* LTA treatment significantly increased the macrophage numbers (Figure 3.6 A-E, P = 0.0240) in lungs post-hatch when compared to that in PBS treated group.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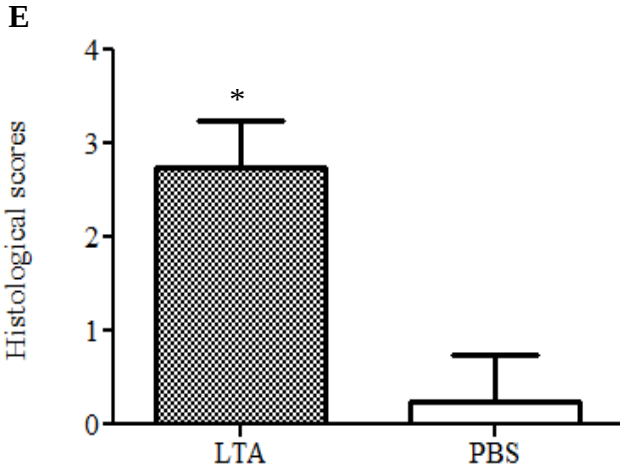

E
Histological scores
0
1
2
3
4
*
LTA
PBS

Figure 3.5. Histological sections showing recruitment of mononuclear cells in lungs post-hatch following *in ovo* LTA delivery. Eggs were treated with 50µg of LTA per egg (n = 4) in a volume of 200µl *in ovo.* The control group received 200µl of PBS (n = 4). The eggs were allowed to hatch and the lungs were collected from chickens on the day of hatch, sectioned and were subjected to H & E staining. (A) and (B) shows a representative parabronchus of a lung section at 40X and 100X magnifications respectively from PBS group with clear lumen (a), inter-parabronchial septum (b) and large number of air exchange areas (c) as compared to that of LTA treated lungs shown in (C) at 40X and (D) at 100X. (E) illustrates the histological scores for cell infiltration between the LTA and PBS groups. Mann-Whitney U test was done to analyze difference between groups. * indicates the significant differences between groups at $P \leq 0.05$. The scale bar of the images is 500µm.

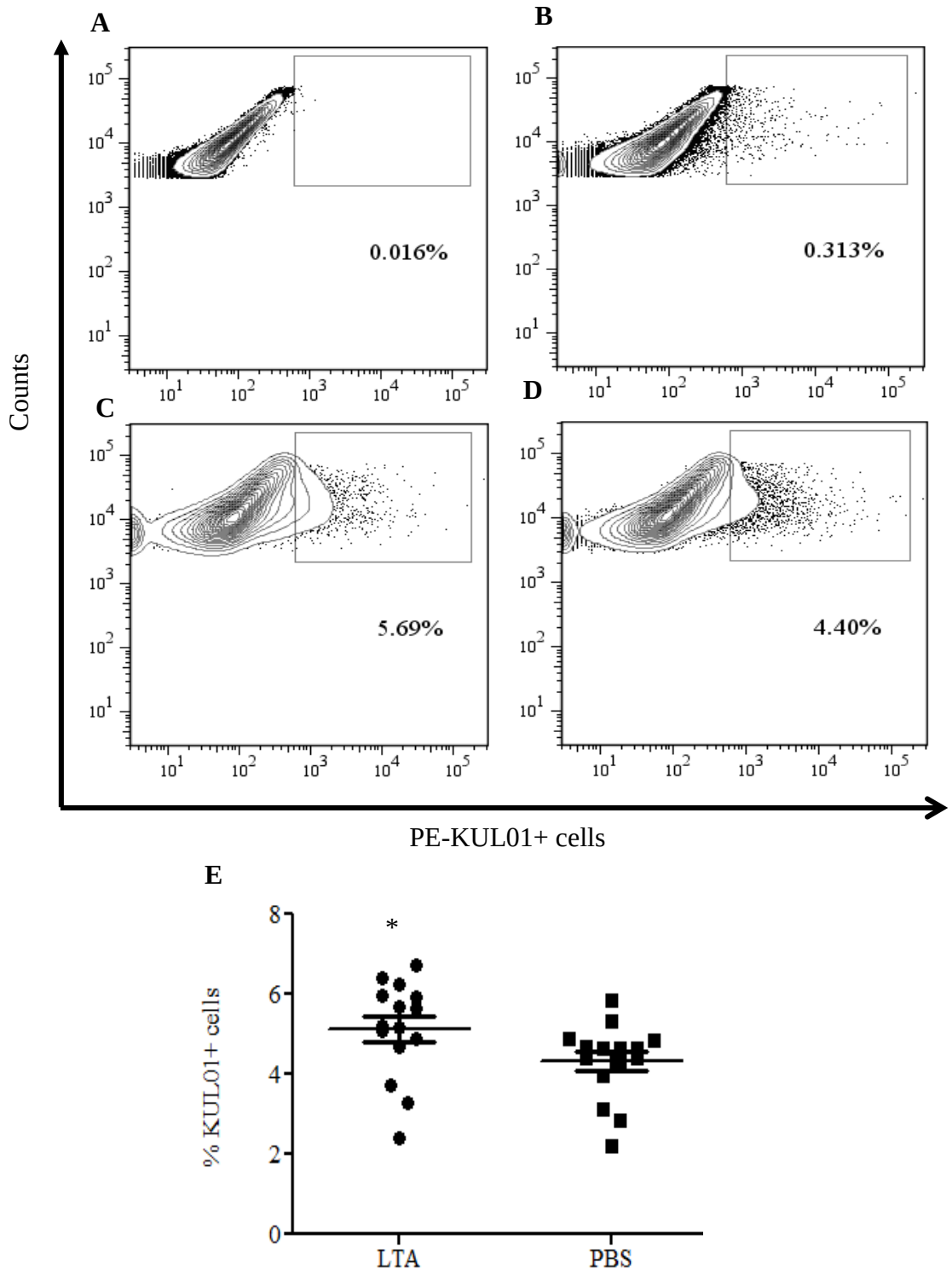


Figure 3.6. LTA expands macrophage numbers in lungs post-hatch. Eggs were injected with 50µg of LTA per egg in a volume of 200µl *in ovo* along with control ED18 eggs receiving 200µl

of PBS. On the day of hatch, the lungs were collected from each chicken in each group and mononuclear cells were isolated as described in the materials and methods section. The cells from each treatment and control groups (n = 6 per group) were then stained for KUL01 expression being marker for macrophage using PE labelled mouse anti-chicken macrophage-monocyte antibody and subjected to flow cytometry analysis. Student's t-test was done to analyze difference between groups. The experiment to characterize macrophages was done three times independently and the data were pooled for the purpose of analysis; LTA (n = 15) and PBS (n = 15). (A) and (B) indicates the unstained and PE-isotype control fluorescence-activated cell sorting (FACS) diagrams. (C) and (D) shows the representative FACS diagrams for percentage of KUL01+ macrophages in post-hatch lung originated from *in ovo* LTA or PBS delivered groups respectively. * indicates the significant difference between groups at $P \leq 0.05$.

3.1.2.3. In ovo delivery of LTA increases mRNA expression of pro-inflammatory mediators induced downstream of TLR-2 signalling pathway in lungs pre-hatch

We observed that *in ovo* delivery of LTA significantly increased the mRNA expression of IL-1β (Figure 3.7 A) at 12 hours (P = 0.0017) and 24 hours (P = 0.0415), MyD88 (Figure 3.7 B) at 12 hours (P = 0.0360) and iNOS (Figure 3.7 C) at 24 hours (P = 0.0070) post-treatment in lungs when compared to that in PBS treated lungs. At 48 hours, the mRNA expression of IL-1β (P = 0.3228) was not significantly different between the two groups. Similarly, differences were not seen for MyD88 at 24 hours (P = 0.4172) and 48 hours (P = 0.2394), and for iNOS at 12 hours (P = 0.3547) and 48 hours (P = 0.1964) post-treatment between LTA treated and control groups.

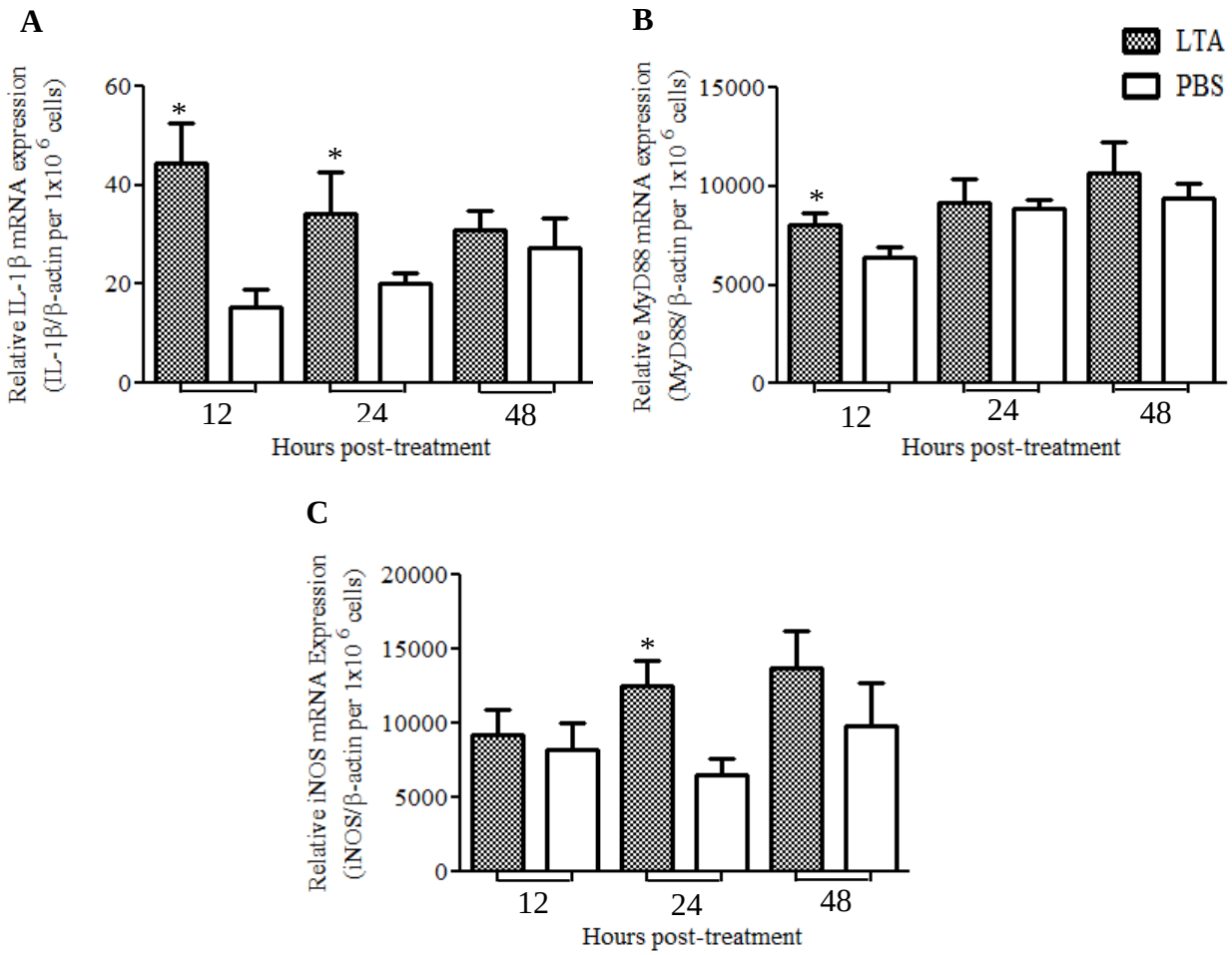


Figure 3.7. *In ovo* delivery of LTA increases mRNA expression of pro-inflammatory mediators induced down stream of TLR-2 signalling in lungs pre-hatch. Eggs were injected with 50µg of LTA per egg in 200µl (n = 5 per time point) along with control ED18 eggs receiving 200µl of PBS (n = 5 per time point). The lungs of embryos from the LTA treated and control eggs were sampled at 12, 24 and 48 hours post-treatment for the extraction of RNA and mRNA expressions of MyD88, iNOS and IL-1β were quantified by real-time PCR technique. The experiment was repeated with *in ovo* delivery of LTA (n = 3–4 per time point) and PBS (n =3 per time point) and sampling of lungs at 12 and 24 hours post treatment for the extraction of RNA. The data were pooled for the purpose of analysis; LTA (12 hours, n = 9; 24 hours, n = 8; 48

hours, n = 5) and PBS (12 hours, n = 8; 24 hours, n = 8; 48 hours, n = 5). Student's *t*-test was performed to analyze the difference between groups at each time point post-treatment. (A)–(C) represent mRNA expression of IL-1β, iNOS and MyD88 respectively. * illustrates the significant differences between LTA treated group and untreated controls at each time point post-treatment at $P \leq 0.05$.

