IMMUNOPROTECTIVE MECHANISMS ASSOCIATED WITH *IN OVO* DELIVERY OF OLIGODEOXYNUCLEOTIDES CONTAINING CpG MOTIFS (CpG-ODN) AND FORMULATION OF CpG WITH NANOPARTICLES TO ENHANCE ITS EFFICACY IN NEONATAL BROILER CHICKENS

A thesis
submitted to the College of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the
Degree of Doctor of Philosophy
in the Department of Veterinary Pathology
University of Saskatchewan
Saskatoon

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ABSTRACT

Oligodeoxynucleotides containing CpG motifs (CpG-ODNs) are known for their ability to stimulate vertebral immune system and provide protection against pathogens. Although CpG-ODN provides protection against bacterial infections in chickens mechanisms of immunoprotection remains elusive. The objective of this study was to identify mechanisms of immunoprotection of CpG-ODN following *in ovo* delivery in chickens .

In the second chapter, we provide the mechanistic insights into CpG-ODN induced protection against *Escherichia coli (E. coli)*. Multiplex cytokine gene analysis using QuantiGene Plex 2.0® technique revealed that CpG-ODN upregulates both Th1 and Th2 cytokines, as well as pro-inflammatory cytokines in both spleen and lung. In our study, Lipopolysaccharide-induced TNF factor-alpha factor (Litaf) stands out in the cytokine profiles of spleen and lungs, underscoring its role in CpG-ODN induced protection mechanisms. Flow cytometry analysis showed a marked increase of T lymphocyte and antigen presenting cells (APC) as well as enhanced expression of CD40 by APCs in spleen and lung after CpG-ODN treatment. This study demonstrated for the first time that CpG-ODN provides protection in neonatal chicks against *E. coli* infections by eliciting cytokine responses and enriching the immunological niches in spleen and lungs.

In the third chapter, we show that CpG-ODN induces a dose-dependent influx of macrophages, $CD4^+$ and $CD8^+$ T-cell subsets in spleen and lungs of chicks that correlates with the immunoprotection against *E. coli*. We observed a dose-dependent enrichment of the immunological niches, wherein 25 µg and 50 µg of CpG-ODN induced significant changes in the immune profile of both spleen and lungs that correlated with their ability to resist *E. coli* infection. In the fourth chapter, we report that *in ovo* delivery of CpG-ODN formulated with CNT or lipid-surfactant potentiate the protective effect against *E. coli* infection. In conclusion, this study provides a greater understanding of cellular and molecular mechanisms for CpG-ODN induced antimicrobial immunity. And this significant advancement in the mechanistic insight will help in utilizing the full therapeutic potential of CpG-ODNs. Our work on CpG-ODN dose-dependent changes and enhanced protection against *E. coli* infection by nanoparticle formulations demonstrate the possible utilitization of CpG-ODN in the poultry industry as an alternative to antibiotics to prevent bacterial infections of neonatal chickens.

ACKNOWLEDGEMENTS

First and foremost, I express my sincere gratitude to my supervisor, Dr. Susantha Gomis, for his continuous support of my graduate studies, his patience, motivation, knowledge and understanding throughout my research program. I consider myself to be very fortunate to complete my PhD with such a supportive advisor and mentor. I would like to thank the other members of the committee, Dr.Marianna Foldvari, Dr. Suresh Tikoo, Dr. Philip Willson, Dr. Beverly Kidney and Dr. Elemir Simko for the assistance they provided at all levels of this research project. I would also like to thank all faculty members of the WCVM for their support and understanding throughout my research program.

A very special thanks goes out to Drs. Khawaja Ashfaque Ahmed, Lisanework Ayalew and Shanika Kurukulasuriya for their help and guidance in all aspects of my thesis. I very much appreciate the continuous support of Shelly Popowich throughout my study. I am also thankful to Betty Chow-Lockerbie and Tara Zachar for all the help provided in successfully completing my research. I also thank Ian Shirley, all the staff members of Animal Care Unit of WCVM and Marina Vaneva Ivanova from University of Waterloo for their technical support. I am grateful to Sandy Mayes, Tyler Moss and Angela Turner for their help in academic and administrative areas.

A special thank you goes to my fellow research team members, Kalhari Goonawardene, Ashish Gupta, Ruwani Karunarathna, Mengying Liu and my all my friends who have supported me in various ways.

My heartfelt thank you goes to my parents, Premasiri Gunawardana and Jean Gunawardana, and my brother Nishantha for their love and support throughout my life. I am very much grateful to my loving husband, Kalpa for his constant support and encouragement, this thesis have never been possible without them. A special thanks to my baby son, Thehan for his lovely smile which was heartening during my thesis writing.

I wish to thank for the financial support provided by Natural Sciences and Engineering Research Council of Canada, Western Economic Diversification Canada, Saskatchewan Chicken Industry Development Fund, Canadian Poultry Research Council, Alberta Livestock and Meat Agency Ltd. and Alberta Chicken Producers.

DEDICATION

I DEDICATE THIS THESIS TO MY LOVING PARENTS FOR THEIR CONSTANT LOVE AND SUPPORT

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LIST OF ABBREVIATIONS

ACU	Animal Care Unit
AF	alexa flour
APC	antigen presenting cell
BBW	bursal weight to body weight ratio
BCG	bacillus calmette-Guérin
CpG-ODN	cyosine phosphodiester guanine - oligodeoxynucleotide
BHI	brain heart infusion
BSA	bovine serum albumin
CCS	cumulative clinical score
CD	cluster of differentiation
CFU	colony forming unit
CNT	carbon nanotube
CpG	cytosine phosphodiester guanine
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
FACS	fluorescence-activated cell sorting
f-CNT	functionalized carbon nanotube
FITC	fluorescein Isothiocyanate
GALT	gut associated lymphoid tissue
HKG	housekeeping genes
Hprt 1	hypoxanthine-guanine phosphoribosyltransferase 1
IEL	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intra-muscular
IRAK	interleukin-1 receptor activated kinase
Litaf	lipopolysaccharide-induced tumor necrosis factor-alpha factor
LPL	lamina propria lymphocytes

LPS	lipopolysaccharide
LSC	lipid surfactant composite
MALT	mucosa associated lymphoid tissue
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
MRC	mannose receptor
mRNA	messenger ribonucleic acid
MWNT	multi walled carbon nanotube
MyD88	myeloid differentiation marker 88
NF-κB	nuclear factor kappa-light chain-enhancer of activated B cells
NK	natural killer
NOD	neucleotide-binding oligomerization domain
NP	nano particle
PAMP	pathogen associated molecular pattern
PCEP	poly [di (sodium carboxylatoethylphenoxy) phosphazene]
PCL	poly-e-caprolactone
PCPP	ploly [di (carboxylatophenoxy) phosphazene]
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PEG	poly ethylene glycol
PKC	protien kinase C
PLA	poly lactic acid
PLGA	poly (lactic-co-glycolic acid)
PO	phosphodiester
PRR	pattern recognition receptor
PS	phosphorothioate
PVP	poly (N-vinyl pyrrolidone)
RBC	red blood cell
RNA	ribonucleic acid
SC	subcutaneous
ssRNA	single stranded ribonucleic acid
SWNT	single walled carbon nanotube

TAK1	TGF beta activated kinese 1
TCR	T cell receptor
TGF	transforming growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
TRAF6	TNF receptor associated factor 6
Tubb 1	tubulin beta 1

CHAPTER 1:INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

There is continuous emergence of infectious diseases in food animal species. Disease prevention and control strategies are focused mainly on immune-based methods such as, vaccines, immunotherapy and immune modulation. Though vaccines are well-known biological preparations to stimulate the immune system for improved disease protection, they are not available for all disease agents and may not be effective when an immediate response is required. Additionally, the routine prophylactic use of antibiotics is controversial because residues may persist and can lead to the emergence of antibiotic-resistant microbes (9). The occurrence and increasing spread of resistant bacteria are rendering current antibiotics progressively less effective. Thus, there is pressure to restrict the use of antibiotics in agriculture, due to the emergence of antibiotic resistance and its implications on treating infectious diseases in humans. Consequently, the Canadian broiler chicken industry voluntarily discontinued the use of Category I antibiotics commencing in May of 2014. Therefore, finding alternatives to antibiotics is a priority in the Saskatchewan and Canadian broiler chicken industries (43). Currently, immune modulators with immune-stimulatory activity are being evaluated for their potential to replace antibiotics. The vertebral innate immune system identifies pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR), inducing immune responses against infections (211). Some common components in pathogens that act as PAMPs include lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acids, mannose-rich glycans, flagellin, and their genomic nucleic acid (8) , Non-vertebral deoxyribonucleic acid (DNA) which contains comparatively higher amounts of unmethylated cytosine phosphodiester guanine (CpG) dinucleotides compared to vertebral DNA, act as a PAMP. Hence, these unmethylated CpG dinucleotides (CpG motifs) induce strong immunostimulatory activity in vertebrates (5). In recent years, synthetic CpG oligodeoxynucleotides (CpG-ODNs) with immunostimulatory properties, have been synthesized and are currently in wide use for anti-cancer therapy, allergy treatment, vaccine adjuvants and as an immune protective agent (162). Thus, CPG-ODNs as immunotherapeutic agents have great potential to replace antibiotics for the prevention of various types of infectious diseases in the poultry industry (105).

1.2 Overview of the avian immune system

Most of the studies on the immune system of avian species have been conducted in chickens (279). The immune system of avian species is composed of a network of immune organs (lymphoid organs), immune cells, humoral factors (antimicrobial immunoglobulins (Ig) and other proteins), cytokines and chemokines (238). The composition and function of the avian immune system is almost similar to that of the mammalian immune system, although there are some important differences (280). The bursa of Fabricius, the key lymphoid organ of B lymphocyte lineage, is one of the unique features in avian species which is absent in mammals (279). Moreover, differences are seen in gut associated lymphoid tissues (scattered lymphoid tissue and cecal tonsils). In addition, the organization of splenic lymphoides and lymphatic nodules are different as compared to that of mammals. There are also differences reported in some avian toll-like receptors (TLR), defensins, cytokines and chemokines (153). Differences in the nature of Igs between avian and mammalian species is another feature that makes the avian immune system unique (88). They also lack functional eosinophils and the functional equivalent to mammalian neutrophils are called heterophils (153).

1.2.1 Avian immune organs

Similar to the mammalian immune system, immune organs of the avian immune system also consist of primary (central) and secondary (peripheral) lymphoid organs (231)

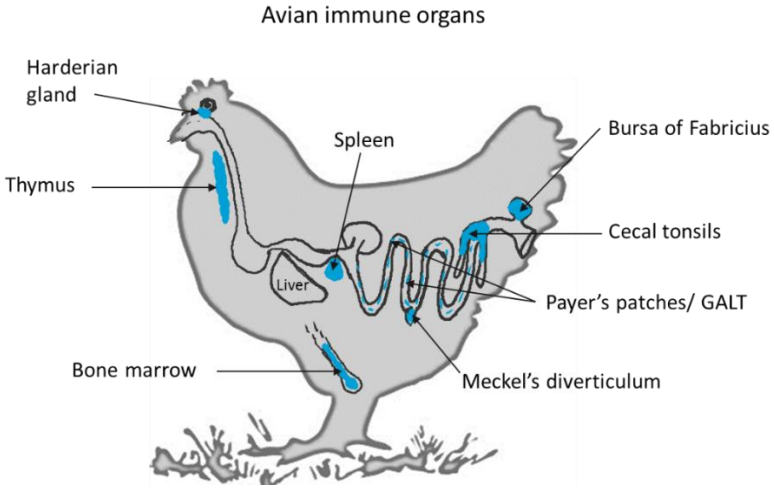

Figure 1-1: Avian immune organs. (GALT = gut associated lymphoid tissue). Adapted from Structure of avian immune system Dr Fares El-Khayat, LinkedIn slide share. www.slideshare.net/ssuser662c3a/structure-of-avian-immune-system-dr-fares-elkhayat. Slide 5.

1.2.1.1 Primary lymphoid organs

Primary lymphoid organs are sites where multiplication and differentiation of immune cells occur resulting in immunologically efficient effector cells (147). The primary lymphoid organs of birds are bursa of Fabricius and thymus where B and T lymphocytes are differentiated, respectively (88). Both organs differentiate from the epithelial anlage of the embryo (231). The clear distinction between B and T lymphocyte population differentiation sites have provided an excellent base for studying immunological concepts of the B and T cell lineages in avian species (59, 70, 87). During embryonic development hematopoietic stem cells enter the bursal and thymic anlages and later differentiate into mature B and T cells respectively (231). The yolk sac is proposed as the site of origin of these hematopoietic cells (87, 242).

The bursa of Fabricius is a sac like structure that is an outgrowth of the cloacal epithelium located at the dorsal end of the hind gut in birds (279). Initially this structure was thought to serve as a receptacle for storing semen in hens (2). However, following many investigations on the role of this organ, Glick *et al*. 1956 and his colleagues reported that the bursa of Fabricius is involved in antibody mediated humoral immunity (98, 99). In contrast, the thymus, emerge as an epithelial outgrowth of the pharyngeal pouches at embryonic development. Chicken thymus is a multilobular organ which can form up to 14 lobes (88, 277) located on either side of the neck continuing into the thoracic cavity (279). The thymus is the site for T lymphocyte maturation and differentiation and is involved in cellular immunity (279).

1.2.1.2 Secondary lymphoid organs

Secondary lymphoid organs of avian species develop from the mesenchymal anlage of the embryo. It is composed of spleen, mucosa-associated lymphoid tissue (MALT), mural lymph node, ectopic lymphoid tissue, pineal gland and bone marrow (231, 261). Antigen induced immune responses take place in secondary lymphoid organs (279), where the differentiation of immunologically active T and B cells mature into antigen specific effector cells (261).

The avian spleen is considered as an important organ in systemic immunity. The major part of spleen development occurs after hatch when birds are exposed to antigenic stimuli (84). It contains red pulp and white pulp which are not clearly defined. In addition, avian spleen contain less red pulp compared to mammals and therefore the contribution to oxygen circulation is less prominent (231). Thus, it is more important in both innate and adaptive immunity as an immune

organ where phagocytosis of damaged cells and antigens occur. It also serves as a site for production, maturation, and storage of lymphocytes (231, 292).