3.1.2.4. In ovo delivery of LTA does not decrease embryo viability and hatchability of eggs

We found that LTA has no detrimental effect on the viability of embryos and the hatchability of eggs. The hatchability was 78% in LTA and 75% in PBS treated groups (Figure 3.8).

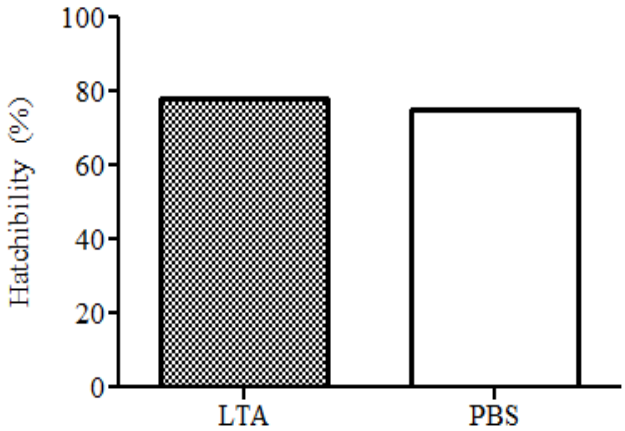


Figure 3.8. LTA has no adverse effect on the hatchability of the eggs. The ED18 eggs were injected with 50µg of LTA per egg in a volume of 200µl (n = 63) *in ovo* along with PBS treated control ED18 eggs (n = 68). At the day of hatch, the hatchability of eggs in each group was recorded. The data is presented as the percentage of hatchability in each group.

3.1.2.5. In ovo delivery of LTA does not provide protection against ILTV caused mortality in chickens

Since we observed that *in ovo* delivered LTA was capable of reducing ILTV genome load in the lungs post-hatch following intra-tracheal ILTV challenge at the day of hatch, we then hypothesised that *in ovo* LTA delivery will lead to protective response against ILTV infection encountered post-hatch. We delivered either LTA (n = 12) or PBS (n = 8) *in ovo* and allowed the eggs to hatch. The chickens were infected on the day of hatch intra-tracheally with ILTV (5×10^4 PFU per chicken). We found that *in ovo* delivered LTA has no effect in decreasing mortality associated with post-hatch ILTV infection (P = 0.8962, Figure 3.9).

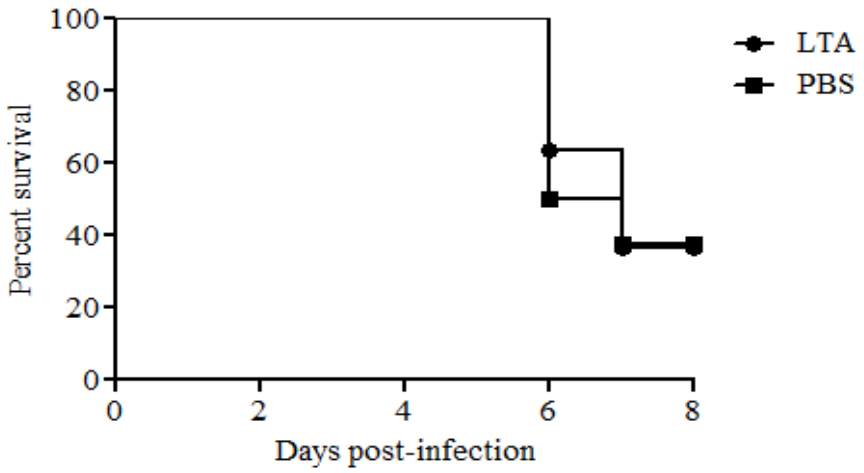


Figure 3.9. *In ovo* delivery of LTA provide no protection against ILTV caused mortality in chickens. SPF eggs were injected with LTA (n = 12) or PBS (n = 8) *in ovo* and chicks were allowed to hatch. On the day of hatch, the chickens were infected with 5×10^4 PFU of ILTV intra-tracheally. The infected chickens were observed for 8dpi for development of clinical signs and determination of the end points as has been indicated in the Appendix 2. The graph shows the survival proportions of the LTA treated and PBS groups. Log-rank test was used to compare the survival proportions between the LTA treated and PBS groups.

3.1.2.6. Peripheral blood monocyte derived macrophages increase the expression of IL-1β and iNOS following LTA treatment

To confirm that the macrophages are the source of IL-1β in the lungs following the *in ovo* LTA delivery, we used the blood monocyte derived macrophages to study the expression of mRNA of IL-1β after stimulation with LTA *in vitro*. Along with IL-1β, mRNA expression of iNOS and NO production by macrophages following the LTA treatment was also observed. There was a significant increase in expression of IL-1β (P = 0.0014, Figure 3.10 A) and iNOS after 24 hours post-treatment with LTA (P = 0.0056, Figure 3.10 B). Similarly, we saw a significant increase in NO production at 12 hours post-treatment with LTA (P = 0.0021, Figure 3.10 C).

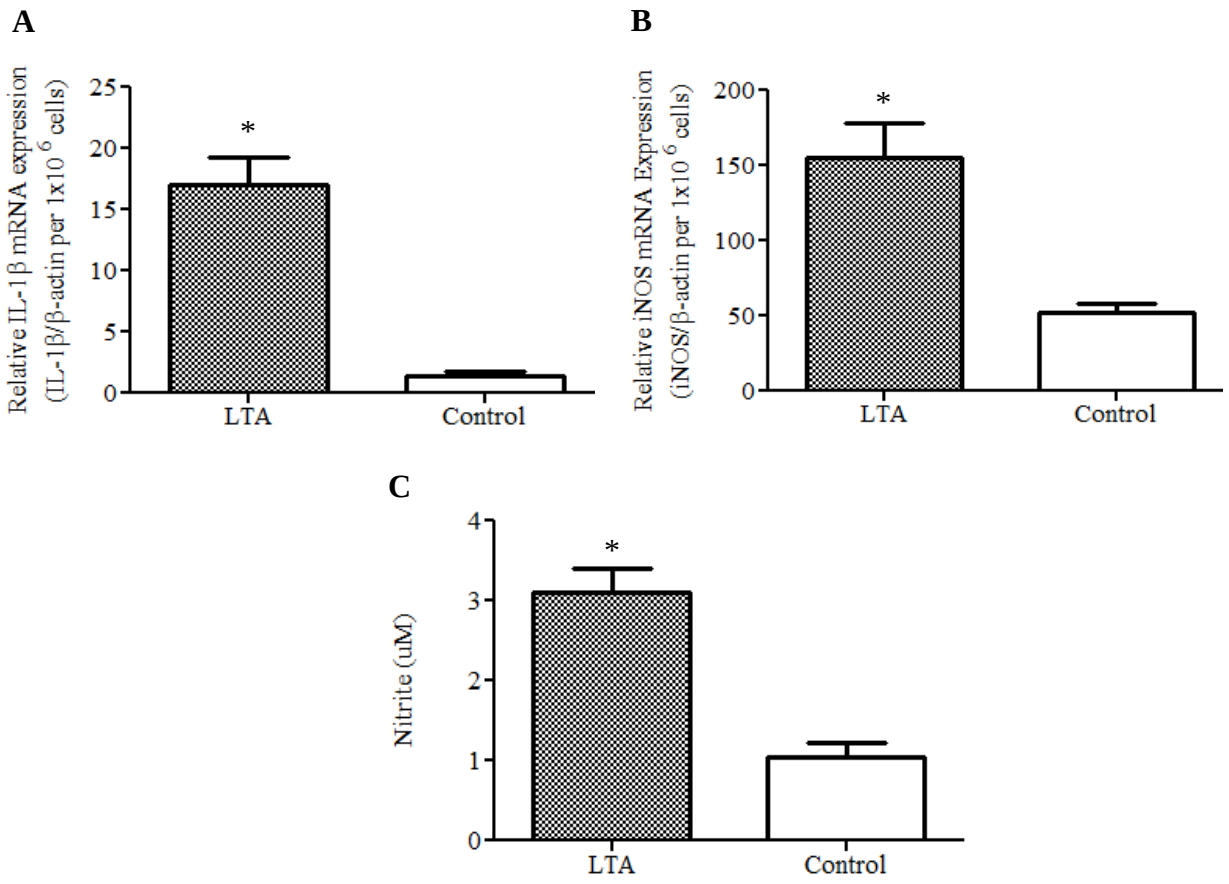


Figure 3.10. Stimulation of primary chicken macrophages *in vitro* with LTA upregulates the mRNA expression of pro-inflammatory mediators and increases NO production. Macrophages were cultured from chicken blood in a 6-well plates overnight with 5×10^5 cells per well. The cells were then treated with LTA (10µg/ml) and untreated cells were kept as control. After 12 hours post-treatment, supernatants were collected for NO assay and cells were collected from the wells using cell scrapper in Trizol reagent for RNA extraction. The experiment was done in triplicate. The quantification of mRNA expression of iNOS and IL-1β was done using real-time PCR technique and are presented as relative to β-actin mRNA expression. For NO production, Griess test was performed with supernatants collected at 12 and 24 hours post-treatment. Data are presented as mean values ± SEM. The NO production and mRNA expression differences between groups were analyzed using Student's t-test. (A) and (B) show the IL-1β and iNOS mRNA expressions respectively and (C) represents the NO production. * indicated significant differences between treatment groups at $P \leq 0.05$.

3.2. *In ovo* delivered CpG DNA mediated antiviral responses against ILTV

3.2.1. Objective 2.1: To investigate whether *in ovo* CpG DNA delivery can mediate antiviral responses against ILTV infection pre-hatch correlating with expression of mRNA of pro-inflammatory molecules and expansion of macrophage population

3.2.1.1. In ovo delivery of CpG DNA reduces pre-hatch ILTV replication in lung of embryos correlating with increased mRNA expression of IL-1β in lungs and expansion of macrophage populations

As CpG DNA was reported to be able to induce antiviral responses against AIV [125, 190] and IBV [196, 197], we hypothesised that CpG DNA may be able to elicit antiviral responses against ILTV *in vivo*. For this experiment, we delivered 50µg of CpG DNA per egg *in ovo* at ED18 with controls receiving non-CpG DNA and infected the embryos with ILTV after 24 hours through the same route. We found a significant decrease in ILTV mRNA expression in the lung of CpG DNA-treated embryos when compared to the controls (P= 0.0190, Figure 3.11).

In order to study the correlates of *in ovo* CpG DNA mediated antiviral response against ILTV infection, we quantified the mRNA expression of the downstream molecules in the lung coinciding with the time of ILTV infection *in ovo*, i.e., 24 hours post CpG DNA treatment. We found that following CpG DNA treatment, IL-1β and MyD88 mRNA expressions in lungs were significantly increased when compared to non-CpG DNA controls (P = 0.0003 and P = 0.0306 respectively, Figure 3.12 A-B) and not the mRNA expression of iNOS (P = 0.3438, Figure 3.12 C).

The immunohistochemistry staining of the lung tissues sampled 24 hours following the *in ovo* delivery of CpG DNA, non-CpG DNA or PBS at ED18 embryos showed a significant increase in the macrophage numbers in CpG DNA treated group (Figure 3.13 A) when compared to the non-

CpG DNA treated group ($P = 0.0266$). There was no significant difference between non-CpG DNA treated and PBS treated groups in terms of macrophage numbers in lung tissues pre-hatch ($P = 0.0984$).

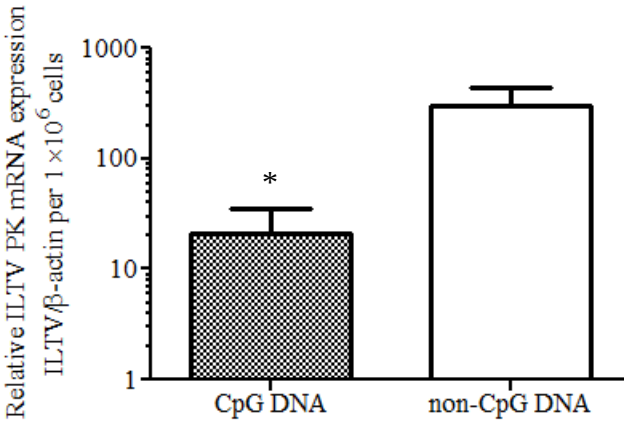


Figure 3.11. *In ovo* delivery of CpG DNA reduces ILTV replication in lungs pre-hatch. ED18 embryos were injected with 50µg CpG DNA in 200µl of PBS (n = 5) *in ovo* and control ED18 eggs received 50µg non-CpG DNA (n = 4). 24 hours post-treatment the embryonated eggs were infected with 1×10^5 PFU of ILTV per egg *in ovo*. 24 hours post-infection, the embryos were euthanized to collect lungs for RNA extraction. Real-time RT-PCR assay was performed to assess mRNA expression of ILTV PK gene. The pre-hatch mRNA of ILTV PK gene in lungs following *in ovo* CpG DNA or non-CpG DNA delivery represent results of two independent experiments pooled (n = 8 CpG DNA group and n = 8 non-CpG DNA group). Mann-Whitney U-test was performed to elucidate the difference between the treatment and control groups. * indicates significance at $P \leq 0.05$.

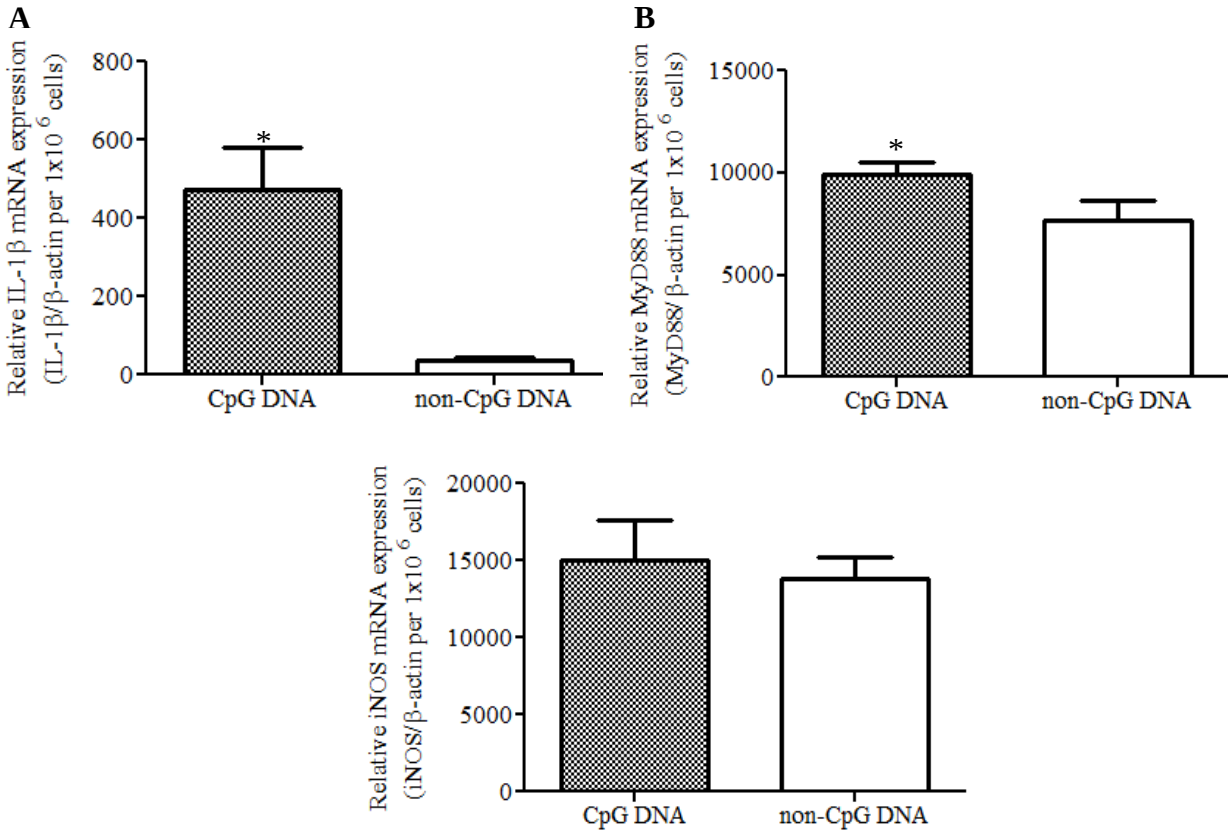


Figure 3.12. *In ovo* delivery of CpG DNA upregulates mRNA expression of IL-1β and MyD88 in lungs pre-hatch. ED18 embryos were injected with 50µg CpG DNA in 200µl of PBS (n = 5) *in ovo* and control ED18 eggs received 50µg non-CpG DNA (n = 4). 24 hours post-treatment, the embryos were euthanized to collect lungs for RNA extraction. Real-time RT-PCR assay was performed to assess mRNA expression of IL-1β (A), MyD88 (B) and iNOS (C) that will be activated downstream of CpG DNA- TLR-21 interaction following *in ovo* CpG DNA delivery. The data represent results of two independent experiments pooled (n = 8 CpG DNA group and n = 7 non-CpG DNA group). Mann-Whitney *U* test was performed to elucidate the differences between the CpG DNA treated and control groups for IL-1β and Student's t-test for both MyD88 and iNOS. * indicates significance at $P \leq 0.05$.

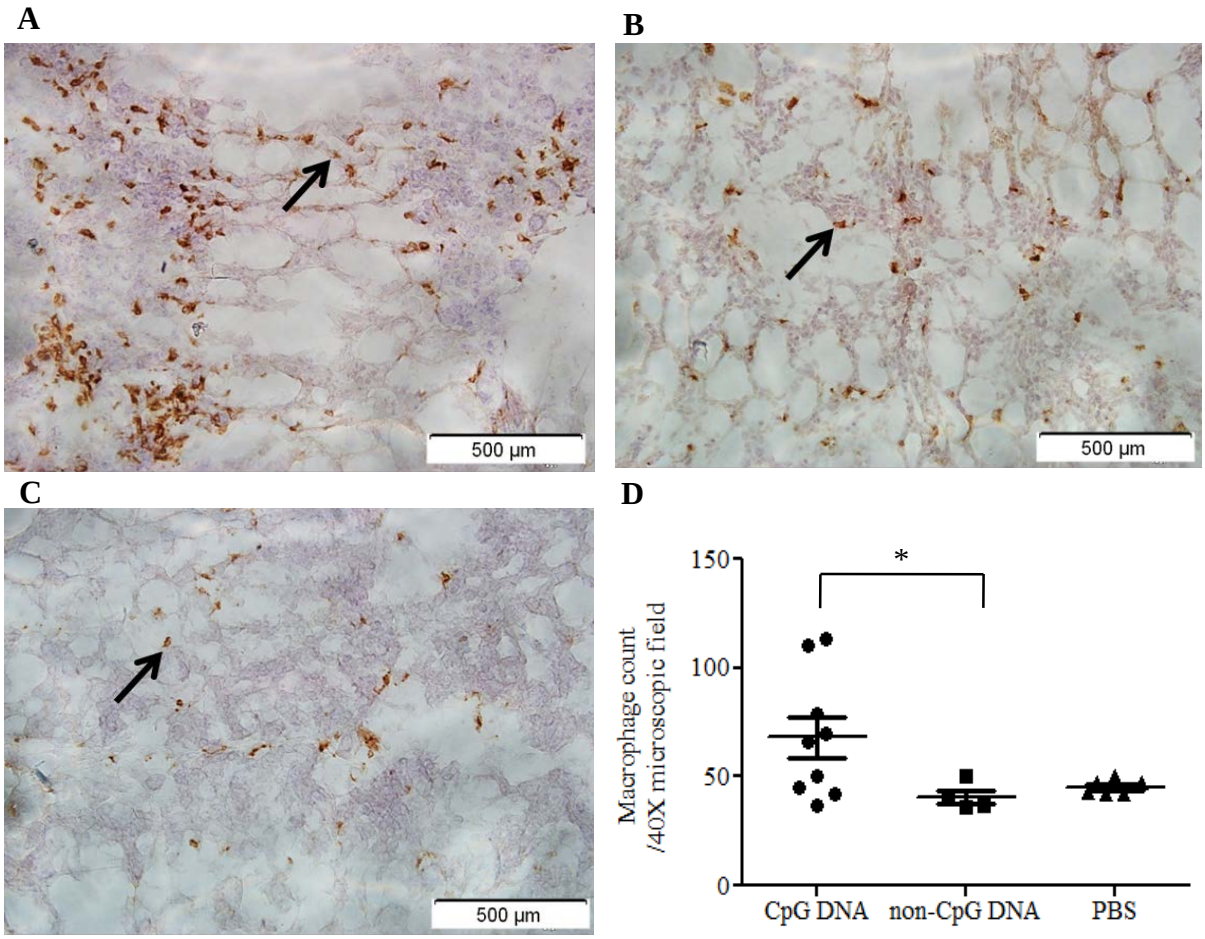


Figure 3.13. *In ovo* delivery of CpG DNA expands macrophage numbers in lungs pre-hatch. ED18 embryos were injected with 50µg CpG DNA in 200µl of PBS (n = 9) *in ovo* and control ED18 eggs received either 50µg non-CpG DNA (n = 4) or PBS (n = 6). 24 hours post-treatment, the embryos were euthanized to collect lungs for immunohistochemistry. The representative images of lung sections stained for macrophages in lungs pre-hatch following *in ovo* delivery of CpG DNA (A), non-CpG DNA (B) and PBS (C) 24 hours post-treatment (ED19) are shown. Arrows show KUL01+ macrophages. Mann-Whitney *U* test was performed. * indicates

significance at $P \leq 0.05$ when CpG DNA treated and non-CpG DNA treated groups were compared. The scale bar of the images is 500µm.

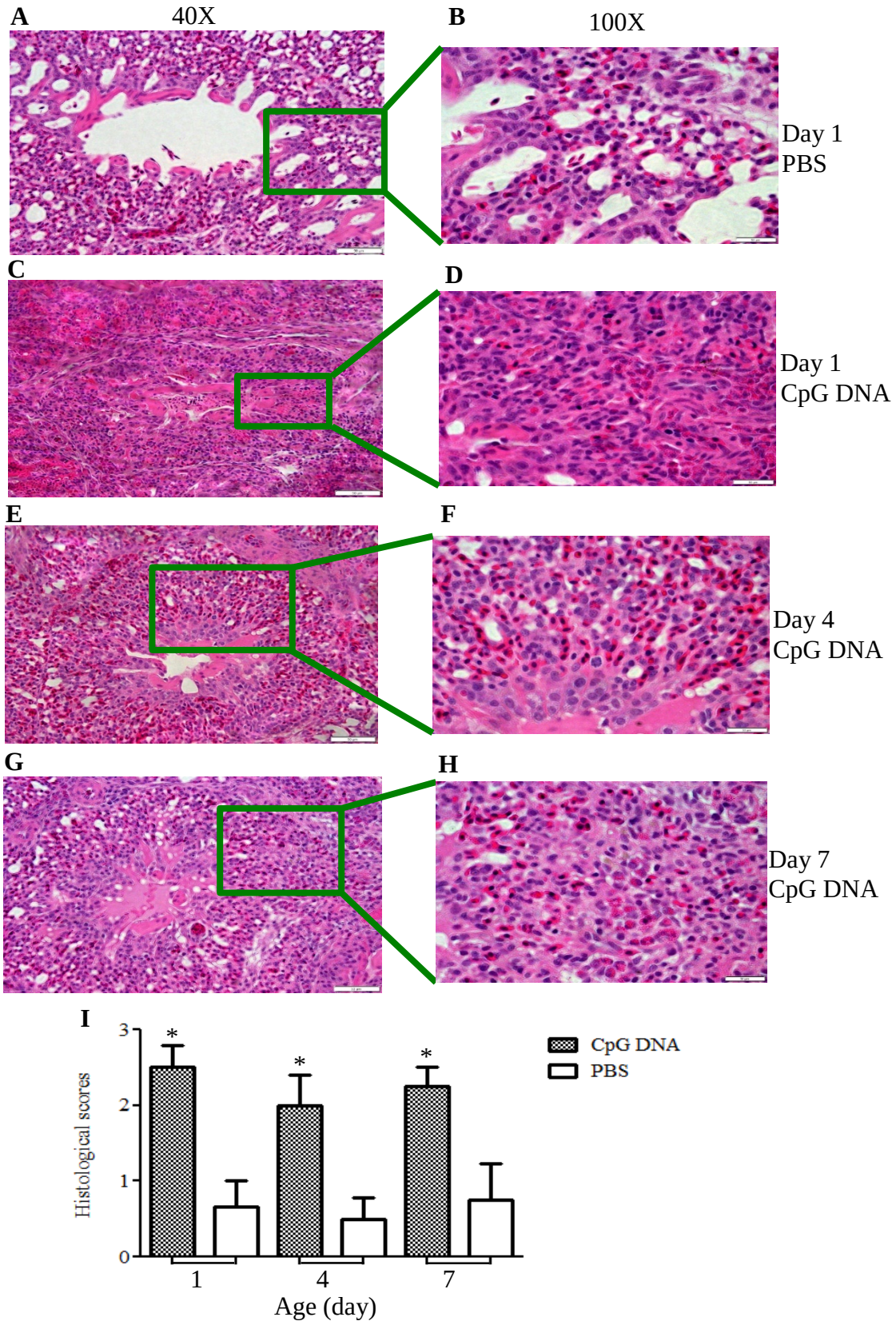
3.2.2. Objective 2.2: To investigate whether CpG DNA when delivered *in ovo* can induce antiviral responses and protection against morbidity and mortality caused by ILTV infection post-hatch and is associated with expansion of macrophage population

3.2.2.1. In ovo delivery of CpG DNA recruits mononuclear cells into lungs resulting in cell infiltration in lungs post-hatch

We examined the lung sections from *in ovo* CpG DNA or PBS delivered chickens at day 1, 4 and 7 post-hatch using routine H & E staining. We observed that *in ovo* CpG DNA delivery recruited significantly higher mononuclear cells into lungs when compared to the PBS treated lungs at day 1 (Figure 3.14 A-D, $P = 0.0228$), 4 (Figure 3.14 E-F, $P = 0.0258$) and 7 (Figure 3.14 G-H, $P = 0.0316$).