As birds lack organised lymph nodes as in mammals, lymphoid tissue is developed at locations where antigenic stimulation occurs. Mucosal surfaces are the initial and main sites where antigens are introduced. Hence, widespread MALT is seen in birds (146). Gut-associated lymphoid tissue (GALT) is an important component of MALT. Small lymphoid accumulations are seen along the intestines. Cecal tonsils, Peyer's patches, Meckel's diverticulum, esophageal and pyloric tonsils, lymphoid accumulations in the roof of pharynx and in cloaca are components of the avian GALT (27, 231). Respiratory, reproductive and head-associated lymphoid tissues, such as the Harderian gland (located in the orbit behind the eye), conjunctival and nasal lymphoid tissue are also parts of MALT (231, 261).

Mural lymph nodules are organized accumulations of secondary lymphoid tissue which are located within or closely to the lymphatic vessels, mainly those of the limbs and neck of the chicken (261). Ectopic lymphoid tissue are seen in non-lymphoid organs such as, liver, pancreas, kidney, endocrine glands, gonads, the brain and spinal cord (231, 241). It has been shown that lymphoid foci are present in these organs as a compensation for the lack of lymph nodes in birds (261) and cause no functional disturbance to the non-lymphoid organs (231). Pineal gland in birds has a lymphoid component that is immunologically active and able to produce antibodies (53).

1.2.2 Immune cells

The immune system is activated and commences responses when there is antigenic stimulation. The response is as a result of teamwork between various immune cells such as, macrophages, B and T lymphocytes (279).

1.2.2.1 Macrophages and dentritic cells

Both macrophages and dendritic cells (DC) originate from circulating monocytes which are derived from myeloblast progenitors in the bone morrow (281).They are both considered professional antigen presenting cells (APC), in addition to B lymphocytes (157).

Chickens do not have macrophages that are constitutively present in serosal membranes and peritoneal cavity as seen in common mammalian species. In chickens, an inflammatory stimulation is needed for macrophages to be recruited to these sites from the circulation (157, 262).

Macrophages are most commonly known for their phagocytic function which contributes to the innate immunity (250). Macrophages with phagocytic activity have also been observed during embryonic development in chicken embryos (146). Monocytes derived from bone marrow mature and differentiate into tissue macrophages once migrated to tissue sites. These macrophages become capable of antigen processing and presenting, leading to adaptive immunity (250). Macrophages may also secrete several immunoregulatory cytokines or metabolites in response to antigenic stimuli. Therefore, chicken macrophages are involved in pathogen recognition and destruction, modulation of innate immune responses and activation of the adaptive immune system.

Immature DC are considered poor T cell stimulators, but can capture antigens by phagocytosis, macropinocytosis or endocytosis. Once they are activated by PAMPs, they migrate out of non-lymphoid tissues into secondary lymphoid tissues where they complete their maturation. These mature DC express up-regulated major histocompatibility complex (MHC) class II and co-stimulatory molecules and are specialized in presenting processed antigens to T-cells to initiate adaptive immune responses (336).

1.2.2.2 Lymphocytes

B lymphocytes are the main cells that mediate humoral immunity. When antigens are processed and presented in association with T helper (Th)-2 cells, it leads to the activation and differentiation of B cells into plasma cells which secrete antibodies specific to the antigen (242, 279). B cells use Ig as their signaling receptors and they are secreted upon activation. Three Ig classes are found in chickens: IgM, IgA and IgY (related to both IgG and IgE in mammals) (242).

T lymphocytes are mediators of cell-mediated immune responses. T lymphocytes possess specific antigen receptors (T cell receptors (TCR)). Unlike B cell receptors, they remain bound to the cell. In birds, TCR1 with γ and δ chains (γ/δ TCR) and TCR-2 and TCR-3 with α and β chains (α/β TCR) have been identified. The majority of T cells bear α/β chains (242, 279). For successful antigen presentation, T cells require certain surface glycoproteins which bind to MHC molecules while the antigen epitope is bound to the TCR. CD4 and CD8 are two main surface glycoproteins on T cells that bind with MHCII and MHCI, respectively. $CD8^+$ T cells are involved in cytotoxic cell lysis and therefore known as cytotoxic T cells (CTL). $CD4^+$ T cells are also known as Th cells. They are further divided to Th1, Th2, Th17 and T regulatory (T reg) cells depending on their effector or regulatory function (242).

1.2.2.3 Natural killer cells

Natural killer (NK) cells are regarded as a third lymphoid lineage (102) which has many features of cytotoxic T cells (152). In chickens NK cells have been described as large lymphocytes with electron dense granula (102). In chickens, NK cells are mainly seen in the intestinal epithelium and less frequently in blood and spleen as is in mammals. NK cell mediated cytotoxicity have been reported in spleen and blood cell populations (152). As in mammals, NK cell activity protects birds against various viruses and virus induced tumors such as Marek's disease (102, 278). Accumulation of these cells in tracheal mucosa during early infection of *Mycoplasma gallisepticum* is another report that proves anti-microbial activity of NK cells in the respiratory system (94).

1.2.2.4 Heterophils

Avian heterophils are equivalent to neutrophils in mammals. They are considered as the first cells to arrive to the site of pathogen invasion. Inflammatory or infectious stimuli cause a significant heterophil influx to sites of interest (125, 152). Heterophils act as phagocytes, but lack myeloperoxidases as seen in mammalian neutrophils. Hence, their bactericidal activity by oxidative burst is insignificant. The granular content of heterophils are also different than in mammalian neutrophils (244). However, antimicrobial beta-defensins found in heterophil granules can kill a wide range of bacterial pathogens (125). Upon activation (by pathogens or cytokines), heterophils induce expression of pro-inflammatory cytokines such as, interleukin (IL)1, IL6 and IL18 (300).

1.3 Role of the immune system in protection against infectious diseases

Similar to mammals, exposure to antigenic stimulation triggers the immune system of birds (279). The immune system is mainly divided as innate and adaptive immunity based on the speed and specificity of immune response (238). Innate immunity act fast and provide protection to the host by a series of defence mechanisms and components. Adaptive immunity on the other hand is not immediate but more precise and develops memory, thus the consecutive exposure to the same antigenic stimuli will result in a strong and quicker immune response (74). However, the synergistic role of both innate and adaptive immunity are important in successfully defending the

host against disease causing agents. Antigens or pathogens are sampled and processed by specialized antigen presenting cells and presented to the adaptive immune system (B and T effector cells) where either the pathogen is destroyed or self-antigens are tolerated (157).

1.3.1 Innate immune responses

Innate immunity is considered the first line of defence followed by adaptive immunity. Even though innate immunity was earlier considered as a non-specific, scavenger system that fought invading pathogens, recent studies have shown that innate immunity is more complex and can be specific (153) and involve in induction and modulation of the adaptive immune system (152). Components of the avian innate immune system are similar to that of mammals. It includes constitute barriers, innate immune cells, pattern recognition receptors, complement system, active chemical responses, antimicrobial peptides, cytokines and chemokines (152, 153).

The physical barriers that contacts with the environment are considered as constitutive barriers. The normal flora present on body surfaces prevents pathogen colonization. In addition, cilia found in airways, fatty acids on skin, peristaltic movement of the gut, gastric acidic pH , mucus and antimicrobial peptides are considered to play an important role in preventing pathogen invasion (152).

1.3.1.1 Pattern recognition receptors

PRR are germline-encoded receptors in the innate immune system. They can recognise both exogenous (pathogens) and endogenous (molecules such as uric acid) antigens (153). PRR recognise conserved microbial products called PAMPs. This recognition of PAMPs allows the immune system to discriminate between self and non-self. And also between harmless non-self and pathogen-associated non-self (209). PRR are generally divided into two types depending on their location, as membrane bound (TLR) and cytoplasmic nucleotide-binding oligomerization domain (NOD) like receptors. These can be further categorised by function as signaling PRR and endocytic PRR (153). Among these PRR, TLRs are among the most studied receptors in immunology. A TLR, is a family of pattern recognition receptors expressed on innate immune cells that is crucial for recognition of microbial patterns (PAMPs) (209). Once a TLR is triggered by a specific PAMP several signaling pathways are induced. The NF-κB pathway, mitogen-activated protein kinase pathways and type 1 interferon (IFN) pathways then initiates production

of pro-inflammatory cytokines, chemokines, type 1 IFNs and also induce co-stimulatory molecules (153). Ten TLR's have been identified in chickens (TLR 1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15, and 21) (309). Among them, TLR2A, 2B, 3, 4, 5 and 7, are orthologs to TLRs found in mammals (40). Chicken TLR21 is an ortholog to TLR21 found in fish and amphibians (40). TLR 1LA, 1LB, TLR 15 and TLR 21 are unique to birds (309).

1.3.1.2 Complement system

The complement system is activated and works in a cascade in response to recognition of microbes. This activation leads to opsonisation which enhance phagocytosis, induce inflammatory responses, enhance B and T cell responses and involves in cytolysis of target cells by forming membrane attack complexes (152).

1.3.1.3 Active chemical response

Active chemical responses in innate immunity includes, acute phase proteins (plasma concentration changes by 25% or more following inflammatory stimuli), C-reactive proteins, amyloid, fibrinogen, Mannan-binding lectin, fibronectin, haptoglobulin, transferrin-ovotransferrin and other components that are involved in destroying pathogens and adaptation of host environment (152). Defensins (only β-defensins) (337) and cathelicidin-like proteins are two types of antimicrobial peptides identified in chickens (152).

1.3.1.4 Cytokines

Cytokines are regulatory peptide mediators which are secreted by cells and act as extracellular signals which affect the actions of other cells, during both innate and adaptive immune responses (95, 153, 154). Cytokines are divided to sub families based on various factors, such as structure, function and receptor usage. In chickens, there are less number of cytokine gene families than in mammals (153). Chicken pro-inflammatory cytokines, which are produced by macrophages as a result of inflammatory response include IL1β, IL6, IL8 and IL17 (95). Th1 type cytokines are IL2, IL12, IL15, IL16, IL18 and IFNγ which are seen in immune responses to intracellular pathogens. Th2 type cytokines include IL3, IL4, IL5, and IL13 which are seen in immunity against extracellular pathogens. In addition, other cytokines such as, transforming

growth factor (TGF)-β, IL10, tumor necrosis factor (TNF)-α known as lipopolysaccharide-induced tumor necrosis factor-alpha factor (Litaf), type 1 IFNs (IFNα, β, κ and ω) are reported (95, 153).

1.3.2 Adaptive immune responses

Adaptive immunity is important to clear infections and for immunological memory when innate immune system is not sufficient. Adaptive immunity can be divided into two main categories as humoral immunity and cell mediated immunity. Once a pathogen is presented by an APC, to naïve T cells they become activated and start proliferating. The type of T cell activated (CD4+ or CD8+) is decided based on the antigenic peptide presented via MHCI or II and signaling of co-stimulatory molecules (153).

1.3.2.1 Humoral immunity

Humoral immunity is directed against extracellular pathogens such as, extracellular bacteria, extracellular protozoa and helminths (153). $CD4^+$ T cells are activated when antigens are presented via MHCII molecules (only on APCs) to the TCR. Activated $CD4^+$ T cells (T helper – Th cells) have either effector or regulatory functions. $CD4^+$ T cells of effector function can be of Th1 or Th2 type. Th2 cells activate B cells and mediate the production of pathogen specific antibodies. IL-4, IL-13 and IL-9 have been shown to be principal Th2 cytokines in chickens (242). Regulatory T cells (T reg) are involved mainly in reducing and suppressing excessive Th1 responses (153).

1.3.2.2 Cell-mediated immunity

Cell mediated immunity is directed against intracellular pathogens such as, viruses, intracellular bacteria and intracellular protozoa (153). Antigens presented through MHCI molecules (found in all cells) bind to TCR of CD8+ T cells and result in the killing of the infected cells (cytotoxic T cell activity). In addition, Th1 cells activate cytotoxic T cells, NK cells, macrophages and other effector cells. Similar to mammals, IFNγ is the predominant cytokine in cell mediated immunity in chickens (242).

1.3.3 Mucosal immunity

Similar to mammals, mucosal surfaces such as the intestinal, respiratory, and urogenital tracts are designed to prevent microbial invasion. The mucosal lining act as a physical barrier, while other defense mechanisms such as a low pH, secretion of soluble antimicrobial molecules like lysozymes, peptides like defensins and cathelicidins, and most importantly the microbiome play an important role in mucosal immunity (242). Intraepithelial lymphocytes (IEL) and lamina propia lymphocytes (LPL) are the two cell types produced in the main gut mucosa associated lymphoid tissue that involve in the first line of host defence. IELs are considered as important components in local cell mediated immunity with NK cell activity. Whereas LPLs are found to be B cell rich areas that produce Ig (47, 191). Similar to mammals, birds produce polymeric IgA (pIgA) by plasma cells in the lamina propria against infectious agents. Then it binds to a receptor at the basolateral surface of mucosal epithelial cells. These receptor-Ig complexes are then endocytosed and eventually transferred to the apical surface where secretory IgA (sIgA) is released into the mucosal lumen (331). Understanding the function and components of mucosal immunity can aid in improving vaccination of poultry as well as developing methods to enhance immune responses.

1.3.4 Neonatal immunity

The first few days after a chick is hatched can be very critical due to its sudden exposure to a large number of antigenic stimuli (87). At the time of hatch, the immune system of a chick is not fully developed and competent enough to provide full protection against pathogens. However, maternal immunoglobulins transferred through yolk will provide short-term protection against pathogens in developing embryos and at the neonatal stage (71).

1.4 Common bacterial diseases in neonatal chickens

Neonatal chickens are most susceptible to invasive pathogens during the first week post hatch due to their functionally inefficient immune systems (168). Several bacterial pathogens cause disease and loss to the poultry industry at this very young age. *Escherichia coli, Salmonella* and *Enterococcus* are a few of the most common bacterial species that cause infections and death in neonatal chickens. These infections can affect the entire flock, particularly in broilers where their life span is short.

E. coli infection (colibacillosis) is one of the main infectious causes of first week mortality. It causes death in newly hatched chicks due to septicemia. Surviving infected chicks develop yolk sacculitis and omphalitis (247), which will result in uneven growth in the chicken flock and more condemnation at processing . Although *E. coli* infections in adult chickens is usually considered as a secondary infection due to immunosuppression or poor management practices (23), it is an important primary infection in neonatal chickens. Most avian pathogenic *E. coli* serotypes have been isolated from intestinal tracts of neonatal chicks compared to chick embryos. (126). This finding shows that *E. coli* spreads horizontally and can be easily transmitted into large number of neonatal chicks with immature immune systems. Therefore, a successful method to prevent infection early in life is very important.

Salmonellosis caused by *Salmonella* Enteritidis is another condition that cause disease which can lead to death mostly in young chicks whose lymphoid organs are not yet fully developed (316). In addition to the negative impact caused on chickens health, *Salmonella* contaminated poultry products pose public health threats (180). A recent study also showed that embryo mortality related infections with *Enterococcus* species have become predominant in western Canadian poultry hatcheries (156). *Enterococcus* is considered as an opportunistic pathogen that is found in the intestinal microflora of humans and birds. However, it can become pathogenic and infect hosts when their immune systems are weak (270). One study presented that *Enterococcus faecalis* together with *E. coli* infections were the main cause for first week mortality in layers (232). Thus, successful methods for controlling bacterial infections in young chickens in the poultry industry without the use of antibiotics have become a major concern to researchers, producers, and veterinarians.

1.5 Immune stimulating agents as antibiotic alternatives for poultry

Many economically important infectious diseases are well controlled by vaccination in the poultry industry. However, vaccination against all disease causing agents is not practical. On the other hand, the use of antimicrobials in food animal species has raised much concern at present. The excessive use of antibiotics to treat and prevent diseases in poultry has been practiced all over the world for many years. This led to the emergence of antimicrobial resistant strains of bacteria leading to health concerns in humans and animals. Taking these points into consideration, the Canadian poultry industry has developed an industry-wide approach to demonstrate responsible

antibiotic use. Preventive use of category I antibiotics (the most important for human medicine) were withdrawn from the Canadian poultry industry commencing on May 2014 (43). Therefore, alternative methods to protect neonatal chicks from bacterial diseases are being explored. At present, more attention is given towards developing immune stimulatory agents as alternatives to antibiotics (192). TLR agonists such as LPS, CpG-ODN (239, 295), flagellin (91) and peptidoglycan, synthetic double stranded ribonucleic acid (RNA) analog poly (I:C) (167) have been identified as potential immune stimulants in chicken.

1.6 History of CpG-ODN

Tumor regression following systemic bacterial infection has been observed for centuries based on personal accounts rather than facts or research. The first attempt of studying this type of effect of a bacterial infection was carried out by Dr.William B. Coley, a surgeon who observed a sarcoma in one of his patients. The tumor then disappeared following a high fever from an *Erysipelas* infection, now known as *Streptococcus pyogenes* (320). First, he used live cultures of streptococci and injected them directly to tumor sites in patients (54). It had some anti-tumor properties, but due to the toxic effect, he then used heat-killed streptococci (55). Though this was still toxic, significant tumor regression was observed. The preparation he used was later referred to as the Coley's toxin and it was identified that the tumor regression was attributed to the bacterial endotoxins in the preparation (332). But as the original organism that Coley used did not produce endotoxins and still had anti-tumor activity, research continued to identify the causes of antitumor activity (320). After continuous work by many researchers, Tokunaga *et al*. 1984, discovered that purified bacterial DNA of *Mycobacterium bovis* (i.e. Bacillus Calmette-Guérin (BCG)) had anti-tumor activity and that DNA from bacteria, but not from vertebrates, can induce the production of interferons, activate NK cells and inhibit tumor growth in mice (312, 341). Later, two other groups reported that treatment of bacterial extracts with nucleases reduced or showed no immune activity, which confirmed that the immune-stimulatory properties are attributed to the DNA molecule (39, 260). The ability of BCG to reduce the incidence of cancer was reported by Bast *et al*. in 1974 after it was given as a vaccine (25). Yamamoto *et al.* 1988 also found that DNA from BCG stimulated mouse spleen cells *in vitro*, resulted in the amplification of NK activity and production of cytokines such as IFN-α/β and –γ (340). Since then, BCG has been used for cancer therapy, more widely for the local treatment of bladder cancer (57, 235). DNA fractions isolated from BCG

and other bacterial species showed similar action. Several hypothesis were considered and tested in order to explain the difference between bacterial and vertebral DNA, such as the differences in (i) DNA molecular size, (ii) guanine (G) + cytosine (C) ratios, (iii) methyl cytosine and, (iv) sensitivity to DNase. Nonetheless, the same group failed to prove any of them. These researchers were able to identify a more unique hexamer palindromic sequences in active DNA than inactive DNA which might be responsible for activation of the immune system (341). Parallel to these findings they reported that particular palindromic sequences with 5'- CG -3' motif (s) can activate NK cells which are more common in bacterial genomes compared to vertebrates or plants (177). Meanwhile, another team reported that the stimulation of murine lymphocytes with highly purified single stranded DNA from *E. coli* triggered B cell proliferation and immunoglobulin secretion than vertebral DNA (215). This was further proven by Krieg *et al.* 1995, who has contributed a great deal in understanding CpG and its immune mechanisms. B cell stimulation by bacterial DNA required unmethylated CpG dinucleotides. In addition, methylation of bacterial DNA with CpG methylase stopped mitogenesis in B cells (174). The fact that cytosines of CpG motifs are highly methylated in vertebrates than in microorganisms were also reported around the same time (35). Wagner *et al.* 1999 reported that unmethylated CpG DNA that is released by pathogens during infection sends a 'danger signal' to the vertebral innate immune system which will provide a protective immune response and clear the pathogen (319). This potential of CpG DNA motivated scientists to produce synthetic CpG DNA. As a result, Krieg *et al.* 2002 and his team synthesized CpG-ODN with a nuclease resistant phosphorothioate (PS) backbone and showed that the synthetic CpG oligonucleotides can induce murine B cells to proliferate and secrete immunoglobulins both *in-vivo* and *in-vitro*. The PS backbone was created by replacing the non-bridging oxygen atoms at each of the phosphodiester (PO) linkages with a sulfur (171). Moreover, they reported that ODNs lost their immune stimulatory activity if the CpG is eliminated. Optimal B cell activation is seen with ODNs containing a CpG motif in which the CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines. This was the first time that unmethylated CpG motifs were identified as vital mediators of immune activation (174). Furthermore, by increasing the number of these stimulatory CpG motifs in an ODN, the activity of the ODN could be increased. Alongside the evolution of immune recognition of CpG DNA, pathogens also adopted counter strategies like reducing or suppressing the level of CpGs in their genomes to reduce immune recognition (171). Since the development of synthetic CpG-ODN, extensive research has

been conducted in various animal species to explore it's mechanism of action and possible routes of application. Four major classes of CpG-ODN are identified depending on the structural and functional characters (Figure 1-2): Class A (type D) = CpG-ODN has a single CpG motif, a central palindromic PO and modified PS backbone, induce IFN-α production and APC maturation; Class B (type K) = CpG-ODN are considered most stimulatory, contains multiple CpG motifs, a full PS backbone, activate B cells and TLR9 dependent NF-kB pathway; Type C CpG-ODN = has combined features of class A and class B; and Type P = CpG-ODN has a PS backbone with multiple CpG motifs (36, 162, 171).

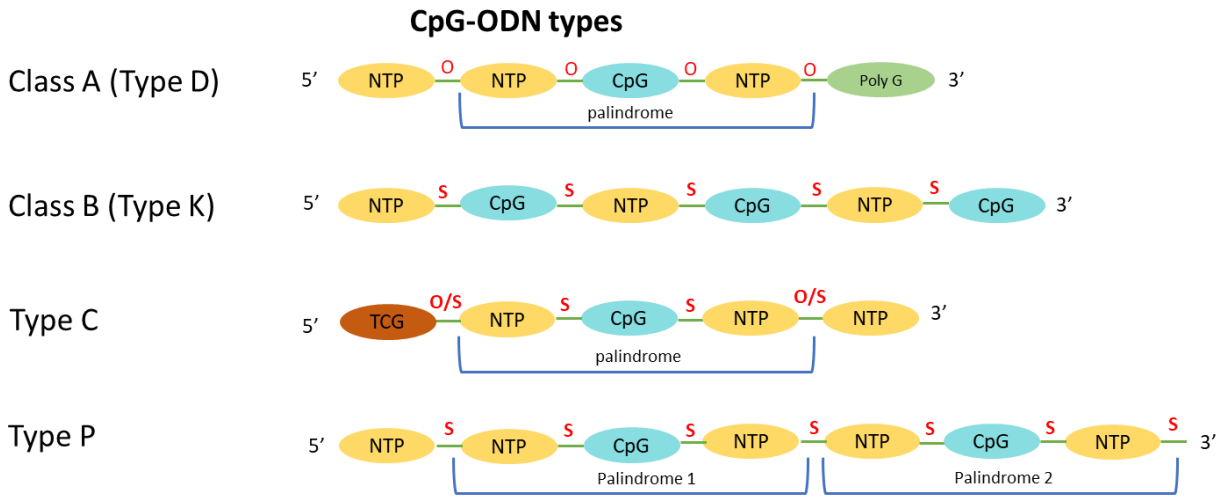

Figure 1-2: Types of CpG-ODN NTP - neucleoside triphosphate, O-phosphodiester, S-phosphorothioate). TCG – TCG dimer. Adapted from Li, Ruiqiao et al 2017, (187).

1.6.1 Mechanism of action of CpG-ODN

DNA from various organisms with little or no methylation of CpG motifs, such as yeast, nematodes, and molluscs induce strong B cell stimulation (298). This led to the theory that immune recognition of CpG motifs trigger protective immune pathways in a host that is similar to detection of endotoxins and other microbial products by PRR (171). Since unmethylated CpG motifs were identified as the immune stimulatory regions of bacterial DNA, research was directed in identifying the innate immune pathways, which recognise and respond to unmethylated CpG.

1.6.1.1 Cellular uptake

When CpG motifs were first reported that they can activate B cells in mice, the molecular mechanism of this activation was unclear. However, Krieg proposed that B cells do not appear to

have a CpG-specific membrane receptor. And when CpG-ODNS were attached to a solid support they did not induce immunity which showed that cellular uptake was a key feature for its activation (171). This was further proven by Manzel *et al.*1999 who reported that CpG-ODN coupled with streptavidin-coated latex, magnetic, gold or agarose beads did not show cellular uptake and showed no immune stimulation. These results proved that CpG-ODN needs to be internalized into cells in order to result in immune stimulation (206). It is known that ODN uptake is different in different subsets of lymphocytes. B cells and monocytic cells has shown the highest rate of uptake while T cells and neutrophils show lower rates (171). The cellular uptake of ODN derivatives is mainly by endocytosis (339) involving phosphatidylinositol 3-kinases (PI3Ks) (142). One study also showed that CpG DNA require endocytosis followed by endosomal/lysosomal compartmentalisation for immune activation. They proved this by using specific inhibitors of endocytosis and endosomal trafficking (5). A more recent study showed that mannose receptor 1 (MRC 1) is involved in CpG ODN uptake and trafficking in mice peritoneal macrophages (220). Studies have shown that uptake of ODNs depends on concentration. Cellular uptake is considered more efficient at low concentrations (<1 μM), where a major amount of ODNs are absorbed on to the cell surface and internalized by absorptive endocytosis in a more efficient manner (339). This endocytosis is suggested to be most possibly clathrin dependent (28). The same group of researchers revealed that, fluid-phase endocytosis (pinocytosis) facilitates cellular uptake of ODNs at higher concentrations (28).Once taken in to cells, ODNs are compartmentalised in endosomes (181, 352). Another study showed that ODNs are endocytosed into acidic vesicles and are transported to the cytosol and nucleus of cells (313)

1.6.1.2 Activation of immune pathways

It was assumed that there was a T cell independent B cell activation pathway that is triggered by CpG-ODN, and that microbial DNA might be providing co stimulatory signals to B cells that are bound to antigens to induce specific antimicrobial immune responses (174). The receptor responsible for this immune response was identified as TLR9. These studies showed that immune responses to CpG DNA, such as splenocyte proliferation, cytokine production from macrophages and maturation of dendritic cells were absent in TLR9 deficient (TLR9 $^{-/-}$) mice. Therefore, it was proposed that this specific toll-like receptor in the vertebral immune system has evolved to distinguish between bacterial DNA from self DNA (135). Concurrent findings of

another group showed that human TLR9 is the receptor playing a critical role in CpG DNA mediated activation of human cells (307). Human TLR9 (hTLR9) is found predominantly in immune cell rich tissues, such as spleen, lymph nodes, bone marrow and peripheral blood leukocytes (51). The same group reported that human cells expressing TLR9 are stimulated by CpG motifs that are active in humans but not in mice and suggested that deviation between TLR9 molecules causes species-specific differences in the recognition of bacterial DNA. TLR 9 receptor has been identified and showed to be involved in CpG DNA recognition in many animal species like, cattle , pig (282, 311), horse (351) and dogs (128). TLR9 is a protein that has a transmembrane domain. Studies have shown that TLR9 is localized in the endoplasmic reticulum (ER) in unstimulated cells and are transferred to endosomes once activated by CpG DNA (181, 183).

Both double and single stranded DNA were suggested to be taken up by endosomal compartment of the cell and have the same intracellular location (30, 353). Once in the endosome, endosomal maturation is required for activation of immune pathways (120).This was proven by using substances such as, chloroquine, bafilomycin or monensin to block or interfere with endosomal acidification and/or maturation, where it resulted in absence in the immune stimulatory activity of CpG DNA (120, 345). All functionally characterized TLRs signal through a common pathway involving myeloid differentiation marker 88 (MyD88), IL1 receptor-activated kinase (IRAK), TNFR-associated factor 6 (TRAF6), TGFb-activated kinase 1 (TAK1), and the kinases of IkB (IKK), IkB, and NF-kB (306). These transcription factors are shown to directly upregulate cytokine and chemokine gene expression (3). As CpG DNA binds to TLR9 in endosomes, their co-localization within the same vesicles induces the recruitment of MyD88 to initiate signaling (306). B cells and plasmocytoid dendritic cells (pDC) are the two main types of cells in humans that express TLR9 and responds to CpG DNA (162). Following activation by CpG DNA these cells will establish an immunostimulatory cascade of events which will result in stimulation, maturation, differentiation and proliferation of other cells such as NK cells, T cells, monocytes and macrophages (21, 162, 296, 297). Subsequently these cells secrete pro-inflammatory cytokines like, IL6, TNF-α and IL18. Moreover, they create a Th1 type immune environment by secreting cytokines such as, IFNγ and IL12 (122, 164) (Figure 1-3). Later, it was also found that CpG-ODN can activate a TLR9-independent pathway initiated by two Src family kinases, Hck and Lyn, which trigger a tyrosine phosphorylation–mediated signaling cascade which leads to cytokine secretion

(269). A very recent study have shown that *in ovo* administration of class B CpG-ODN has the ability to induce cytokine responses in neonatal chicken spleen (266).