3.2.2.2. In ovo delivery of CpG DNA expands macrophage numbers in lungs post-hatch

Since *in ovo* delivered CpG DNA in ED18 eggs resulted in macrophage population expansion in embryonic lungs pre-hatch after 24 hours post-treatment, we hypothesized that the increased macrophage numbers following *in ovo* CpG DNA delivery may be maintained in lungs post-hatch. For the investigation of expansion of lung macrophage numbers post-hatch following *in ovo* CpG DNA delivery, we quantified the macrophages in lungs post-hatch using flow cytometry technique. We found that *in ovo* CpG DNA treatment significantly increased the macrophage numbers post-hatch when compared to that in non-CpG DNA treated control chickens (Figure 3.15, $P = 0.0041$).


A
40X
B
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Day 1
PBS
C
D
Day 1
CpG DNA
E
F
Day 4
CpG DNA
G
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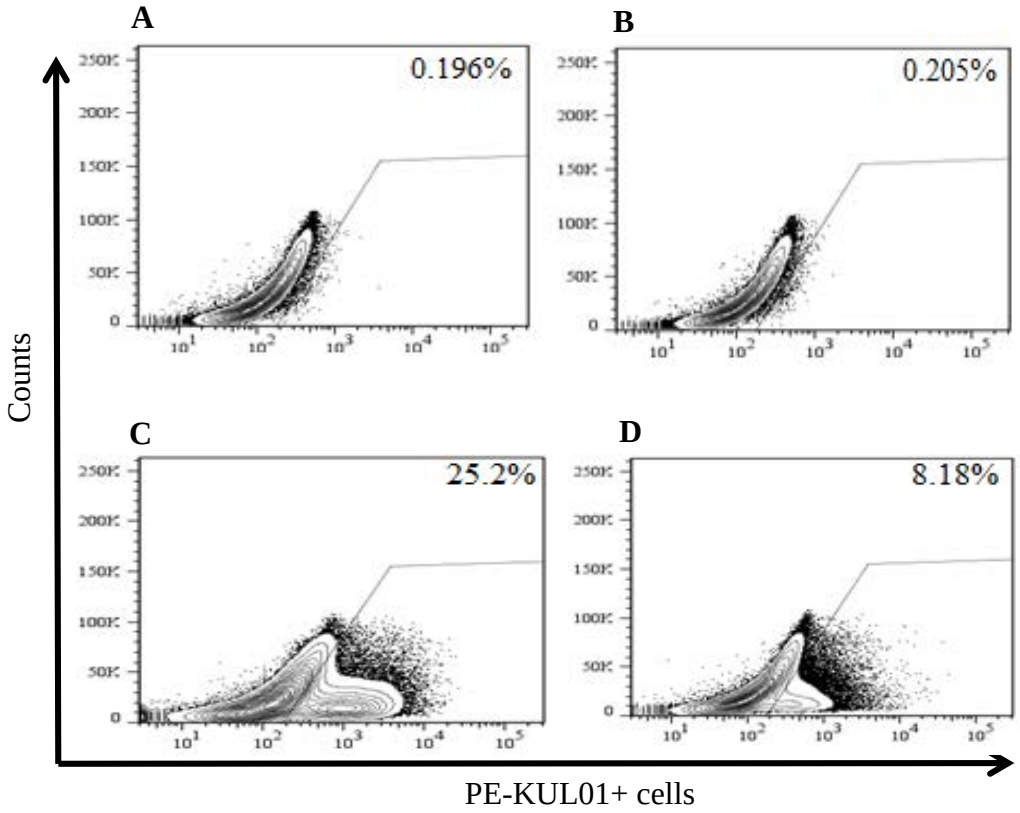
Day 7
CpG DNA
I
Histological scores
0
1
2
3
*
*
*
1
4
7
Age (day)
CpG DNA
PBS

Figure 3.14. *In ovo* CpG DNA delivery increases mononuclear cells in lungs post-hatch. Eggs were treated with 50µg of CpG DNA per egg (n = 12) in a volume of 200µl *in ovo.* The control group received 200µl of PBS (n = 12). The eggs were allowed to hatch and the lungs were collected from chickens on day 1, 4 and 7, sectioned and subjected to H & E staining. (A) and (B) show a representative parabronchus of a lung section at 40X and 100X magnifications from PBS group with clear lumen, inter-parabronchial septum and large number of air exchange areas at day 1 as compared to that of CpG DNA treated lungs shown in (C) and (D) at day 1, (E) and (F) at day 4 and (G) and (H) at day 7 at 40X and 100X respectively. (I) illustrates the histological scores for cell infiltration between the CpG DNA and PBS treated groups. Mann–Whitney U test was done to analyze difference between groups. * indicates the significant differences between groups at $P \leq 0.05$. The scale bar of the images is 50µm.



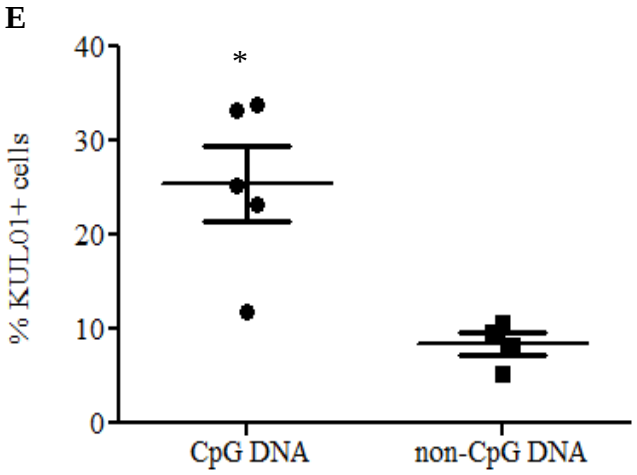


Figure 3.15. *In ovo* delivery of CpG DNA expands macrophage numbers in lungs post-hatch. Eggs were treated with 50µg of CpG DNA per egg ($n = 5$) in a volume of 200µl *in ovo.* The control group received 200µl of PBS ($n = 4$). The eggs were allowed to hatch and the lungs were collected from chickens on the day of hatch for flow cytometry. (A), (B), (C) and (D) indicate the representative FACS diagrams showing unstained control, iso type control, percentage of KUL01+ macrophages in lungs post-hatch originated from *in ovo* CpG DNA and non-CpG DNA delivered groups respectively. (E) illustrates the percentage of KUL01+ macrophages in lungs post-hatch originated from *in ovo* CpG DNA or non-CpG DNA delivered groups. Student's t-test was employed to compare two groups. * indicates the significant difference between CpG DNA treated and control group at $P \leq 0.05$.

3.2.2.3. In ovo delivery of CpG DNA induces protection against ILTV caused morbidity and mortality reducing viral replication in the respiratory tract of chickens

Since we observed that *in ovo* delivered CpG DNA was capable of causing cellular infiltration and increasing lung macrophages post-hatch, we then hypothesised that the chickens that received *in ovo* CpG DNA leading to increased macrophages post-hatch may be able to elicit protective response

against ILTV infection encountered post-hatch. For this part of the study, we *in ovo* delivered either CpG DNA or PBS, allowed the eggs to hatch and then infected the hatched chickens intra-tracheally with ILTV on the day of hatch. We found that *in ovo* delivered CpG DNA; 1. decreases mortality associated with post-hatch ILTV infection (P = 0.0003, Figure 3.16 A), 2. decreases clinical signs associated with post-hatch ILTV infection (Figure 3.16 B), 3. protects chickens from loss of body weights associated with post-hatch ILTV infection at 11dpi (P = 0.0316, Figure 3.16 C) and 4. decreases ILTV replication as assessed by mRNA expression of ILTV PK gene (P = 0.0342 at 7dpi, Figure 3.17 A) and absolute ILTV genome load in tracheal swabs at 7dpi (P = 0.0138 at 7dpi, Figure 3.17 B).

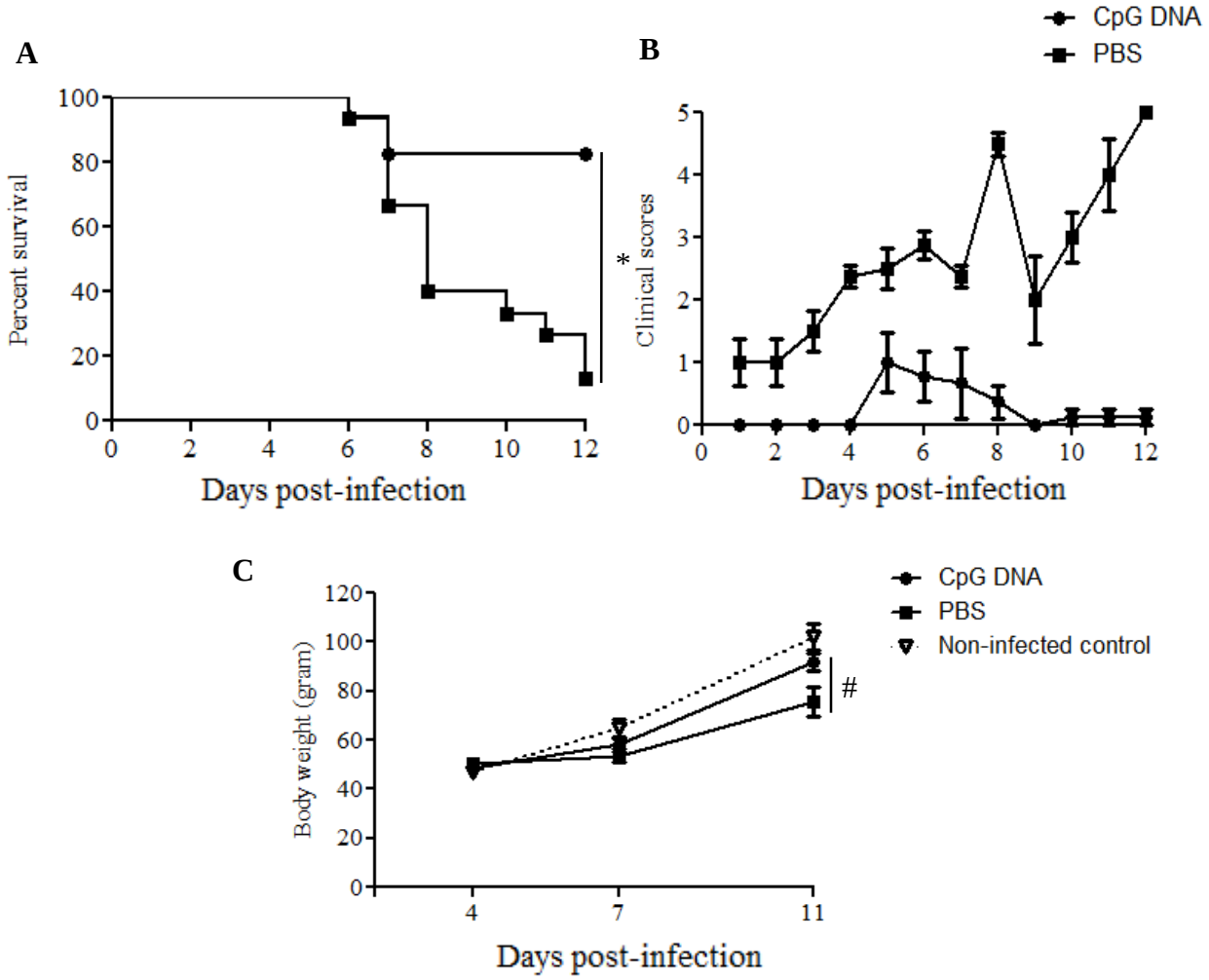


Figure 3.16. *In ovo* delivery of CpG DNA induces protection against ILTV caused morbidity and mortality in chickens. SPF eggs were injected with CpG DNA (n = 9) or PBS (n = 8) *in ovo,* allowed to hatch and hatched chickens were infected with 5×10^4 PFU of ILTV per bird intra-tracheally. The infected chickens were observed for 12dpi for development of clinical signs and determination of the end points as has been described in the Appendix 2. The data represent results of two independent experiments pooled (n = 17 CpG DNA group and n = 15 non-CpG DNA group). (A) survival proportions, (B) clinical scores and (C) body weights. Log-rank test was used to compare the survival proportions and Mann-Whitney *U*-test for body weight between the LTA treated and PBS groups.

* indicates the significant differences between CpG DNA and PBS groups at $P \leq 0.05$ and # indicates the difference between CpG DNA and PBS groups at 11dpi.

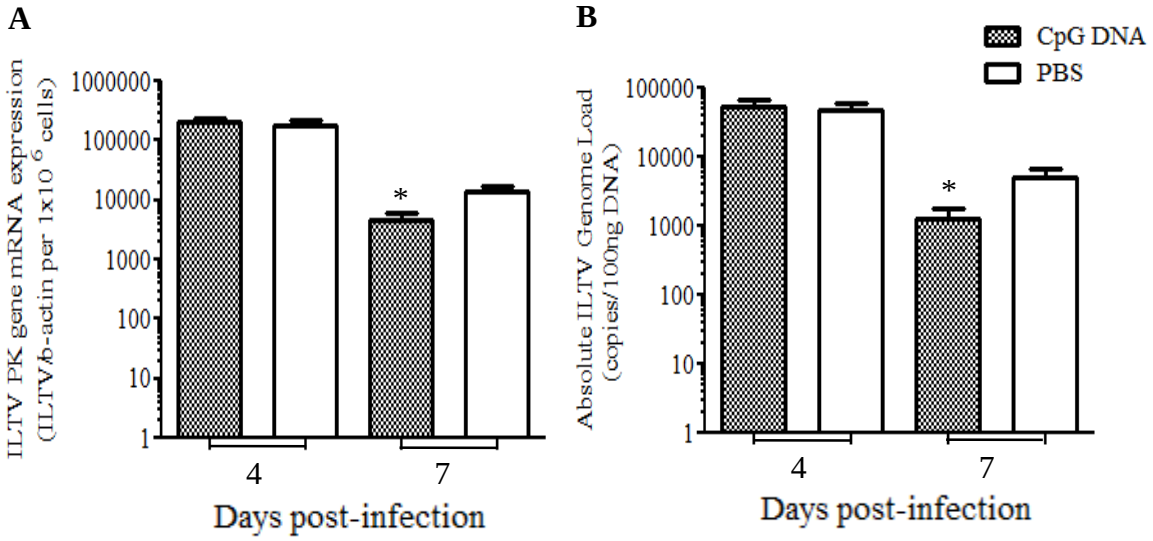


Figure 3.17. *In ovo* delivery of CpG DNA reduces viral replication in the respiratory tract of chickens. SPF eggs were injected with CpG DNA (n = 9) or PBS (n = 8) *in ovo,* allowed to hatch and hatched chickens were infected with 5×10^4 PFU of ILTV intra-tracheally. The infected

chickens were observed for 12dpi for development of clinical signs and determination of the end points as has been described in the Appendix 2. The tracheal swabs (4 and 7dpi) were collected for quantification of ILTV PK gene mRNA expression and genome load using real-time RT-PCR technique. The data represent results of two independent experiments pooled (n = 17 CpG DNA group and n = 15 PBS group) (A) ILTV PK gene mRNA expression as assessed in tracheal swabs using cDNA as template and (B) ILTV genome load in tracheal swabs using DNA as template. * indicates the significant differences between two groups at $P \leq 0.05$ when Mann-Whitney U test was used.

3.2.2.4. In ovo delivery of CpG DNA does not affect the hatchability of eggs

We recorded the hatchability rates of 86% and 75% following *in ovo* delivery of CpG DNA and PBS respectively (Figure 3.18).

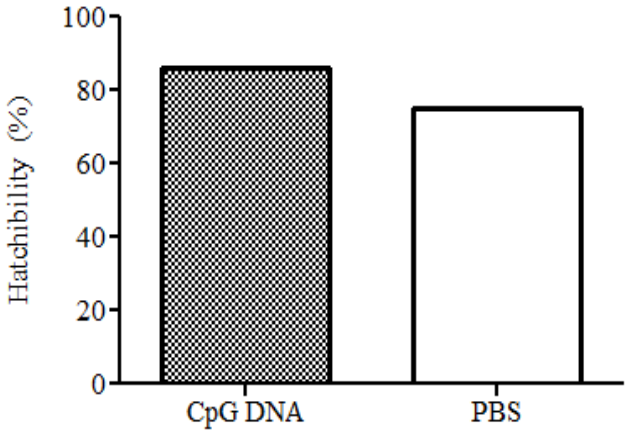


Figure 3.18. CpG DNA has no adverse effect on the hatchability of the eggs. The ED18 eggs were injected with 50µg of CpG DNA per egg in a volume of 200µl (n = 22) *in ovo* along with PBS treated control ED18 eggs (n = 68). At the day of hatch, the hatchability of eggs in each group was recorded. The data are presented as the percentage of hatchability in each group.

3.2.2.5. Stimulation of macrophages in vitro with CpG DNA up regulates mRNA expression of pro-inflammatory mediators and increases NO production

Since we observed *in ovo* delivery of CpG DNA increased macrophage numbers and IL-1β mRNA expression in lungs pre-hatch, in order to clarify a potential source of IL-1β mRNA, we conducted an *in vitro* experiment stimulating an avian macrophage cell line (MQ-NCSU) with CpG DNA and non-CpG DNA. Following the stimulation, we quantified the mRNA expression of iNOS which leads to NO production and MyD88 in addition to IL-1β. The NO production was found to be significantly high in MQ-NCSU cells following CpG DNA treatment when compared to that received non-CpG DNA at 3 (P = 0.05), 6 (P = 0.05) and 12 (P = 0.0195) hours post-treatment (Figure 3.19 A). There was a significant increase in the mRNA expression of MyD88 in cells treated with CpG DNA when compared to the controls at 3 hours post-treatment (Figure 3.19 D, P = 0.0095). The mRNA expressions of IL-1β at 3 (P = 0.05), 6 (P = 0.0286) and 12 (P = 0.0286) hours post-treatment were also significantly higher than that in controls (Figure 3.19 C).

Since we found the CpG DNA mediated increase in the expression of mRNA of IL-1β as well as NO production with no iNOS mRNA up regulation by the avian macrophage cell line, MQ-NCSU, we confirmed these findings with primary chicken blood monocyte derived macrophages. We hypothesized that blood monocyte derived macrophages increase in the expression of mRNA of IL-1β along with NO production following the CpG DNA treatment when compared to non-CpG DNA treatment. We observed a significant increase in iNOS mRNA expression at 12 hours post-treatment (Figure 3.20 B, P = 0.0286). Similarly, there was a significant increase in the expressions of IL-1β (Figure 3.20 C, P = 0.0003) and NO production (Figure 3.20 A, P = 0.0286) after 12 hours post-treatment.

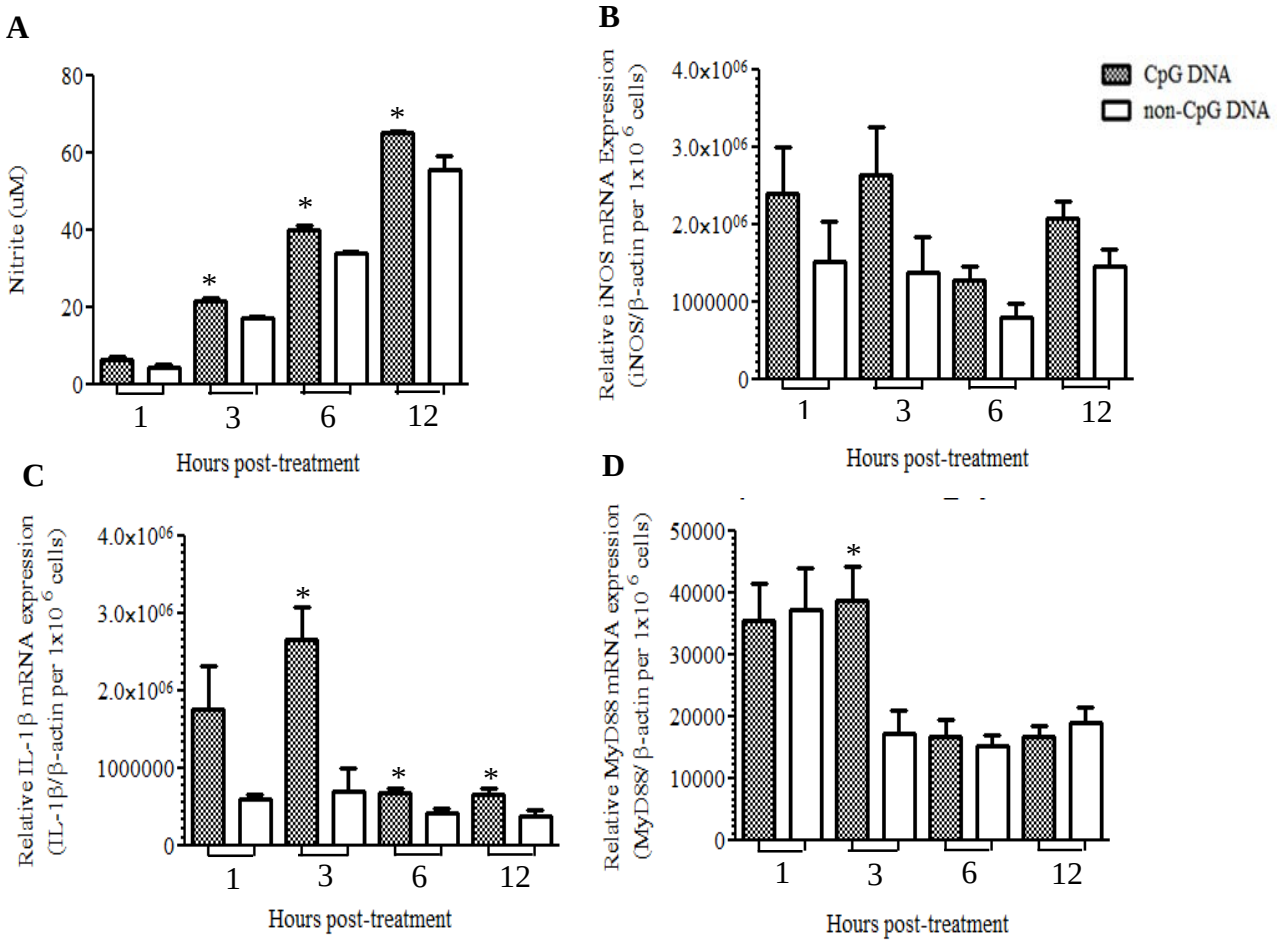


Figure 3.19. Stimulation of chicken macrophage cell line *in vitro* with CpG DNA up regulates mRNA expression of pro-inflammatory mediators and increases NO production. Avian macrophage cell line, MQ-NCSU were seeded in 6 well plates (2×10^6 cells/well), cultured overnight, growth medium was removed and washed with 1× HBSS. CpG DNA stimulation was done at 10µM in phenol red free complete RPMI 1640 without antibiotics. Control for the CpG DNA treatment was non-CpG DNA treatment done at the same concentration. The plates were incubated for 1, 3, 6 and 12 hours at 40°C under 5% CO_2 before collection of culture supernatants for NO (A) assay and cells in Trizol for RNA extraction and real-time RT-PCR for the quantification of iNOS (B), IL-1β (C) and MyD88 (D) mRNA. Each treatment was done in

triplicate and the experiment was done twice under same conditions. *= significant at $P \leq 0.05$ when Mann-Whitney *U* test was used.