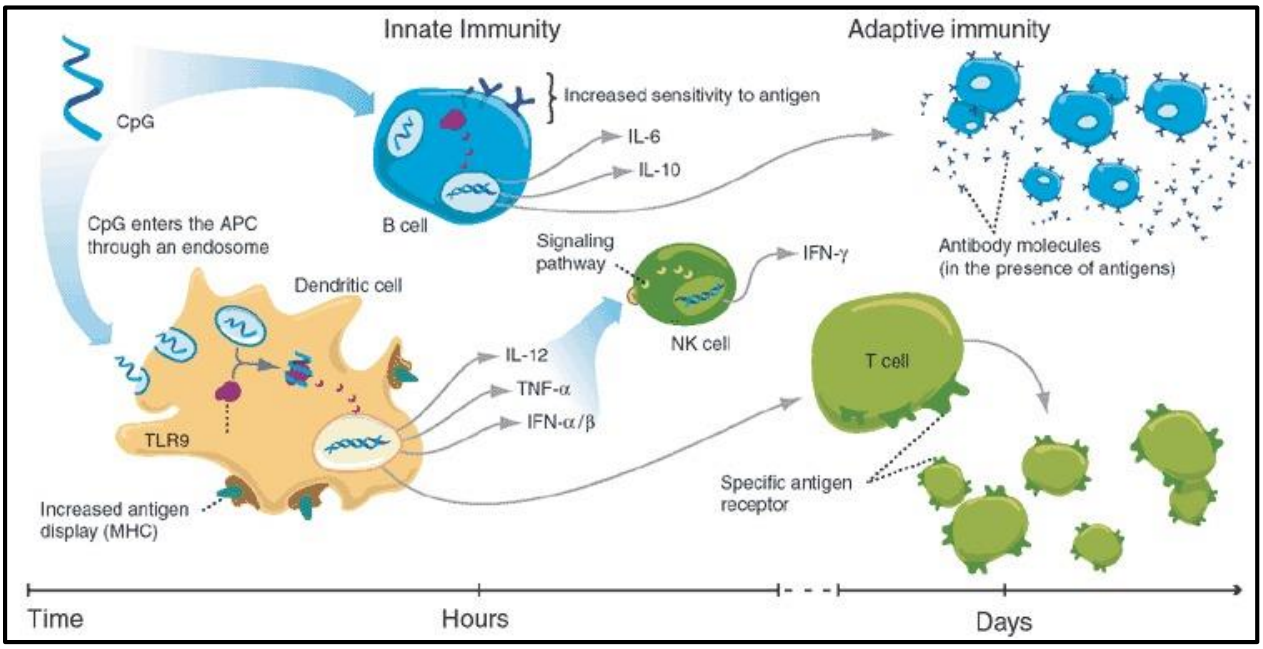

Figure 1-3: CpG-ODN mediated activation of the immune system. Reprtinted from AM Kreig, 2003.

1.6.2 CpG-ODN safety

The immunostimulatory properties of CpG-ODN could lead to safety concerns if not used cautiously. The main potential risks can be development of organ-specific or systemic autoimmune diseases and septic shock (162). Studies show that production of autoantibodies against double stranded DNA is accelerated with high doses of bacterial DNA in animals prone to autoimmune diseases like lupus (170). In addition, bacterial DNA (CpG DNA) stimulates the production of IL6 cytokine and reduce the apoptotic death of activated lymphocytes leading to persistence of self-reactive lymphocytes progressing to autoimmune diseases (96, 196, 344). The increased production of pro-inflammatory cytokines (TNF, IFN-γ) due to CpG-ODN could result in septic shock if reached extreme levels (162).

Several studies focused to investigate the safety concerns of CpG-ODN. The toxicities observed in these studies all appear to be related to the chemical class, PS, and dose administered. Cardiovascular collapse and death was reported in primates due to complement activation and hemodynamic changes when very high doses of PS ODN was given (184). Another study where

bacterial DNA containing unmethylated CpG motifs were injected to knee joints of mice showed that it can induce arthritis (75). Similarly, a different group of scientists reported that when CpG-ODN were administered daily up to three weeks, disruption of lymphoid tissue, hepatic damage and hemorrhagic ascites could be seen in mice (134).

However, repeated administration of CpG-ODN (two to four times/month) protected mice from challenge by *Listeria monocytogenes* and *Francisella tularensis* and these ODN-treated animals remained healthy and developed neither macroscopic nor microscopic evidence of tissue damage or inflammation. (163). Also, when CpG DNA was injected in doses equal or above the levels normally used as vaccine adjuvants every week up to four months to BALB/c mice, animals did not show any adverse effects and remained physically healthy (166). This indicates that even repeated CpG-ODN administration was safe in mice when used with caution. No toxicities or adverse effects were recorded in normal animals when CpG ODN was used as an immune protective agent, vaccine adjuvant or an anti-allergen.(162). A study that compared several adjuvants claimed that CpG DNA induces stronger immune responses with less toxicity than other adjuvants (327).

1.6.3 CpG-ODN applications

Though CpG-ODN was initially identified as an anti-tumor agent, at present, other potential applications of CpG-ODN are under investigation such as; induction of protective immunity against infectious diseases, stimulation of the immune system as a vaccine adjuvant and induction of anti-allergy immunity (162) (Figure 1-4).

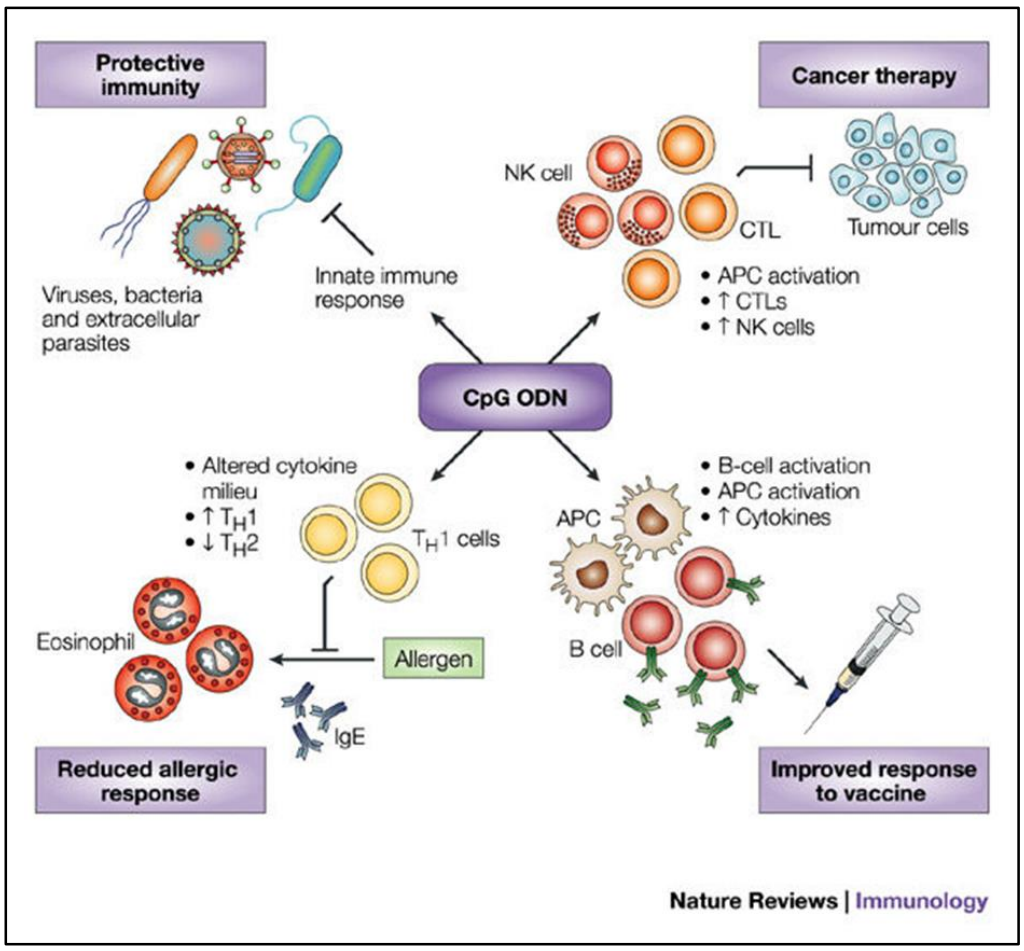

Figure 1-4: Therapeutic uses of CpG-ODN. Reprinted from DM Klinman, 2004.

1.6.3.1 CpG-ODN as an immune protective agent

Earlier, the innate immune system was considered of less importance in the prevention of infectious diseases. However, at present the importance of innate immunity is studied vastly (210). CpG-ODNs can induce an innate immune response that can protect hosts from infectious agents. CpG DNA can elicit plasmacytoid dendritic cells (pDCs) to secrete type 1 IFNs which has a negative effect on a broad range of viruses and bacteria (141, 199). Therefore, the possibility of using CpG-ODN alone to reduce infections were considered (162). Many studies have been directed towards studying this theory in many animal species, especially using murine animal models. Mice were injected with CpG-ODN or bacterial DNA prior to challenge with an infectious agent of interest. These studies have shown that mice are protected from many bacterial infections like, Pulmonary *Klebsiella* (76), listeria and tularaemia (163, 173), acute polymicrobial sepsis (329) viruses like, herpes (18, 249), cytomegalovirus (144), influenza (79) and parasites like,

leishmania (321, 354) and malaria (109). This immune protection lasted for about two weeks in mice when only a single CpG-ODN dose was injected (85). However, repeated administration of CpG-ODN showed significantly improved host resistance to infection for a prolonged period (163). A recent study showed that type-A CpG-ODN can induce high amounts of IFN-α in pDCs and thereby significantly reduced disease severity in experimental autoimmune encephalomyelitis which is a condition that mimics human multiple sclerosis. It also delayed onset of neurological deficits and reduced spinal cord demyelination, while increasing the percentage of splenic regulatory T cells (60).

Many studies done in different species of animals have now shown the immune protective effect of CpG-ODN (38, 105, 182, 193, 228). Yet, studies on immune protective effect of CpG-ODN in humans are not reported due to limitations of conducting challenge studies. However, Non-human primate studies gained interest by researchers, one study showed that susceptibility of normal rhesus macaques to infection with leishmania was significantly reduced by treatment with CpG-ODNs (317).

1.6.3.2 CpG-ODN as a vaccine adjuvant

Many of the successful vaccines are live, attenuated versions of the targeted pathogen where there can be mild disease when administered, or killed, recombinant or synthetic vaccine antigens that cannot act alone and need co-stimulation using an adjuvant (52, 245). CpG-ODNs activate cells that express TLR9 and induce an innate immune response by the production of pro-inflammatory cytokines and Th1 type immunity. In addition, CpG-ODNs improve the maturation and activation of professional antigen-presenting cells and enhance the generation of humoral and cellular vaccine-specific immune responses when used as a vaccine adjuvant (36).

Currently, CpG-ODN is being used as an adjuvant in vaccines produced for many animal species including humans (58, 69, 121). It has been used as an adjuvant in vaccines developed against toxins (tetanus, diptheria), bacteria (Brucella, anthrax, tuberculosis), viruses (measles, hepatitis, cytomegalovirus, influenza) and parasites (malaria, leishmania, trypanosome) [reviewed by (162)]. Inclusion of CpG-ODN as an adjuvant with leishmania promastigotes increased the level of IFNγ production, CD4+ and CD8+ T cells by 2–3 fold in mice compared to the mice that got the promastigotes alone (212). Another study showed that combining CpG-ODN with anthrax vaccine adsorbed (AVA - the licensed human vaccine) generates high levels of toxin neutralizing

antibodies very rapidly and challenged macaques (with anthrax spores) immunized with AVA plus CpG-ODN mounted a 3-fold stronger immune response than animals immunized with AVA alone (165). Furthermore, CpG-ODN have proven to enhance both mucosal and systemic immune responses with intra nasal mucosal administration of a hepatitis B (208) and influenza (218) virus antigens in mice. Some studies showed that CpG-ODN can induce an effective Th1 type immunity when used as an adjuvant in neonatal mice, which is a challenging age for vaccination as neonatal immune systems are not well developed (216). The safety of using CpG-ODN as a vaccine adjuvant has also been taken in to consideration. A study that evaluated several commonly used adjuvants found that CpG-ODN induces a stronger immune response with less toxicity than other adjuvants (327). Several studies reported about the safety of using CpG-ODN as an adjuvant (121, 248). For example, safety evaluation of a CpG 7909, a class B CpG-ODN which was used as an adjuvant together with a commercial trivalent killed split influenza vaccine for humans showed normal values on physical evaluation, laboratory blood assays, and assays for DNA autoimmunity. The vaccine was well tolerated and induced a good immunity resulting in significantly higher levels of IFNγ secretion from peripheral blood mononuclear cells recovered at 4 weeks (58). Several studies in humans have shown the successful immune enhancement of vaccines with the use of CpG-ODN 7909 (VaxImmune™). A phase 1 clinical trial demonstrated the AMA1 (asexual blood-stage merozoite surface antigen Apical Membrane Antigen 1)-C1/Alhydrogel + CPG 7909 vaccine provide an adequate safety profile and was highly immunogenic in malaria-naïve individuals (223). More phase 1 clinical trials showed the striking improvement of the immune response to BioThrax® (Anthrax Vaccine Adsorbed) AVA by CPG 7909 in healthy volunteers (138, 263). Adding CpG-ODN to the human influenza vaccine Fluviral® have shown to induce higher virus-specific antibody titers resulting in better protection in ferrets as manifest by a 20% reduction in viral load when compared to Fluviral® alone (86).