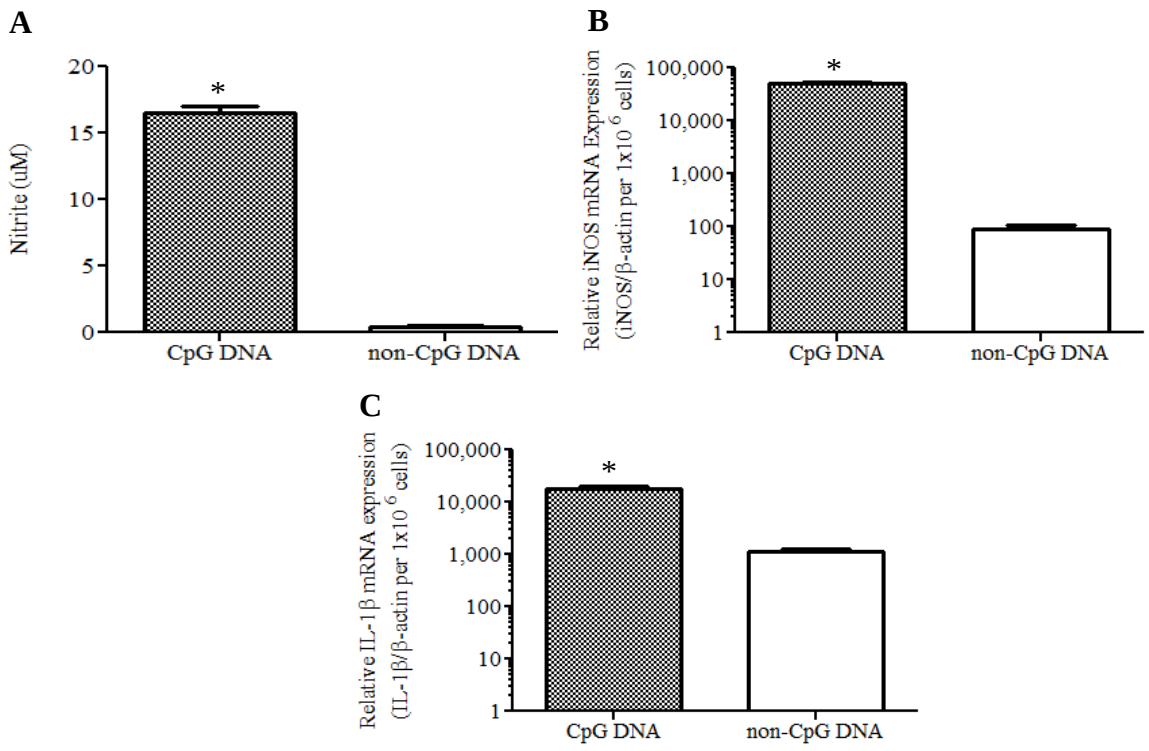


Figure 3.20. Stimulation of primary chicken macrophages *in vitro* with CpG DNA up regulates mRNA expression of pro-inflammatory mediators and increases NO production. Blood derived macrophages (1.5×10^6 cells per well) were stimulated with 10µM of CpG DNA in phenol red free complete RPMI 1640 without antibiotics and same concentration of non-CpG DNA treatment was used as control for the determination of NO (A) and the mRNA expression of iNOS (B) and IL-1β (C) at 12 hours post-stimulation. Differences between CpG DNA treated macrophage groups and non-CpG DNA treated groups were analysed using Mann-Whitney *U* test for NO and iNOS and Student's t-test for IL-1β. *= significant at $P \leq 0.05$.

CHAPTER FOUR: DISCUSSION

4.1. *In ovo* delivered LTA mediated antiviral responses against ILTV infection

For LTA, the significance of the findings of the current study is four fold. Firstly, we have shown that *in ovo* delivery of LTA induces antiviral response against ILTV pre-hatch and this antiviral response was associated with expansion of macrophage populations and higher expression of MyD88 and IL-1β mRNA in lungs. It is known that TLR-2 signaling leads to the production of pro-inflammatory cytokines through the activation of adaptor molecule, MyD88. The activated MyD88 mainly regulates NFκ-B dependent transcription of pro-inflammatory cytokines [215]. In agreement with these observations, in the current study, we established that *in ovo* LTA treatment up regulates mRNA of MyD88 and pro-inflammatory cytokine, IL-1β in ED19 chicken embryos. Some studies suggested that IL-1β can lead to direct or indirect antiviral response against viruses such as, West Nile virus [216] and human respiratory syncytial virus [217]. IL-1β signaling in the neurons complementing with type I IFN suppressed West Nile virus infection in an indirect manner [216]. Our observation of up regulation of IL-1β indicates that *in ovo* route can be used for the induction of antiviral response against ILTV. Interestingly, we observed significant decrease in ILTV genome load *in vivo* along with significant increase in the IL-1β mRNA expression in LTA treated lungs. However, further investigation is needed to confirm whether LTA mediated antiviral effect of ILTV is dependent on the expression of IL-1β. Secondly, *in ovo* delivery of TLR-2 ligand LTA decreases post-hatch ILTV genome load in lungs and that indicates that *in ovo* route can be used for the induction of protection against ILTV in post-hatch chickens. This is in agreement with observations recently made by other researchers [188, 218] who have demonstrated protective responses against bacterial infections in chickens when TLR-21 ligand, CpG was delivered *in ovo*.

Our findings on antiviral response of LTA against ILTV infection in chickens is also consistent with previous studies that showed evidence of antiviral effects of TLR-2 ligands (LTA and Pam-2-Cys) against another herpesvirus, CMV, and RNA viruses such as influenza virus and HBV infections [165, 167, 219, 220]. However, we recorded that *in ovo* delivered LTA does not result in a strong innate response leading to protection of birds from mortality associated with post-hatch ILTV infection. Thirdly, we observed that LTA-mediated reduction in ILTV infection in lungs was associated with infiltration of mononuclear cells, predominantly macrophages post-hatch. Our finding of increased recruitment of macrophages is in agreement with the observations made in mammals previously [221, 222]. Increased recruitment of macrophages in lungs in the current study indicates that *in ovo* LTA delivery could enhance innate immunity in the respiratory mucosa of chicken post-hatch leading to reduction in the ILTV viral load. Fourthly, induction of antiviral innate immunity post-hatch using *in ovo* delivered LTA was associated with enhanced expression of MyD88, IL-1β and iNOS genes pre-hatch. We observed that *in ovo* TLR-2 ligand delivery modulated the pro-inflammatory mediators pre-hatch eliciting cellular responses post-hatch. Finally, we found that *in ovo* delivery of LTA was safe and did not negatively influence the embryo viability and the hatchability of the incubated eggs.

4.2. *In ovo* delivered CpG DNA mediated antiviral responses against ILTV infection

Our investigations using CpG DNA led to three major findings. Firstly, CpG DNA decreases ILTV replication in lungs pre-hatch correlating with increase in mRNA expression of IL-1β in lungs and expansion of macrophage populations. Secondly, we found that avian macrophages could be stimulated with CpG DNA *in vitro* to produce NO and increase in mRNA expression of iNOS and IL-1β genes. Finally, CpG DNA elicits protective responses against mortality and morbidity

resulting from ILTV infection in chickens encountered post-hatch and this protection was associated with a reduction in ILTV replication in the lungs at a late stage of infection in the *in ovo* CpG DNA delivered chickens as well as a significant expansion of macrophage populations in lungs post-hatch.

In ovo route has been investigated for the delivery of CpG DNA against bacterial infections such as *E. coli* and *S. Typhimurium* encountered post-hatch [187, 188] and found to be protective against morbidity and mortality caused by these bacterial agents. It has also been shown that *in ovo* delivered CpG DNA is efficacious as an antiviral agent against pre-hatch IBV infection [196, 197]. The data we gathered in the current study show that *in ovo* delivery of CpG DNA could protect chickens from morbidity and mortality of ILTV infection encountered post-hatch implying the potential applicability of our findings for ILTV control.

It has been shown that host responses against ILTV infection may represent initial innate responses characterized by expression of pro-inflammatory cytokine and chemokine genes [223]. It has also been observed that protective host responses against ILTV infection is associated with cell-mediated rather than antibody-mediated immune responses [224, 225]. Infiltration of cells such as polymorphonuclear cells, macrophages and lymphocytes in trachea following ILTV infection has been recorded [226]. In fact, it is not clear what lung cellular components of the innate and adaptive arms are involved in the protection against ILTV infection in chickens. In the current study we observed that the *in ovo* delivery of CpG DNA related protection against morbidity and mortality induced by ILTV infection is associated with the expansion of macrophage populations in lungs pre- and post-hatch. It has been shown that CpG DNA act as a ligand for TLR-21 in chickens [135, 141]. The activation of TLR-21 leads to the production of pro-inflammatory mediators [130]. In agreement with this observation, in the current study, we observed that CpG DNA treatment *in*

vivo increases the mRNA expression of IL-1β in lungs pre-hatch. Il-1β is a known chemotactic factor for immune cells such as macrophages [126]. It is possible that the recruitment of macrophage populations in lungs pre- and post-hatch following *in ovo* CpG DNA delivery in our study could be due to the chemotactic function of IL-1β. In a different context, it has been recorded that CpG DNA treatment in mouse models increase recruitment of macrophages [227, 228] and these records confirm our finding of increased macrophage populations in lungs following *in ovo* CpG DNA delivery. Secondly, it is also possible that CpG DNA treatment increases survival of macrophages increasing the number of macrophages accumulate in a tissue over a period of time. Our view of increased survival of macrophages leading to increased number of macrophages overtime could be supported by the finding that CpG motifs present in *Leishmania donovani* is able to inhibit programmed cell death in macrophages [229].

There are three potential circumstances to clarify the observed difference in ILTV PK gene transcripts and genome load between CpG DNA treated and controls in the present study. Firstly, there would have been a possible phagocytic role of the recruited macrophage in clearing some of the ILTV infected cells since it has been shown previously that macrophages could involve in clearing influenza virus infected cells in mice through phagocytosis [230]. Secondly, it is possible that differential NO production in macrophages between CpG DNA treated and control groups contributed to the observed difference in viral replication. To support our second view, we conducted an *in vitro* experiment using a macrophage cell line and primary macrophages and found increased production of NO by macrophages following CpG DNA treatment when compared to controls. In agreement with this explanation it has been shown previously that NO is inhibitory against avian herpes viruses including ILTV [51, 118, 231]. Thirdly, it is possible that differential cytokine production in macrophages between CpG DNA treated and control groups contributed to

the observed difference in viral replication. It has been shown that macrophages in chickens are a known source of pro-inflammatory cytokines such as IL-1β [120] and IL-1β is known to possess direct and indirect antiviral effects that have been recorded in other host-virus models [126, 216]. We also found in our study that the mRNA of IL-1β gene was up-regulated in the lungs following *in ovo* delivery of CpG DNA. We recorded that macrophages could be a source of IL-1β mRNA following stimulation with CpG DNA. Altogether, macrophages appears to be involved in the reduction of pre- and post-hatch ILTV infection in the respiratory tract following *in ovo* CpG DNA delivery.

Although we observed a reduction in the ILTV replication in lungs at a late time point in chickens originated from CpG DNA treated embryos, at 4dpi we did not observe a difference in ILTV replication between CpG DNA treated and control chickens. Similarly, we did not see a difference in body weights between the CpG DNA treated and control chickens at 4dpi but a significant difference between these two groups were observed at late time point following ILTV infection. These observations suggest that protection mediated by *in ovo* delivery of CpG DNA is not entirely depend on early innate immune responses characterized by lung macrophage population expansion and increase of expression of pro-inflammatory mediators such as NO and IL-1β. One potential mechanism may be the rapid development of adaptive immune responses in chickens that received *in ovo* CpG DNA when compared to the chickens that received PBS *in ovo*. In agreement with this view, it has been shown that CpG DNA could protect neonatal piglets from *E.coli* infection induced by enterotoxigenic strains correlating with development of adaptive immune responses, particularly mucosal antibody responses [232]. Further, CpG DNA has been shown to stimulate B cells leading to antibody production in mammals [233]. Secondly, it is possible that ILTV may be using macrophages for viral replication at the same rate of clearing ILTV infected cells using

various mechanisms in the CpG DNA treated chickens at the early time points. In supporting our view, it has been shown that ILTV could utilize macrophages for its replication [39].

4.3. Implications of results, limitations and future directions

Implications: *In ovo* delivery of antigens is extensively investigated as poultry disease control method since it is vital, due to ubiquitous nature of pathogen distribution in poultry barn environments, to employ control methods pre-hatch. Induction of host responses pre-hatch empowers the birds' immune system at hatch and minimizes the window of susceptibility. In fact, non-pathogenic avian herpes viruses and attenuated virulent viruses have been routinely used as *in ovo* vaccines most notably for the control of MD in chickens [70]. These *in ovo*-administered MD vaccines have proven to be efficacious in preventing morbidity and mortality induced by the causative virus. In the present study we observed that *in ovo* delivery of LTA and CpG DNA could expand macrophage populations pre-hatch as well as post-hatch strengthening the innate arm of the immune system of the hatched chickens. We also showed that the CpG DNA induced host responses such a way that was capable of reducing mortality and morbidity resulted from ILTV infection encountered post-hatch. Although the innate responses induced by LTA was strong enough to reduce ILTV infection pre- and post-hatch, the induced response was not strong enough to reduce the mortality of ILTV infection encountered post-hatch. The anti-ILTV activity LTA and CpG DNA was also found to be associated with the up regulation of mRNA of IL-1β pre-hatch. Finally, we showed that *in ovo* delivery of LTA and CpG DNA found to be safe in terms of hatchability of the incubated eggs. It is imperative to investigate whether this pre-hatch stimulation of innate immune responses with CpG DNA also leads to the quick and solid antigen specific adaptive immune response against ILTV.

Limitations: To achieve our objectives to investigate the antiviral role of TLR-2 and -21 ligands via immunomodulation, we used several laboratory techniques. The major methods we used were *in ovo* delivery of TLR ligands into ED18 embryos, real-time PCR and RT-PCR assays for quantification of ILTV genome loads and relative quantification of mRNA expression of host immune genes, NO assay based on Griess reagent system, flow cytometry and immunohistochemistry techniques for staining and quantification of macrophages.

We used the *in ovo* delivery technique to distribute the TLR ligands into the respiratory mucosa of the developing embryos. *In ovo* delivery has been shown to deposit the injected vaccines into the amniotic compartment of the ED18 eggs, from where the injected vaccines is distributed to the respiratory mucosa in addition to gastrointestinal tract of developing embryo [201]. Although we made certain that the *in ovo* delivery is directed to the amniotic cavity by a preliminary dye injection into ED18 eggs, we did not investigate the distribution of LTA and CpG DNA into the respiratory mucosa following *in ovo* injections. As such it is not feasible us to say whether induction of innate immune response characterized by macrophage recruitment and increased pro-inflammatory mediators in our study is directly a result of distribution of LTA and CpG DNA on to the respiratory mucosa.

ILTV replication rates between *in ovo* TLR ligands delivered and controls is one of the parameters we observed in our experiments and we explored the potential techniques available for experiments before the experiments were conducted. Viral protein-based methods such as immunofluorescence assay and immunohistochemistry technique have been utilized for ILTV detection [234, 235], we opted out these assays in our investigations since these techniques are semi-quantitative at their best. Viral replication in tissues can be quantified using plaque and end-point dilution assays [236, 237]. While the plaque assay supports the detection of infective viral particles in the primary cell

cultures systems, the end-point dilution assay detects viral titre based on 50% of embryos mortality rates in SPF eggs (50% Embryos Infective Dose (EID50). Although, these two techniques are ideal in determining ILTV replication in lungs in our experiments, we chose real-time PCR and RT-PCR techniques for few reasons. Firstly, ILTV cannot form plaques in cell cultures unless it is adapted to cell culture system through multiple passages [48]. Secondly, embryo inoculation to quantify EID50 requires lot of animals and is laborious. Thirdly, both techniques are less sensitive when compared to currently available molecular assays. Considering all these limitations, we used real-time PCR technique to quantify ILTV genome loads in tissues and tracheal swabs in our investigations. The SYBR green based real-time PCR assay has been previously described for quantification of ILTV genome load [238]. It has also been reported that real-time PCR based virus quantification is more sensitive and correlates with active ILTV infection [239, 240]. One of the disadvantages of real-time PCR based ILTV genome quantification is that the assay is unable to discriminate replicating virus from non-replicating virus particles. To overcome this issue, we quantified mRNA of ILTV PK gene using real-time RT-PCR technique to target active replicating virus in addition to quantifying ILTV genome load using real-time PCR assay.

To determine the pro-inflammatory mediators activated down stream of TLR-2 and TLR-21 signaling pathways, we quantified the mRNA expression of MyD88, IL-1β and iNOS. Although mRNA is ultimately code for the protein synthesis correlating between the concentrations of mRNA expression and translated protein, measuring protein expression rather than mRNA expression would have provided more information in our experiments. Similar to mammals, IL-1β expression in chickens undergo post-translational modification [241] suggesting that correlation between IL-1β mRNA and protein expressions in chickens may be weak. The existence of post-translational modification mechanisms when turning mRNA of avian iNOS and MyD88

has not been defined. We resort to the quantification of mRNA expressions rather than protein expressions since reagents are not available for the quantification of these pro-inflammatory molecules in avian species.

Despite the novelty of the study investigating the protective role of *in ovo* delivered TLR ligand against ILTV infection presented in this thesis, there are indeed limitations in our experimental approaches that need to be addressed. Firstly, we demonstrated ILTV infection in lungs, although trachea is also a major site for establishment of ILTV infection [242, 243]. The main reason for not considering to include tracheal tissue in our investigations is the cartilaginous nature of trachea, unlike lung which we thought may contribute to majority of total RNA rather than the epithelial cells and other infiltrating immune cells. Previously our group has documented that the RNA deriving from cartilage component of trachea may mask the gene expression derive from the tracheal mucosa leading to higher variation [210]. Secondly, it is possible that the difference in distribution of immune cells such as macrophages between trachea and lung (<1% in trachea vs >8% in lung in healthy chickens of one week age) [124] may reflect better group differences in tested parameters with lungs rather than trachea.

Secondly, ILTV infection was performed to investigate the efficacy of *in ovo* delivered TLR ligands pre-hatch and on the day of hatch. In the field situations, the distribution of respiratory viruses are ubiquitous, can cause infections during first few days of placement of day old chickens in the barn and our investigations were not extended to see the efficacy of TLR ligands against ILTV infection encountered later than day 1 post-hatch. In a different context, it has been shown that maximum protection is conferred by TLR ligands, particularly CpG DNA in chickens if bacterial challenge is done within 3 days of the ligand administration, after which decreased protection pattern has been observed [186]. To address the issue, our group has investigated

duration of persistence of macrophages post-hatch following the *in ovo* delivery of CpG DNA and found that higher number of macrophages persist for up to 4 days post-hatch (data not shown). Although further investigations are needed, our finding of persistence of macrophages at day 4 post-hatch, we expect the ligand may confer protection against ILTV challenge up to day 4 post-hatch. Our group is in the process of investigating *in ovo* delivery systems that sustain innate immune response further than 4 days post-hatch following *in ovo* delivery of TLR ligands particularly the ligands that binds endosomal TLRs. Delivery via polymer microspheres that encapsulate these endosomal TLR ligands is one such system that may enhance the slow release and uptake of these ligands by macrophages via endocytosis, particularly phagocytosis (particle size of 0.5-10 µm) [244]. In this way the delivered ligands will be available for longer period to induce persisting innate immune responses.

We attempted to investigate certain aspects of the role of macrophages in the TLR ligand mediated protection against ILTV infection but not the role of other cells, especially NK cells being the most active innate immune cells during the viral infection [245]. Moreover, the antiviral molecules like Type I IFNs (IFN-α and IFN-β) were not investigated in the work described in this thesis [246]. Of the TLR signalling pathways in chickens, only TLR-3 is known to signal via MyD88 independent TRIF pathway leading to type I IFNs production [152, 247]. Thus, TLR-2 and -21 in chickens which signal only through MyD88 dependent pathway do not lead to the induction of type I IFNs.

The virus we used in the investigations, ILTV, is a herpesvirus that is known to establish latency with the development of host immunity in the host and may reactivate following a stress situation. Although, we observed that *in ovo* delivered TLR ligands, particularly TLR-21 ligand in decreasing clinical signs and ILTV load in the post-hatch experiments, we did not observe whether

latent phase of the ILTV virus is established in the process. Williams *et al* have demonstrated that the latency of ILTV is established in trigeminal ganglion [40] and it is remained to be seen whether ILTV control using TLR ligands leads to latency establishment and later reactivation.

Future directions: Given the limitations of the studies described in the thesis, future studies should be conducted in number of directions to dissect out the exact mechanisms behind the antiviral responses mediated by *in ovo* delivered LTA and CpG DNA against ILTV infection. Firstly, in the studies described in the thesis, we focussed our studies entirely on certain aspects of macrophages, however, there are other innate cells such as, NK cells and heterophils which are active during viral infections. In order to elucidate the role of innate immune cells other than macrophages in the antiviral response mediated by LTA and CpG DNA, it is imperative to extend the studies further. Secondly, although we focussed our investigations entirely on the innate arm of immune system, it is possible that subsequent to innate immune responses, adaptive arm of the immune system also become activated in the protection mediated by these TLR ligands, particularly CpG DNA against ILTV infection. Thirdly, when we designed our experiments, we did not pay our attention to the fact that ILTV is a herpesvirus with the capability to establish latency and reactivation following development of immune responses. Future studies should be focussed on investigating the influence of *in ovo* delivered TLR ligands on the establishment of ILTV latency and also reactivation capability of ILTV. Fourthly, we have recognised the importance of persistence of innate responses for extended periods following *in ovo* delivery of TLR ligands. Polymer microspheres encapsulation could be used for the *in ovo* delivery of TLR ligands extending the persistence of TLR ligands hence the duration of innate immune responses and this is another area our efforts should be directed . Finally, we investigated the innate immune

responses mediated by *in ovo* delivered LTA and CpG DNA in the lungs, however, we do not know whether the innate immune responses induced following *in ovo* delivery of TLR ligands are systemic. Future studies should also be directed to elucidate the innate responses that are activated in other mucosal surfaces such as gastrointestinal tract following *in ovo* delivery of TLR ligands and this will allow us to investigate the potential applicability of *in ovo* delivered TLR ligands against pathogens that use mucosal surfaces other than the respiratory tract.

CONCLUSIONS

***In ovo* delivered LTA mediated antiviral responses against ILTV infection:** In conclusion, *in ovo* delivered LTA significantly reduces ILTV infection pre-hatch correlating with the expression of IL-1β and increase of macrophage numbers in lungs. As assessed *in vitro*, LTA stimulated avian macrophages could be a potential source of IL-1β and other pro-inflammatory mediators. Since we also found that *in ovo* LTA delivery maintains increased macrophage numbers in lungs post-hatch, we infected the chickens on the day of hatch with ILTV. We found that *in ovo* delivered LTA significantly reduces ILTV genome load in lungs until 5dpi however it does not reduces mortality in chickens resulting from ILTV infection encountered post-hatch. According to our knowledge, this is the first time an *in ovo* delivered TLR-2 ligand has been shown to induce antiviral activity against a virus infecting avian species.

***In ovo* delivered CpG DNA mediated antiviral responses against ILTV infection:** We found that *in ovo* delivered CpG DNA significantly reduces ILTV infection pre-hatch correlating with the expression of IL-1β and increase of macrophages in lungs. As assessed *in vitro*, CpG DNA stimulated avian macrophages could be a potential source of IL-1β and other pro-inflammatory mediators. Since we also found that *in ovo* CpG DNA delivery maintains increased macrophages in the lungs post-hatch, we infected the chickens on the day of hatch with ILTV. We found that *in ovo* delivered CpG DNA significantly reduces mortality and morbidity resulting from ILTV infection encountered post-hatch. Thus, CpG DNA can be a candidate innate immune stimulant worthy of further investigation for the control of ILTV infection in chickens.

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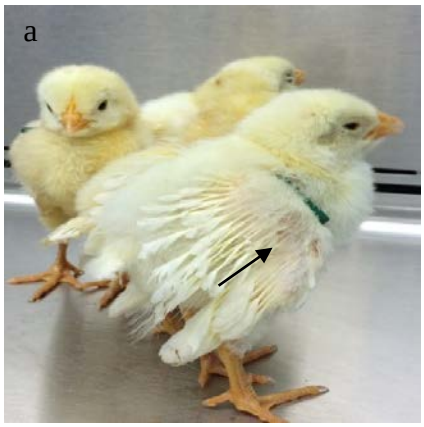
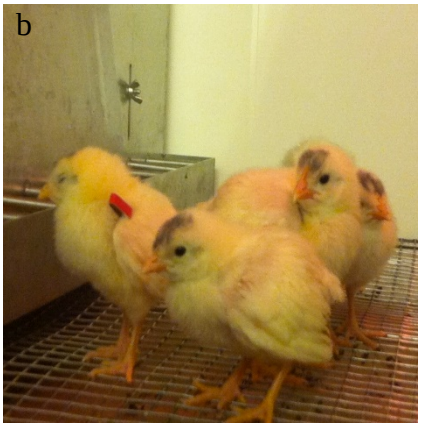
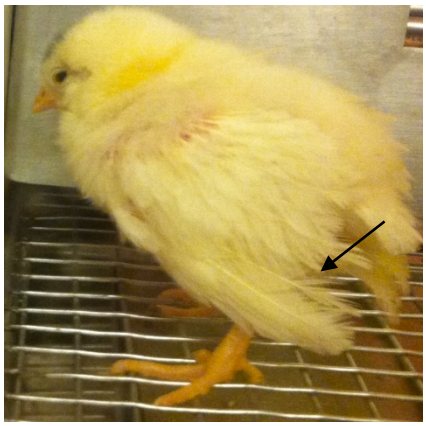
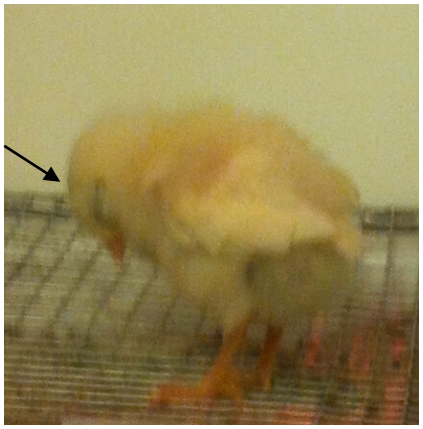
APPENDICES

Appendix 1. Scoring criteria used for evaluation of histological changes in lungs [124].