1.6.3.3 CpG-ODN as an anti-tumor agent

The accidental discovery of tumor curing ability of bacterial extracts was a significant milestone in the discovery of CpG-ODN. Over the years since its first discovery, many scientists have worked on understanding the mechanism of cancer curing ability of CpG-ODN. The well-established theory is that the immune cascade that is induced by CpG-ODNs activate NK cells and CTLs which destroys cancer cells and facilitates the remission or treatment of cancer (162). One

study where mice were implanted with syngeneic GL261 glioma (a brain tumor model) showed that CpG-ODN induced TLR 9 down-regulation, followed by apoptosis of GL261 cells *in vitro* and *in vivo*. They also showed that CpG-ODN enhanced the antigen presenting capability of microglia and pronounced CD8+ T cell mediated immunity suggesting that CpG-ODN could be used for immunotherapy for treating malignant glioma (16). Another study using a colon carcinoma mice model also showed that CpG-ODN stimulated DC maturation and T cell activation which resulted in delayed or reduced tumor growth (42). Similar results were published by another group where peritumoral CpG-ODN injections to mice with colon carcinoma elicits a strong CD8 T cell response and innate effector mechanisms that could cure tumors (133). In addition to its prophylactic and therapeutic use, CpG-ODN has been evaluated as an adjuvant in tumor vaccines. Mice immunized with CpG-ODN as an adjuvant were protected from tumor challenge (38C13 B cell lymphoma), similar to mice immunized with complete Freund's adjuvant. However, with CpG-ODN less toxicity and a higher titer of antigen-specific Immunoglobulin gG2a (IgG2a) was observed than with complete Freund's adjuvant (330). Similarly, human exosomes combined with CpG-ODN as an adjuvant efficiently activates naive CTLs leading to tumor (melanoma) rejection in transgenic mice (48). A number of preclinical studies showed that intra-tumoral injections of CpG-ODN elicited a stronger protective response than did systemic delivery of the same agent (44, 284). A very recent study showed promising results with reduction in tumor sizes in patients with B-cell lymphoma with a novel Class C CpG-ODN (SD-101) given with low dose radiation in a phase 1/2 trial (185). The development of an inhaled CpG-ODN, DV281 as immunotherapy for lung cancer is suggested very recently (92).

1.6.3.4 CpG-ODN for anti-allergy therapy

Allergic reactions, such as allergic asthma is caused by overproduction of Th2 type cytokines. The capability of CpG-ODNs to stimulate a Th1 immune responses that inhibits/balances the development of Th2 cell-mediated allergic response has been the key to using CpG-ODN as an anti-allergic agent (162). Several studies have been conducted on asthma models of mice to investigate the potential use of CpG-ODN for treatment of asthma. Sur and his group examined the ability of unmethylated CpG-ODN, to prevent the allergic asthma in mice sensitized to ragweed allergen. They reported that when CpG-ODN was administered 48h before allergen challenge there was an increase in the ratio of IFNγ to IL4 secreting cells, reduced allergen-induced

eosinophil recruitment, and less ragweed allergen-specific IgE producing cells. Furthermore a strong Th1 response resulted in less peribronchial and perivascular lung inflammation, and inhibition of bronchial hyper responsiveness 6 weeks after administration of CpG-ODN (299). In another study, mice were sensitized with conalbumin (model of asthma) and they reported the potential use of CpG-ODN for treatment of allergic asthma by suppressing Th2 responses during IgE dependent allergic airway reactions (276). A similar study that sensitized mice to *Schistosoma mansoni* eggs also looked at the effect of CpG-ODN. They reported that the group which received CpG-ODN + *Schistosoma* eggs showed less eosinophilia and reduced inflammation than the group that received the *Schistosoma* eggs alone. They also suggested that CpG-ODN is desirable than cytokines, such as IL12, as a therapy for asthma, due to its low cost, stability, safety, and induction of prolonged expression of cytokines (161). CpG-ODN has also been shown to be effective in controlling both early and late phases of allergic conjunctivitis in mice when administered either systemically or mucosally (203). CpG-ODN has been considered to be used as an adjuvant in allergen specific immunotherapy. A clinical trial conducted in humans reported that subcutaneous application of house dust mite allergen, together with A-type CpG-ODN packaged into virus like protein, reduced rhinitis and allergic asthma significantly in patients and was safe to use (275). A recent study showed that targeted treatment of allergic rhinitis with intra-nasal CpG-ODN reduces lower airway inflammation (186).

1.6.4 CpG-ODN application in veterinary species

Many studies have proven that bacterial DNA is recognized by immune systems of fish, birds and a variety of mammalian species [reviewed by (162)]. One group studied a number of important livestock, companion and laboratory animal species (sheep, goat, horse, pig, dog, cat, chicken, cotton rabbit, mouse and rat) and reported the active CpG motifs for the first time in each species. Moreover, based on their data, it was clear that CpG motif recognition is highly conserved in a broad variety of species. In all species they tested, the "GTCGTT" motif appeared to have stimulatory potential. The chemical nature of the backbone of the CpG motif also played a role in stimulatory capacity (254). Many studies have shown the potential use of CpG-ODN as an immune protector, vaccine adjuvant, anti-allergy and anti-tumor therapy in veterinary species.

As neonatal immune systems are immature and highly vulnerable to diseases, one study investigated the ability of CpG-ODN to protect newborn lambs following aerosol challenge with

bovine herpesvirus-1, a respiratory pathogen. They showed that CpG-ODNs effectively activate innate immune responses in newborn lambs and induce antiviral responses which correlated with a reduction in viral shedding (229). Most studies on CpG-ODN immune activation and possible uses have been conducted in mouse models (16, 68, 161, 354). Similarly, immune activation by CpG-ODN and its various potential uses have been investigated in other animal species such as, cattle (255, 326) , pigs (175, 195), horses (200), rabbits (140), dogs (178), cats (257), non-human primates (150, 317) and birds (105, 202, 302). Several studies have also shown the immune effects of CpG-ODN in fish (38, 45, 308) and in fish vaccines (155).

1.6.5 CpG-ODN applications in chickens

Despite the absence of TLR9 in birds, which identify CpG-ODN in mammalian species, immune stimulation by CpG-ODN has been shown by several studies in chickens (63, 105, 301, 324). This led to studies directed towards identifying the receptor which is responsible for CpG-ODN mediated immune stimulation.

DNA high in unmethylated CpG motifs is immunostimulatory through avian TLR21 which is absent in mammals. This was confirmed by showing expression of TLR21 resulting in marked NF-kB activation upon stimulation with exogenous ODN and confirmed that chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG ODN (41). A similar study on TLR21 by another group also recognised chicken TLR21 as an innate CpG DNA receptor that is distinct from mammalian TLR9 (159). Chicken harderian gland cells have shown up regulated expression of TLR21 and IFNγ with class B CpG-ODN stimulation (50). Immune stimulatory effects of CpG-ODN in chickens have been demonstrated in many *in vitro* and *in vivo* studies (240, 318). Immune modulation, as an immune protector or a vaccine adjuvant by CpG-ODN in chickens have been investigated related to common bacterial diseases such as, *E. coli* (103-105) , *Salmonella* (131, 132, 202, 301), *Campylobacter* (304, 305); viral diseases such as, infectious bronchitis (65, 66), infectious bursal disease (204, 324), Newcastle disease (194, 350), avian leukosis virus (80) avian influenza virus (289-291), infectious laryngotracheitis (310); protozoan diseases such as, coccidiosis (63, 64). Intra pulmonary delivery of CpG-ODN has shown protective immunity against *E. coli* in neonatal chickens suggesting its use as an alternative to antibiotics (106).

1.6.5.1 CpG-ODN as an immune modulator in chickens

In modern poultry practices, broiler chickens reach market weight at about 35-42 days of age, therefore, the first days of a broiler chickens life is very important. During these first days of life neonatal chicks are exposed to a new environment and their immune systems are not fully functional, causing them more susceptible to diseases. Therefore, improving the immune response and disease resistance of these immature chicks is vital for survival and performance up to their market age (347). Additionally, stimulating the immune system and taking prophylactic measures rather than having to use therapeutic dosages is superior from a food safety and public health viewpoint (105). Thus, CpG-ODNs were considered as an ideal immune modulator. Gomis *et al.* 2003 reported the immunoprotective effect of CpG-ODN against an extracellular bacterial infection in chickens for the first time. At 22 days of age, broiler birds received CpG-ODN by either the subcutaneous (SC) or intramuscular route (IM). Three days later, a virulent isolate of *E. coli* was applied to a scratch site on the caudal abdominal skin and examined for 10 days. Groups that received CpG-ODN by either SC or IM injection had significantly higher survival rates compared to the control group. In addition, the cellulitis lesion caused by *E. coli* was also smaller in CpG-ODN received group. This was the first time the immune protective effect of CpG-ODN was demonstrated in any food animal species (105). Similarly, the potential use of CpG-ODN as an immune protective agent against an *E. coli* infection was demonstrated by the same group in neonatal broiler chickens (104). Thereafter, the immune protective effect of CpG-ODN was demonstrated by several studies by many other researchers in chickens (63, 65, 301).

1.6.5.2 *In ovo* CpG-ODN administration in chickens

The first automated egg injection system was introduced in 1992 (Inovoject). This method of directly injecting substances like a vaccine or drug in to the egg replaced the conventional method of vaccinating newly hatched birds by subcutaneous injection. There are many advantages of *in ovo* vaccination compared to post-hatch vaccination, which include earlier immunity (in vaccines like Marek's), reduced bird stress, precise and uniform injection, reduced labor costs, and reduced contamination (258).

As *in ovo* technology was already in use in the poultry industry. CpG-ODN delivery by this method was considered to activate immune systems of neonatal chickens and prepare them to fight diseases in their first weeks of life. Thus, for the first time Gomis *et al.* 2004 showed that *in ovo*

delivery of CpG-ODN protected neonatal chicks against a bacterial infection. (104). Similarly the immunoprotective effect of CpG-ODN when given by the *in ovo* route has been investigated in several studies (65, 202, 301, 310). The potential use of CpG-ODN as an *in ovo* vaccine adjuvant in chickens was also studied (64, 78). These findings directed researchers to further explore methods to develop efficient CpG-ODN delivery systems in chickens that could be applied in the modern poultry industry.

1.7 Nano particle based drug delivery systems

The development of novel drug/gene delivery systems involving nanotechnology has become a popular field over the years. These are delivery systems of nanometer scale complexes (10–1000 nm) consisting of an active therapeutic ingredient and a carrier material (73). Nanoparticles (NP) based drug delivery systems are well known for their controlled and targeted release of a drug/gene (256). In these delivery systems, the nanoparticle act as a vehicle where the drug of interest is dissolved, entrapped, adsorbed, attached and/or encapsulated into or onto a nano-matrix (288). Many categorization systems are used to classify different nanoparticle types by many researchers. However, depending on the material composed these nanoparticle types can be simply divided as (i) metal based, (ii) lipid based, (iii) polymer based (iv) carbon nanotubes and (v) biological nanoparticles (56) .

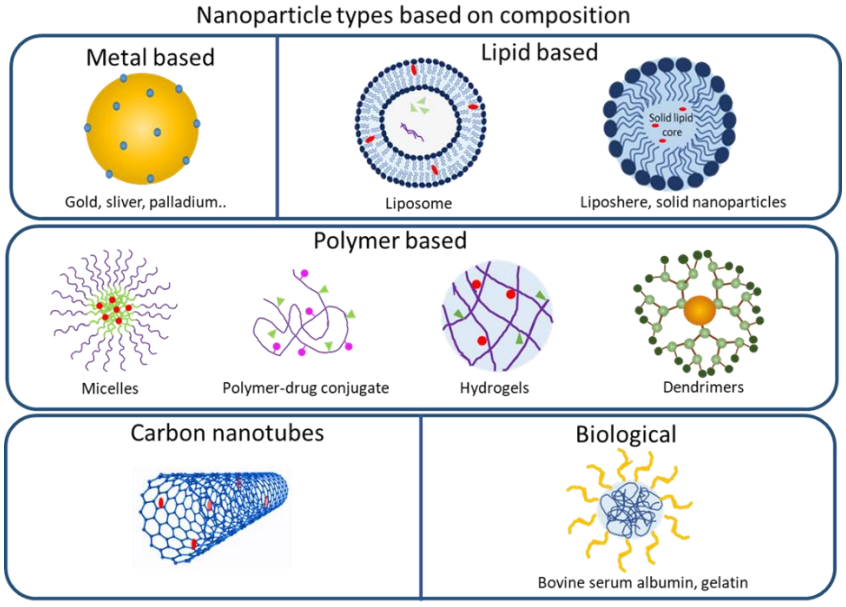

Figure 1-5: Commonly used nanoparticle types based on composition. Adapted from Conniot et al 2014,(56).

1.7.1 Nanoparticle systems

1.7.1.1 Metal based

Metal based systems – metals such as, gold, silver, palladium and platinum are commonly used for drug delivery (139). The extreme small size and vast surface area to carry large dose of drug are considered as main advantages of these systems (346). Drug delivery for cancer treatment using metal nanoparticles such as gold nanoparticles are common and shown to be successful (176, 234). Gold nanoparticles have also been helpful in diagnosing cancer conditions (243).

1.7.1.2 Lipid based

Lipid based systems – they are composed of chemical compounds possessing both hydrophilic and lipophilic properties (amphiphiles), which self-assemble into liquid crystalline phases and can be dispersed into nano-sized particles. Hydrophobic drugs can be encapsulated into hydrophobic regions and hydrophilic drugs can be incorporated to the hydrophilic part (265). These lipid based systems are broadly classified into three types , emulsion based systems (microemulsions, nanoemulsions), vascular systems (liposomes, niosomes) and lipid particulate systems (liposheres,solid lipid nanoparticles) (285).

1.7.1.3 Polymer based

Polymer based systems – A polymer is a large molecule that is composed of many repeated subunits (236). These biologically active polymers can be merged with drugs and provide therapeutic benefits. As they are biodegradable, it improves release and prevent accumulation. Thereby, these can modify transport and circulation half-life which allow efficient passive and active targeting in delivering therapeutics (190). Some commonly used polymeric material for drug delivery are cellulose derivatives, poly (ethylene glycol) PEG, PLA: poly(lactide), poly-e-caprolactone (PCL), PLGA: poly (lactide-co-glycolide) and poly (N-vinyl pyrrolidone) PVP (190, 219). Polymer based drug delivery systems can be broadly divided as hydrogels (hydrophilic polymer networks able to trap large amounts of water or biological fluids), micelles, dendrimers, polyplexes, or polymer-drug conjugates (190).

1.7.1.4 Carbon nanotubes

Carbon nanotubes (CNT) – These are nano-sized carbon cylinders composed of benzene rings capable of drug encapsulation (265).These CNTs are either single-walled CNT; (SWNT) produced by rolling up a single layer of graphene sheet or multi-walled CNT; (MWNT) by rolling up many layers to form concentric cylinders (33). Functionalized-CNT (f-CNT) are created by linking active molecules such as, peptides, proteins, nucleic acids and other therapeutic agents (33). These f-CNT have the ability to penetrate into the cells and act as vehicles for the delivery of small drug molecules (237).

1.7.1.5 Biological nanoparticles

Biological nanoparticles – Bovine serum albumin (BSA) is one of the common biological material used for generation of nanoparticles in a drug delivery system (73, 252) . Gelatine is another material considered as a biological nanoparticle as the amino acid side-chains of the gelatin matrix molecule offer multiple modifications, making gelatin nanoparticles a great carrier for drug delivery intravenously with few surface modifications (286).

1.7.2 Safety of nanoparticle based delivery systems

Proper evaluation of the safety of a nanoparticle based drug delivery system is important before introducing it as a vehicle. As the field of NP based drug delivery is immense and composed of a large number of various materials, it is impossible to make generalized statements about the safety of all nanoparticles in general. Several nano-therapeutics are currently approved such as, PEGylated (polyethylene glycol bound), liposomal doxorubicin (Doxil®/Caelyx®), albumin-bound paclitaxel are considered safe with fewer side effects, while other nanoparticles (e.g. metallic and carbon-based particles) tend to show more toxic effects in certain conditions (334). Therefore, the potential toxicity of a nanoparticle based drug delivery system greatly depends on the actual composition of the nanoparticle formulation (73). In safety studies, the physicochemical features of a NP and the alterations that it may undergo in different environmental conditions should be considered. The size, surface, chemical composition, bioavailability, solubility, agglomeration, dissociation and adsorption of environmental substances are factors that may have an impact on the ultimate toxicity of the NPs (83). It has been reported that smaller particles (approximately 20 nm) were more penetrative into lung tissue with more acute inflammatory

reaction than larger ones (less than 200 nm) after intratracheal instillation in mice (230). Certain nanoparticles have caused reactive oxygen species generation, oxidative stress, mitochondrial perturbation, inflammation, uptake by reticulo-endothelial system, protein degradation/denaturation, brain/peripheral nervous system injury, DNA damage, endothelial dysfunction/blood clotting, and alternation of cell cycle [reviewed by (328)]. Metallic and carbon nanotube based drug delivery systems are believed to be associated with toxicities compared to lipid and polymer based systems. The toxicity of CNTs may vary depending on their structural characteristics, surface properties and chemical composition (322) However, f-CNTs are proven to be non-cytotoxic and preserve the functionality of primary immune cells (82). Pristine CNTs are considered highly toxic, mainly due to their insolubility, therefore it is important to verify the solubility of f-CNT in physiological media before used in drug delivery (33)

When NP based drug delivery systems are considered, scientists and drug developers should carefully study several aspects of a NP. In 2004, the US Food and Drug Administration (FDA) has introduced the critical path initiative with the intent of safe drug development (335). According to this report, three important points to be considered are safety, medical utility, and industrialization of a drug. In a NP based drug delivery system, the NP with its drug should be safe to be used in humans and animals, effective as a therapeutic agent and able to be produced in a large scale (328). A conceptual understanding of biological responses to nanomaterials is needed to develop and apply safe nanomaterials in drug delivery in the future (73).

1.7.3 Lipid/surfactant based CpG-ODN delivery

CpG-ODN is an effective immunostimulant. However, delivery of CpG-ODN alone to cells have come across some challenges with suboptimal in vivo stability, toxicity, unfavorable pharmacokinetic/ bio distribution characteristics, lack of specificity for target cells and the requirement for intracellular uptake. Because of this, the use of lipid-based NPs have been suggested to improve ODN stability, enhance ODN delivery to cells, improve cellular uptake by target cells and reduce toxicity (333). Many *in vivo* studies have proven that liposome-entrapped drugs and vaccines are better than conventional drugs, mainly in the areas of cancer chemotherapy, antimicrobial therapy, vaccines, diagnostic imaging and the treatment of ophthalmic disorders (110). As CpG-ODN is negatively charged, use of positively charged cationic lipids can efficiently encapsulate and improve the stability of CpG-ODN (207). The immune stimulation of CpG-ODN

could be enhanced by encapsulating them in lipid particles for efficient delivery to antigen presenting cells (222). There are many studies that have shown these enhanced effects of CpG-ODN when incorporated in to lipid based delivery systems (114, 189). The enhanced adjuvant effect of CpG-ODN was also reported in many studies when incorporated in to a lipid based delivery system. One study showed that pigs were protected significantly against pleuropneumonia infection when CpG-ODN was used as an adjuvant in a lipid based delivery system (12). Another study done in mice showed that liposomes co-encapsulating HCV NS3 (hepatitis C virus non-structural protein 3) and CpG is a good candidate vaccine which induce strong Th1 immune responses against hepatitis C viruses (149). Enhanced adjuvant and anti-tumor activity of CpG-ODN was presented in another study. They showed that encapsulation in liposomal nanoparticles specifically targeted CpG-ODN for uptake by immune cells which resulted in potent innate immunity as well as specific immunity (72). Liposomes are also effective delivery vehicles for CpG-ODN as an immune adjuvant for T cell independent (TI) antigens (188). A vaccine formulated with respiratory syncytial virus fusion protein, CpG-ODN and innate defense regulator peptide in polyphosphazene microparticles showed enhanced immune responses and protection in mice. The study also revealed that mice received this formulation developed significantly higher levels of virus-neutralizing antibodies in the sera and lungs, as well as higher numbers of IFNγ secreting cells than mice which received the protein alone (93).

1.7.4 Carbon nanotube based CpG-ODN delivery

Regardless of the well-known immune properties of CpG-ODN, delivery of CpG-ODN faces the challenge of low cellular uptake because of the negative charge of cell membranes, and often needs repeated administrations or high doses of ODN (224). DNA could be bound to cationic f-CNTs by electrostatic interactions between the phosphate backbone of DNA and the cationic functional groups. Therefore, cationic f-CNTs have been considered as an excellent candidate carrier to deliver genes (90). Bianco and group reported that cationic carbon nanotubes bound to CpG-ODN can enhance their immunostimulatory properties in vitro. They stated that f-CNT neutralizes negatively charged CpG-ODN reducing the repulsion by the negatively charged cell membrane and therefore, the cellular uptake of CpG-ODN is facilitated. Furthermore, they showed that f-CNT did not exert any mitogenic nor any toxic effect on activated or non-activated lymphocytes (34).

1.7.5 Enhanced CpG-ODN delivery to neonatal chickens

As described before CpG-ODN has shown promising immunostimulatory properties in chickens as an immune modulator and also as a vaccine adjuvant. However, NP based delivery of CpG-ODN to chickens have shown enhanced immune properties in several studies. In ovo delivery of CpG-ODN formulated with polyphosphazenes (PCPP-Poly [di (carboxylatophenoxy phosphazene)] and PCEP-Poly [di (sodium carboxylatoethylphenoxy) phosphazene]) has shown enhanced immunoprotective effect against an *E. coli* infection in neonatal broiler chickens. In the same study, birds that received CpG-ODN or CpG-ODN formulated with PCPP or PCEP has shown significantly higher survival rate compared to groups that received non-CpG-ODN or saline (302).

1.8 Hypothesis

The economic loss associated with neonatal chicken mortality due to infectious agents is very difficult to estimate as losses vary with the type of infectious agents and the degree of virulence. In general, if the first week mortality of chicks exceeds 2%, the overall performance of the flock will be very poor. Hence, it is important to devise strategies to curtail first week mortality. As prophylactic antibiotic use is withdrawn in the Canadian poultry industry, other methods of disease prevention and control are being researched. CpG-ODN have gained much attention and shown success in immune stimulation and providing protection against infections in many species (18, 38, 124, 182, 228, 229), including chickens (65, 105, 132). In chickens, immune stimulation is mostly required at the time of hatch when they are the most susceptible. Therefore, we hypothesize that the *in ovo* route is an ideal route of CpG-ODN administration to chicken embryos so that the immune system is ready at hatch providing protection against infectious disease related morbidity and mortality. In addition, understanding the immune protective mechanisms of CpG-ODN, optimizing and extending its bioavailability and duration of action will increase its therapeutic value in neonatal chickens. Furthermore, the effective delivery of CpG-ODN by *in ovo* route can be enhanced by using efficient drug delivery methods.

1.9 Objectives

1. To study the immune mechanisms of *in ovo* CpG-ODN in neonatal broiler chickens:
    a. Kinetics of cytokine gene expression involved in immune protective effect of CpG-ODN (multiplex assay for mRNA gene expression)
    b. Cellular mechanisms involved in immune protective effect of CpG-ODN (flo cytometry)
2. Evaluate CpG-ODN induced changes in immune cells in spleen and lung of neonatal chicken, and investigate if immune profiles correlate with the CpG-ODN induced protection against *E. coli* infection.
3. Study immune protective effects and safety of CpG-ODN formulated with CNT or lipid based delivery systems for *in ovo* delivery.

PREFACE TO CHAPTER 2

Immune stimulation in newly hatched chickens has become a main concern in the poultry industry. Since the immune system is still developing, young chickens are more susceptible to bacterial infections such as *E. coli* and *Salmonella* infections (131). CpG-ODN has been studied and proved to be an ideal candidate for immune stimulation in chickens (104, 132, 301, 302). Moreover, *in ovo* delivery of CpG-ODN has shown promising results in immune protection in neonatal chickens (65, 104, 202). Though several studies have investigated the mechanism of action of CpG-ODN as an immune protective agent in chickens (240, 338), detailed studies on cellular changes are scarce. In both innate and adaptive immunity, immune cells plays the main role in successful pathogen suppression and elimination. APCs like, macrophages and DCs identify pathogens via PRR, process and present antigens to T cells. Once lymphocytes are stimulated, necessary adaptive immune responses takes place to fight the pathogen (273). As CpG-ODN act as a PAMP, which can be identified by PRR (TLR9 and TLR21 in chickens) the preparation of the immune system can be achieved even without a real pathogen invasion (319). Studies have shown the cellular changes caused by CpG-ODN in different species, such as mice (119, 174) and humans (116, 307). However, studies on CpG-ODN mechanism in chickens are exiguous. CpG-induced intracellular *Salmonella* Enteritidis bacterial killing was demonstrated in a chicken macrophage cell line by Xie *et al.* 2003 (338). Meanwhile, another group reported similar findings in the same macrophage cell line and peripheral blood mononuclear cells (PBMCs). In addition they evaluated the ability of CpG-ODN to produce cytokines (IL1β, IFN-γ) in these cells (130). *In vivo* studies on cellular changes in chickens, due to CpG-ODN are not studied extensively so far. In the following chapter (Chapter 2) we discuss the cellular changes, cytokines involved and expression of cellular markers on immune cells following *in ovo* CpG-ODN administration to chicken embryos in detail. Flow cytometric analysis of macrophage/monocyte cell population, their maturation markers and T lymphocyte populations will be described. In addition, cytokine gene expression, which was analysed by QuantiGene plex assay will be explained.

CHAPTER 2: SYNTHETIC CpG-ODN RAPIDLY ENRICHES IMMUNE COMPARTMENTS IN NEONATAL CHICKS TO INDUCE PROTECTIVE IMMUNITY AGAINST BACTERIAL INFECTION

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This work has been submitted to *Scientific Reports* journal.
"Synthetic CpG-ODN rapidly enriches immune compartments in neonatal chicks to induce protective immunity against bacterial infection". Thushari Gunawardana, Khawaja Ashfaque Ahmed, Kalhari Goonewardene, Shelly Popowich, Shanika Kurukulasuriya, Ruwani Karunarathana, Ashish Gupta, Betty Chow-Lockerbie, Marianna Foldvari, Suresh Tikoo, Philip Willson, and Susantha Gomis

2.1 Abstract

CpG-ODN are known to induce innate immunity and protection against bacterial infections. Despite recent advances, how CpG-ODN alone protects against bacterial infections remained elusive. Here, we report that CpG-ODN regulates the enrichment of immune compartments in chickens to orchestrate protective immunity against pathogenic insults. In this study, eighteen-day-old embryonated eggs were injected with either 50 µg of CpG-ODN or saline (n≈90 per group for three experiments). In the first experiment, four days after CpG-ODN treatment, chicks were challenged subcutaneously with either 1×10^4 or 1×10^5 colony forming units (cfu) of a virulent strain of *E. coli* and mortality was monitored for 8 days. We found significant protection, and reduced clinical scores in CpG-ODN treated chicks. To gain insights into mechanisms of protection induced by CpG-ODN, first we investigated cytokine expression kinetics elicited by CpG-ODN. The expression profiles were examined using QuantiGene Plex 2.0®, which employs probes to detect mRNA of multiple target genes in a single assay. The spleen and lung were collected from three embryos or chicks per group at 10 time points post-CpG-ODN inoculation. Multiplex gene analysis (IL18, IFNα, IFNγ, IL1β, Litaf, IL4, IL6, and IL10) revealed a significantly higher expression of pro-inflammatory cytokines following CpG-ODN treatment compared to the saline controls. In our study, Litaf stands out in the cytokine profiles of spleen and lungs, underscoring its role in CpG-ODN-induced protection. The third experiment was designed to examine the effects of CpG-ODN on immune cell populations in various organs. We collected spleen, lungs, and thymus from embryos or chicks (n=3 or 4 per group) at 24, 48 and 72 hours (thymus only collected at 72 hours) after CpG-ODN administration. Flow cytometry analysis was conducted to examine the changes in CD4+ and CD8+ T-cell subsets, monocyte/macrophage cell populations and their expression of maturation markers (CD40 and CD86). Flow cytometry data indicated a significant enrichment of macrophages, CD4+ and CD8+ T-cell subsets in both spleen and lungs of CpG-ODN treated embryos and chicks. Macrophages in spleen and lungs showed an upregulation of CD40 but not CD86, whereas thymocytes revealed significantly high CD4 and CD8 expression. Overall, the present study has demonstrated for the first time that in ovo delivery of CpG-ODN provides protection in neonatal chicks against *E. coli* infection not only by eliciting cytokine responses and stimulating immune cells but also through enriching immunological niches in spleen and lungs.