Score	Criteria
0	No cellular infiltration. Parabronchi are clearly visible surrounded with many airspaces, clear septa surrounding the parabronchi and presence of clearly defined air exchange areas.
1	Mononuclear cell infiltration in the area of lung was less than 25%. Parabronchi are clearly visible surrounded with airspaces, clear septa surrounding the para-bronchi and presence of air exchange areas.
2	Mononuclear cell infiltration in the area of lung is at 25-50%, particularly at the air exchange areas near the lumen of the parabronchi, reduced parabronchial lumen and unclear septa surrounding the parabronchi.
3	Mononuclear cell infiltration in the area of lung is more than 50% with some cells visible in the lumen of the parabronchi. Air exchange areas are lost with few or no such areas near the lumen and towards the periphery of the para-bronchi. Unclear septa surrounding the parabronchi.

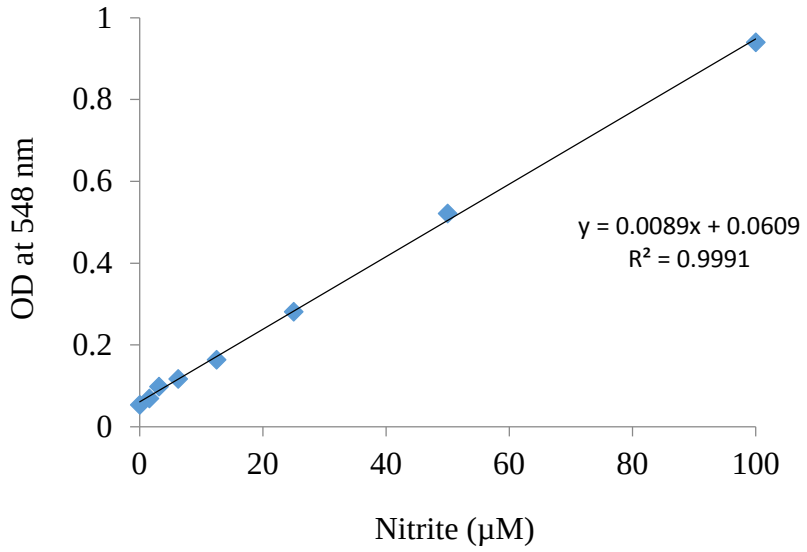
Appendix 2. Clinical scoring criteria used for evaluation of chickens infected with ILTV intra-tracheally on the day of hatch.

Scoring	Clinical sign	Representative images of clinical signs
1 2 3	Increased respiratory rate • Mild (slightly increased breathing rate) • Moderate (highly increased breathing rate) • Severe with open mouth breathing or gasping (condition shown by arrow)	 
1	Ruffled feathers (shown with arrow in a) and huddling together (b)	

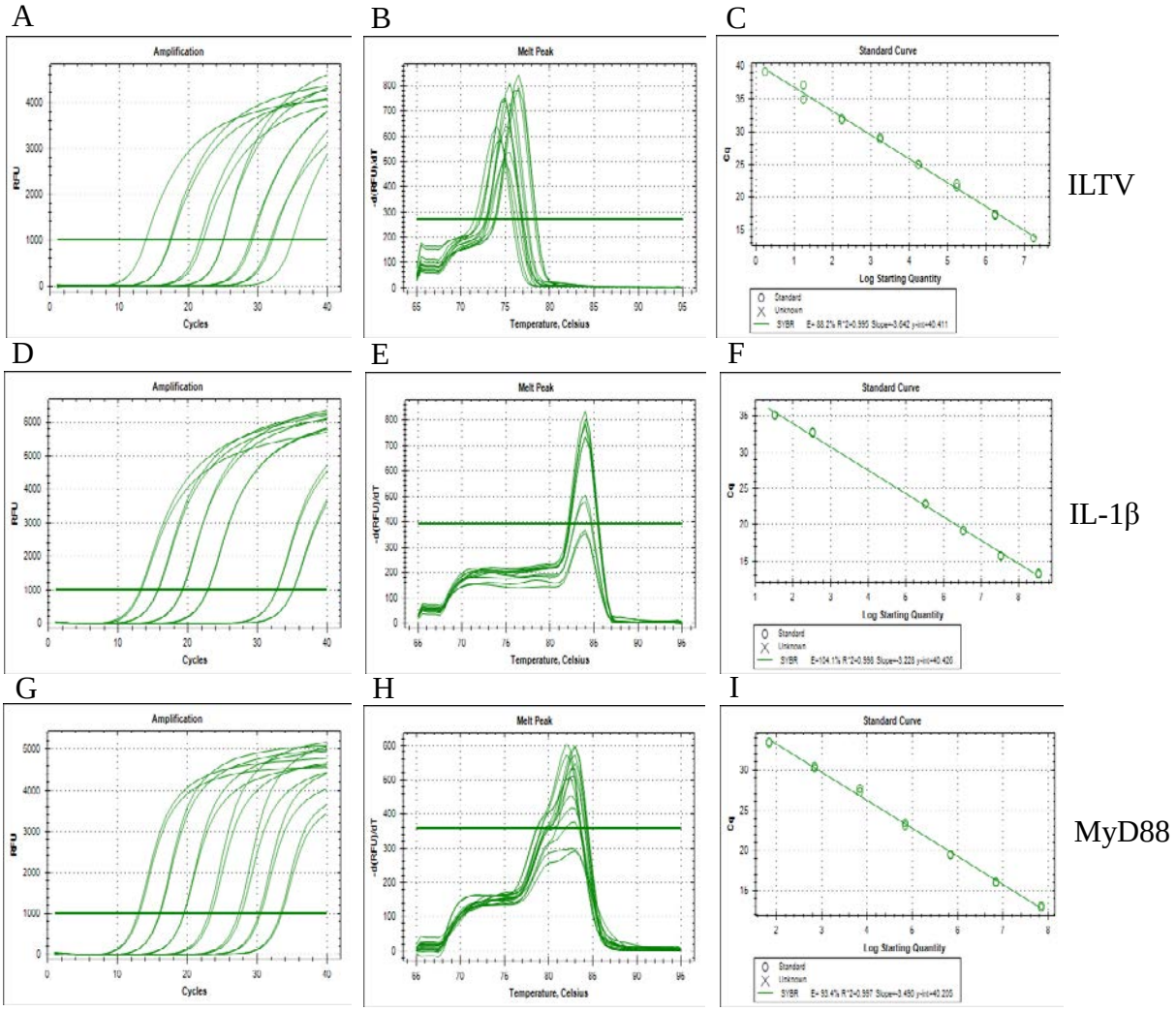
Scoring	Clinical sign	Representative images of clinical signs
		
1	Droopy wings (condition shown by arrow)	
1	Depression or head lowered with no movement (indicated by arrow)	

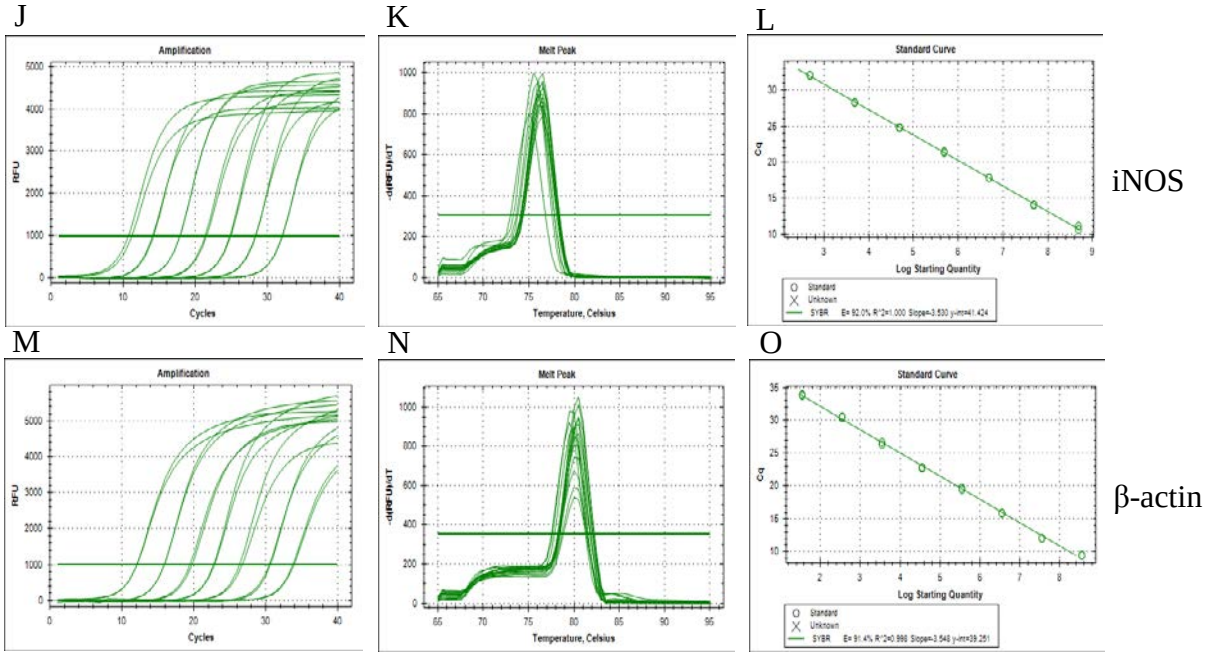
Scoring	Clinical sign	Representative images of clinical signs
1	Body weight loss rather than gain	

Appendix 3. The standard curve for NO assay using the different concentrations of nitrite. The optical density (OD) value for each nitrite concentration was averaged and plotted in y-axis with nitrite concentration in x-axis. The equation shows the function of "y" in terms of "x". R^2 indicates the reaction coefficient.



Appendix 4. Standard curves optimized for the gene expression analysis using real-time PCR. The dilution series of plasmids with gene of interest were subjected to real-time PCR in duplicates. The amplification plots, melting curves and standard curves were generated for ILTV (A-C), IL-1β (D-F), MyD88 (G-I), iNOS (J-L) and β-actin (M-O). The efficiency of the standard curves ranged from 88-105% and reaction coefficient (R^2) from 0.995-1. The melting curves with single peak indicated towards single specific product production without any primer dimer formation.




J
K
L
Amplification
RFU
Cycles
Melt Peak
-d(RFU)/dT
Temperature, Celsius
Standard Curve
Cq
Log Starting Quantity
Standard
Unknown
SYBR
iNOS
M
N
O
β-actin

Appendix 5. Permission from the co-authors of the articles included in this thesis.

As the co-authors, we give our consent to use the data in the paper " *In ovo* delivery of CpG DNA reduces avian infectious laryngotracheitis virus induced mortality and morbidity " published in the Viruses 7(4): 1832-52 in 2015 as part of Simrika Thapa's thesis entitled "Induction of antiviral response against avian infectious laryngotracheitis virus infection" to be submitted to the Faculty of Graduate Studies at University of Calgary in June 2015.

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As the co-authors, we give our consent to use the data in the paper "*In ovo* delivery of toll-like receptor 2 ligand, lipoteichoic acid induces pro-inflammatory mediators reducing post-hatch infectious laryngotracheitis virus infection" published in the Veterinary Immunology Immunopathology 164(3-4): 170-8 in 2015 as part of Simrika Thapa's thesis entitled "Induction of antiviral response against avian infectious laryngotracheitis virus infection" to be submitted to the Faculty of Graduate Studies at University of Calgary in June 2015.

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As the co-authors, we give our consent to use the data generated by Simrika Thapa in the paper "Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection *in vitro* and *in vivo*" published in the Developmental and Comparative Immunology 48(1): 22-32 in 2015 as part of her thesis entitled "Induction of antiviral response against avian infectious laryngotracheitis virus infection" to be submitted to the Faculty of Graduate Studies at University of Calgary in June 2015.

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