2.2 Introduction

CpG-ODNs are short segments of DNA representing a type of PAMP that stimulate immune cells and trigger immune responses to infections (61, 115, 158, 198, 233). CpG-ODNs act as immunostimulatory agents in many vertebral species including mice (49), human (1, 213, 330), fish (151), cattle and sheep (228), and chickens (63, 104, 301). CpG-ODNs are recognized by TLR9 and TLR21 in mammals and avians, respectively. Human TLR9 and avian TLR21 are very similar in CpG-ODN motif recognition; and both respond to CpG-ODNs containing GTCGTT motifs, whereas murine but not human TLR9 recognizes GACGTT motifs (127, 343). Human TLR9 and avian TLR21 have similar intracellular localization, signaling cascades, immune activation, and cytokine induction (40, 41, 159, 343). Studies reported that CpG-ODNs support innate and adaptive immunity by activating immune cells and inducing cytokine secretion (123). CpG-ODNs have great promise as vaccine adjuvants and immunotherapeutic agents against infectious diseases and cancer (36, 123, 283, 349).

Our lab has provided the first *in vivo* evidence for an immunoprotective effect of CpG-ODN against bacterial infection in chickens (105). We demonstrated protection against *E. coli* infection in chickens administered CpG-ODN via SC or IM (105) or *in ovo* routes (104). We recently reported that intrapulmonary delivery of CpG-ODN micro-droplets could also protect neonatal chicks against *E. coli* septicemia in a dose-dependent manner (106). The immunoprotective effect of CpG-ODN in chickens was further supported by the studies demonstrating protection against *Salmonella* Enteritidis infection following CpG-ODN administration via intraperitoneal (131) or *in ovo* (202) routes. We also demonstrated protective effects of CpG-ODN against *Salmonella* Typhimurium infection (301). The immune protective effect of CpG-ODN can further be improved by formulating it with nanoparticles (112, 302, 304). CpG-ODN was shown to stimulate strong induction of IL6 and nitric oxide secretion in chicken macrophage cell line (HD11 cells) leading to an increased intracellular killing of *Salmonella* Enteritidis in the activated HD11 cells (338). CpG-ODN can activate several signaling pathways including protein kinase C (PKC), NF-kappaB and mitogen-activated protein kinases (p38 MAPK and MEK1/2) (129). Another study reported a significant increase in the heterophil degranulation and oxidative burst following an intraperitoneal administration of CpG-ODN that caused an enhanced resistance to *Salmonella* Enteritidis infection in neonatal chickens (132). The resistance to intracellular bacterial infection may be the result of a Th1 biased immune response in chickens

that was recently reported to be elicited by CpG-ODN (240). Several recent studies showed enhanced expression of cytokines and chemokines in response to CpG-ODN in chickens (66, 304). Despite these advances, the mechanisms by which CpG-ODN alone provides protection in chickens against bacterial infections are not completely understood. A greater understanding of cellular and molecular mechanisms for CpG-ODN induced antimicrobial immunity will help in harnessing the full therapeutic potential of CpG-ODNs against diseases.

In the present study, we investigated immunity induced by *in ovo* administered CpG-ODN including response to *E. coli* challenge, cytokine kinetics by using QuantiGene Plex (multiplex assay for mRNA gene expression), and effects on the immunological niches in spleen and lungs using flow cytometry.

2.3 Materials and methods

2.3.1 Synthetic CpG-ODN

The sequence of CpG-ODN (class B CpG 2007) used was 5'-TCGTCGTTGTCGTTTTGTCGTT- 3'. ODNs were produced with a phosphorothioate backbone (Operon Biotechnologies, Inc. Huntsville, AL).

2.3.2 Bacteria

The bacterial strain for challenge was obtained from a field isolate of *E. coli* from a turkey with septicemia as described in previous studies (104, 105). This *E. coli* was serogroup O2, non-hemolytic, serum-resistant, produced aerobactin, with a K1 capsule and Type 1 pili. Aliquots of bacteria were stored at -80 C in 50% brain heart infusion broth (BHI; Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific, Inc., Montreal, Quebec). Bacteria used for challenge were cultured on Columbia sheep blood agar plates for 18-24 hr at 37 C. One colony was added to 100 mL of Luria broth in a 250 mL Erlenmeyer flask. The culture was grown at 37 C for 16 - 18 hr with shaking at 150 rpm. Stationary phase culture contained approximately 1×10^9 cfu of bacteria per mL. The cultures were further diluted in sterile saline so the concentration of bacteria required for challenge (1×10^5 or 1×10^4 cfu/bird) was obtained. Viable bacterial counts were determined by plating serial dilutions of the diluted culture in duplicate on Columbia sheep blood agar plates, incubating for 18-24 hrs at 37 C; then counting the number of colonies.

2.3.3 Chicken embryos

For all experiments **(***E. coli* challenge study ,gene expression and flow cytometry analysis), fertilized hatching eggs were obtained from a commercial broiler breeder operation in Saskatchewan, Canada. Eggs were incubated until hatch at the Animal Care Unit (ACU) at the Western College of Veterinary Medicine, University of Saskatchewan. This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.3.4 Animal model for *E. coli* challenge

Hatched chicks were allocated into an animal isolation room at the ACU. An identification tag was placed on their neck in order to identify birds that received *in ovo* CpG-ODN or saline. Water and commercial broiler ration were provided *ad libitum* throughout the trial. The animal room was ventilated with filtered, non-recirculated air at a rate of 10–12 changes/hr. In addition, air pressure differentials and strict sanitation were maintained in this isolation facility. One day post-hatch, the birds received either 1×10^4 or 1×10^5 cfu of stationary - phase *E. coli*, in a total volume of 250 µL per bird, by subcutaneous injection in the neck, resulting in *E. coli* septicemia. In this model, *E. coli* septicemia with airsacculitis, pericarditis, perihepatitis or polyserositis develops in 60%–90% of birds that are not protected by treatment intervention. The two different doses of *E. coli* were given to groups of birds to simulate field conditions where all birds are not exposed to a consistent dose of *E. coli* in a barn. Birds were assessed three times daily at the critical stage (first four days post challenge) and twice thereafter up to 8 days post challenge. Birds were observed for clinical signs and each individual bird was assigned a daily clinical score: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1= depressed, reluctant to move; 1.5 = reluctant to move, may take a drink and peck some; 2 = unable to stand or reach food or water; and 3 = found dead. Birds that received a clinical score of 2 were humanely euthanatized by cervical dislocation. Chicks that were found dead or euthanatized were necropsied immediately. On day 8 post *E. coli* challenge, the remaining birds were euthanatized by cervical dislocation. Bacterial swabs were taken from the air sacs and cultured on Columbia sheep blood agar using a typical method of inoculation and streaking on four quadrants of the plate of medium. A semi-quantitative estimate of *E. coli* isolation was conducted on Columbia sheep blood agar. Growth on these plates were recorded on a scale from 0 to 4+, where 0 = no growth; 1+ = growth of bacteria on the area

1; 2+ = growth of the bacteria on areas 1 and 2; 3+ = growth of bacteria on areas 1, 2, and 3; and 4+ = growth of bacteria on areas 1, 2, 3, and 4.

2.3.5 Cells for flow cytometry

Cell preparation and antibody staining for flow cytometry was done as previously described with some modifications (6, 113, 179). Briefly, spleen, lung and thymus tissues were collected at 24, 48 and 72 hr post *in ovo* injection from chicken embryos and processed for cell isolation (thymus only at 72 hr). Each spleen was gently pushed through a metal strainer by manual pressure to obtain a single cell suspension with ~3 mL of phosphate buffered saline (PBS) and collected in a 15 mL centrifuge tube. For lung and thymus, each tissue was manually dissected and incubated with ~1 mL of collagenase (1 mg/mL) dissolved in Dulbecco's Modified Eagle Medium for 30 min in 37 C. After incubation these tissues were pushed through a metal strainer to obtain a single cell suspension and washed twice with PBS. Then all spleen, lung and thymus cells were incubated with red blood cell lysis buffer to lyse red blood cells. Following three washes with wash buffer (PBS containing 2% fetal bovine serum and 0.1% sodium azide) cells were used for the flow cytometry after staining with appropriate antibodies.

2.3.6 Antibodies for flow cytometry

Monoclonal antibodies against chicken monocyte/ macrophages (mouse anti-chicken monocyte/ macrophages KUL01-PE), CD4 (mouse anti-chicken CD4-PE) and CD8 (mouse anti-chicken CD8α-FITC) were purchased from Southern Biotechnology (Birmingham, Ala, USA). Mouse anti-chicken CD40 and mouse anti-chicken CD86 monoclonal antibodies were used as primary antibodies (purchased from Bio-Rad, Raleigh, NC, USA). Anti-mouse-FITC IgG antibody was used as secondary antibody. Goat anti-mouse IgG, Streptavidin-PerCP/Cy5.5 and Mouse IgG1 isotype control were purchased from Bio Legend (San Diego, CA, USA).

2.3.7 Experimental design

2.3.7.1 Delivery of CpG-ODN by the *in ovo* route

Embryonated eggs that were 18-days old were injected with 50 µg of synthetic CpG-ODN dissolved in sterile pyrogen-free saline, in a total volume of 100µL/egg (n≈90 for all three experiments) or 100 µL of sterile saline (n≈90). The air cell side of each egg was cleaned with an

alcohol wipe and injections were administered into the amniotic cavity through the air cell using a 22 gauge, 1 inch hypodermic needle. Following injections, a drop of melted wax was placed on the pore created to seal the egg. All eggs were then transferred to the incubator until hatch or taken for tissue sample collection.

2.3.7.2 *E. coli* challenge

Four days after *in ovo* injections (day-1 post-hatch), either 1×10^4 or 1×10^5 cfu of a virulent strain of *E. coli* was inoculated subcutaneously in the neck in all remaining (after sample collection for flow cytometry and gene analysis) hatched birds [bird numbers per group; CpG-ODN 50 µg: n = 32; saline: n=36]. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for 8 days following challenge with *E. coli*.

2.3.7.3 Sample collection.

For QuantiGene Plex assay- spleen and lung samples from three embryos or newly hatched chicks per group were collected at each of 10 time points post CpG-ODN inoculation (0, 3, 6, 12, 24, 32, 48, 72 hr, day 5 and day 7) into 1.5 mL tubes and flash frozen in dry ice until stored in -80 C.

For flow cytometry-three embryos or newly hatched chicks from each group were humanely euthanized at 24, 48 and 72 hr post *in ovo* injections by cervical dislocation and necropsied for spleen, lung and thymus tissue collection (thymus only at 72 hr post *in ovo* injections).

2.3.7.4 Gene expression analysis

The mRNA gene expression of IFNα, IL18, IFNγ, IL1β, Litaf, IL4, IL6 and IL10 cytokine genes in the spleen and lung were measured by commercially available probes for avian cytokines (Panomics /Affymetrix Inc., Fremont, CA, USA) by QuantiGene Plex 2.0® technique. The genes of interest and their accession numbers are listed in Table 2-1.

Table 2-1: Genes of interest. (* housekeeping genes- HKGs)

Gene	Gene name	Accession number
IL18	Interleukin 18	NM_204608
IL4	Interleukin 4	NM_001007079
IFNα	Interferon alpha	NM_205427
IL10	Interleukin 10	NM_001004414
IFNγ	Interferon gamma	NM_205149
IL1β	Interleukin 1, beta	NM_204524
Hprt 1*	Hypoxanthine-guanine phosphoribosyl transferase 1	NM_204848
Litaf	Lipopolysaccharide-induced TNF factor-alpha factor	NM_204267
Tubb 1*	Tubulin, beta 1	NM_205445
IL6	Interleukin 6	NM_204628

Frozen spleen and lung tissues were processed and tissue homogenates were prepared following the manufacturer's instructions with some modifications. Briefly, 5 mg of tissue was excised, manually broken down and added to 300 µL of homogenization solution and 3µL of proteinase K. The tissue lysate was digested at 65 C to release the mRNA and centrifuged to precipitate the debris. The supernatant of tissue lysates were collected and stored in -80 C if not used the same day. The oligonucleotide capture probes for mRNA and label probes were designed by the manufacturer as requested. The tissue homogenates were added to a 96-well plate (40 µL/well) that was pre-loaded with 20 mL of the capture reagent and the respective probe set. After overnight hybridization at 54±1 C, hybridizations with bDNA pre-amplifier 2.0, bDNA amplifier 2.0, biotinylated label probe and finally substrate were subsequently carried out according to the manufacturer's instructions. Luminescence was quantified using a Luminex instrument (Bio-Rad, USA). Signals, which is the mean fluorescence intensity (MFI) generated from each bead, are proportional to the amount of each mRNA captured on the surface of each generated specific probe set. (348). The expressions of these genes were normalized with the gene expression of hypoxanthine-guanine phosphoribosyl transferase 1 (Hprt 1) and tubulin beta 1 (Tubb1) genes which were used as housekeeping genes.

2.3.7.5 Flow cytometry

The lung and spleen cell populations were stained for the presence of monocyte/macrophages, $CD4^+$ and $CD8^+$ T cells. The monocyte/macrophages were further analysed for the expression of maturation markers (CD40 and CD86). Briefly, ~$5x10^5$ cells were incubated with mouse anti-chicken monocyte/macrophage phycoerythrin (PE) antibody at 4 C for 30 min for detecting APCs. For checking maturation state of monocyte/macrophages, the cells from previous step were washed three times and incubated with either mouse anti-chicken CD40 or CD86 primary antibodies separately at 4 C for 30 min. After three washings with PBS, the cells were stained with PerCP/Cy5.5 goat anti-mouse IgG secondary antibody at 4 C for 30 min. Another set of ~$5x10^5$ cells were also incubated with mouse anti-chicken CD8 (FITC) and CD4 (PE) together at 4 C for 30 minutes to determined $CD4^+$ and $CD8^+$ T cells. Lastly, the washed cells were suspended in 300 µL buffer in flow tubes and processed for flow cytometric analysis. Flow cytometry data were acquired by Epics XL (Beckman Coulter) and FACS Caliber (BD Bioscience), and data were analyzed with FlowJo software (Tree Star).

2.3.7.6 Statistical analysis

Survival pattern, cumulative clinical scores (CCS), bacterial percentages and cell populations from flow cytometry analysis were graphed and analyzed with the use of Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) with a significance level of $P<0.05$. The survival patterns were compared using the log-rank test and chi-square statistic. For calculating CCS, the clinical score for each bird was summed over the 8 day observation period and the significance of differences among groups were tested with Kruskal-Wallis nonparametric analysis of variance. Dunnett's test following ANOVA testing was used to test for significant differences of gene expression between the CpG-ODN group and the saline control group at each time point. For testing difference of APC percentages, CD4+ and CD8+ expression between groups, we used a two way ANOVA followed with Bonferroni post-test and Student-t test with Welch's correction for unequal variance, with a significant difference of $P<0.05$.

2.4 Results

2.4.1 *E. coli* challenge

Survival following *E. coli* challenge was significantly higher in groups of birds that received 50 µg of CpG-ODN compared to the saline control group (P 0.04). (Figure 2-1 A). The CCS of chicks following *E. coli* challenge with either 1×10^4 or $1x10^5$ cfu of *E. coli* showed that the chicks that received 50 µg CpG-ODN had a significantly lower CCS compared to the saline group (P=0.03). (Figure 2-1B). Samples collected from the challenged chicks revealed heavy bacterial growth more frequently in samples from chicks in the control group than in the CpG-ODN group (Figure 2-1C). Moreover, the reduction in the relative risk of mortality following *E. coli* challenge was 46.4% compared to the saline control group. The data of groups which received 1×10^4 and 1×10^5 cfu of *E. coli* were combined for clarity of presentation and analysis.

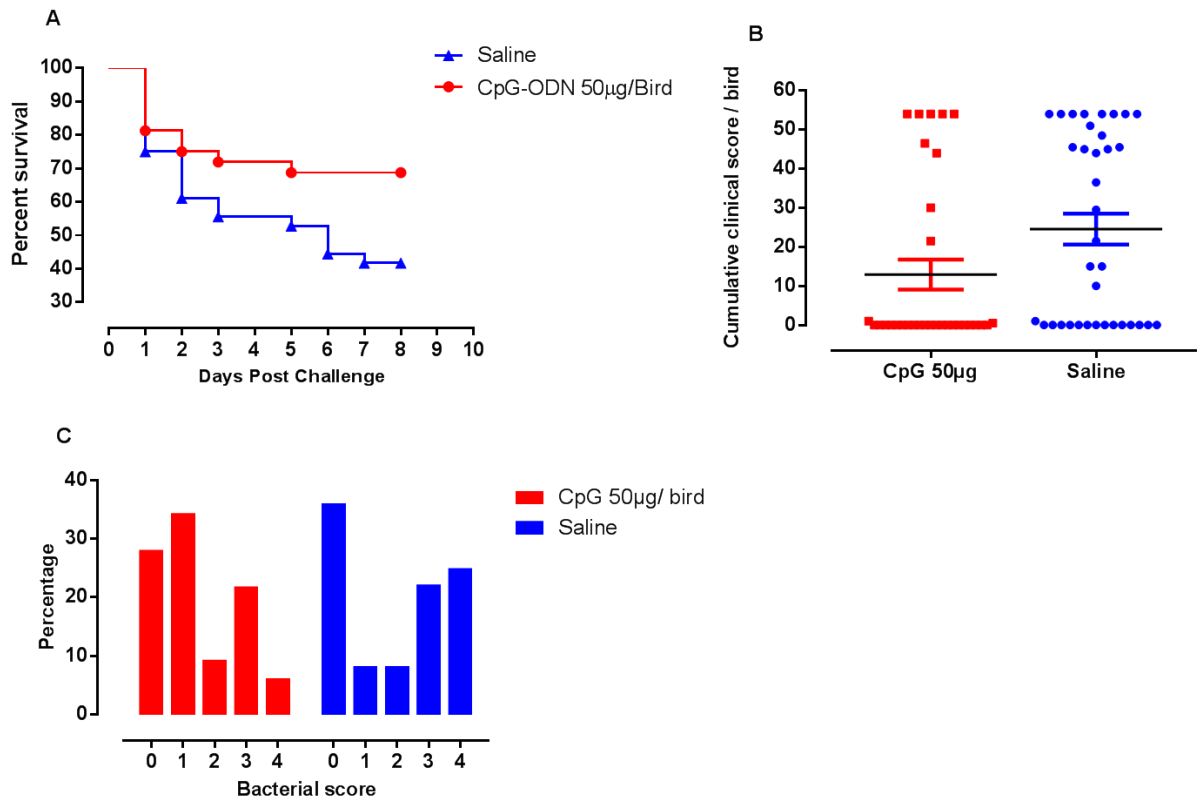

Figure 2-1: Survival percentages, CCS and bacterial scores. (A) Survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day 18 of incubation were injected with 50µg of CpG-ODN or saline by the in ovo route (n = 32 CpG-ODN, n= 36 saline group). Birds that received CpG-ODN were significantly protected against *E. coli* infection compared to the saline control group (P= 0.04). **(B)** CCS of broiler chickens following *E. coli* challenge with either 1×10^4 or 1×10^5 cfu of *E. coli*. Groups of birds that received 50 µg CpG-ODN CpG-ODN had a significantly lower CCS compared to the saline group, (P=0.03). Bar = mean. **(C)** Heavy bacterial growth was observed more frequently in lesions from birds in the saline group than in the CpG-ODN group. The substantial number of '0' or 'no growth' of bacteria in both groups is a common finding in such experiment**.**

2.4.2 Gene expression analysis

The mechanism of action of CpG-ODN in these chicks was also elucidated by measuring gene expression patterns of pro-inflammatory (IL1β, IL6, Litaf, IL18), Th1 type (IFNγ, IFNα) and Th2 type (IL4, IL10) cytokines in spleen and lungs at several hours post CpG-ODN treatment. Fold changes of mRNA levels were 1 or >1 at almost all time points in birds of CpG-ODN treated group compared to the saline control group. Levels of gene expression were not consistent nor followed a specific pattern over time. However, most significant differences in gene expression

occurred in the 32 to 48 hour period after treatment. Normalized gene expression of pro-inflammatory cytokines (IL18, IL1β and IL6 and Litaf) tended to show a relatively higher level in CpG-ODN treated group compared saline treated. The expression of IL1β and IL6 in the lungs of chickens was many fold higher compared to spleen at 6 through 48 hours and 12 through 48 hours, respectively. IL18 and Litaf expression mainly increased between 12 through 72 hours and 32 through 72 hours, respectively in both organs. (Figure 2-2). The gene expression levels of Th1 type cytokines (IFNγ, IFNα) were higher in lungs compared to spleens in CpG-ODN treated birds. Similarly, Th2 type cytokine (IL4, IL10) gene expression was higher in lung compared to spleen in CpG-ODN treated birds. (Figure 2-3).

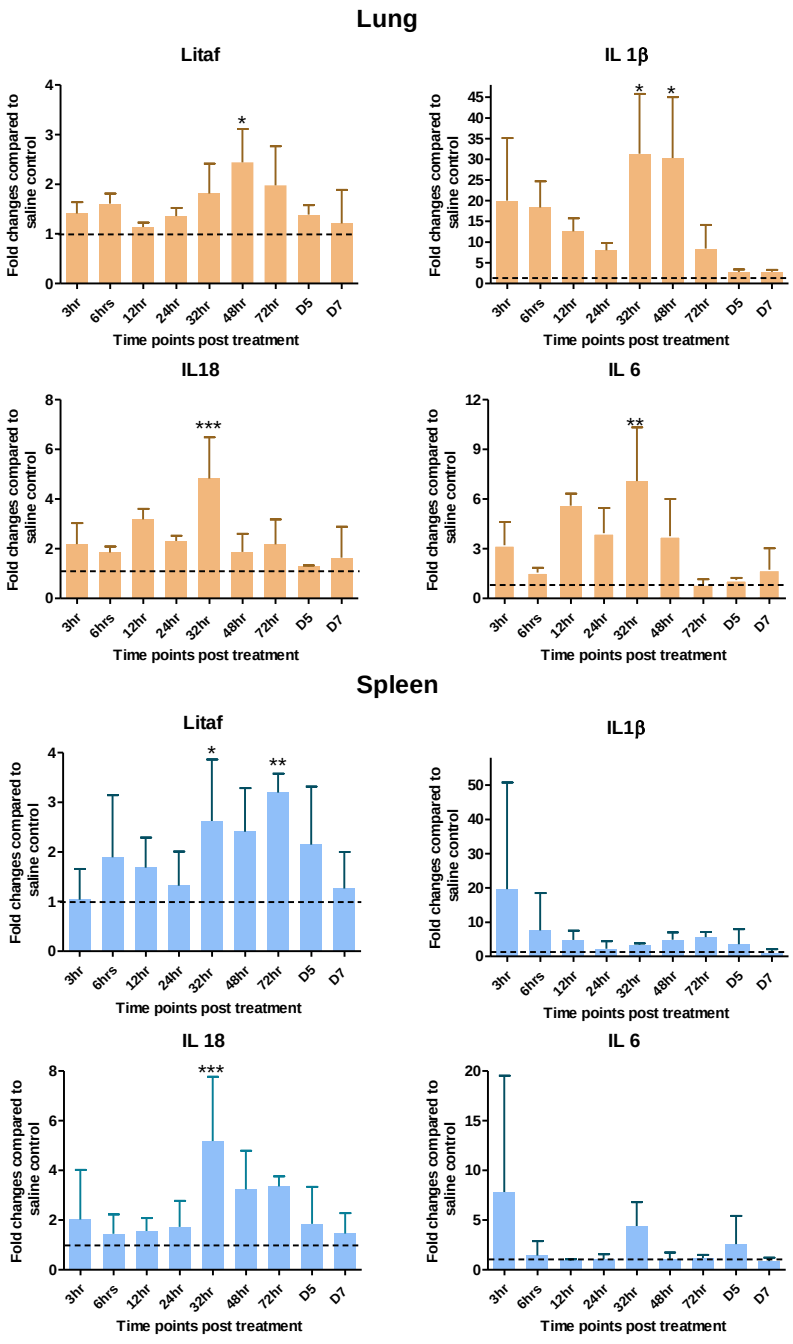

Figure 2-2: Pro-inflammatory cytokine profiles in lungs and spleen. MFI of each gene was normalized to the average of house keeping genes (HKGs - Hprt 1 and Tubb 1), then fold changes were calculated against the saline control at each time point and the means were graphed. (Dotted line shows a fold change of one, which indicates no change compared to the control group and the vertical line and horizontal bar show the standard error of mean-SEM). Dunnett's test following ANOVA testing was used to test for significant differences of gene expression between CpG-ODN group and the saline control group at each time point. Asterisks indicate groups that were significantly different from control group, * = P<0.05, ** = P<0.01 and *** = P<0.001.

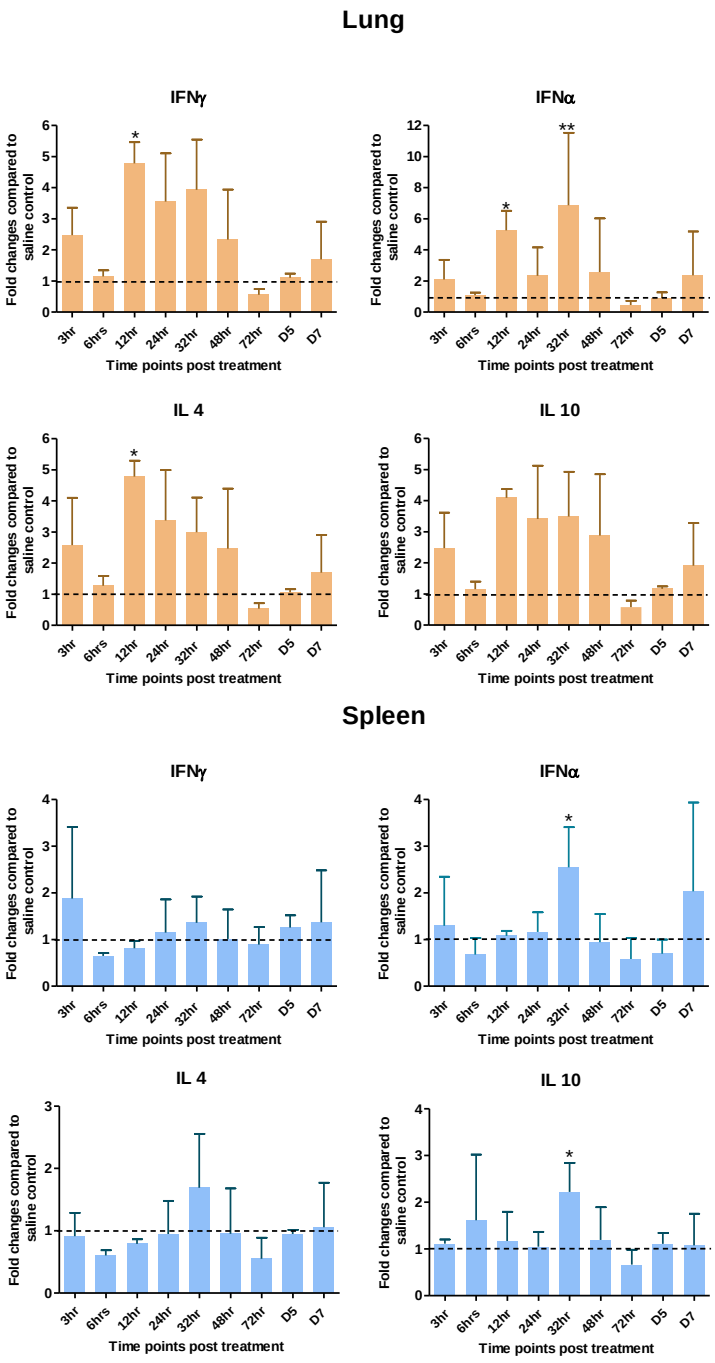

Figure 2-3: Interferon and regulatory cytokine profile in lung and spleen. MFI of each gene was normalized to the average of HKGs – (Hprt 1 and Tubb 1), then fold changes were calculated against the saline control at each time point and the means were graphed. (Dotted line shows a fold change of one, which indicates no change compared to the control group and the vertical line and horizontal bar show the standard error of mean-SEM). Dunnett's test following ANOVA testing was used to test for significant differences of gene expression between CpG-ODN group and the saline control group at each time point. Asterisks indicate groups that were significantly different from control group, * = $P<0.05$ and ** = $P<0.01$.

2.4.3 Flow cytometry analysis

Flow cytometric analysis of spleen and lung cells isolated from birds of saline control and CpG-ODN treated groups showed a marked influence of CpG-ODN on APCs (monocyte/macrophage), expression of costimulatory markers on APCs and T lymphocyte populations.

2.4.3.1 Antigen presenting cells

The overall cell number was low at 24 hours post treatment, where the chicken embryos were 19 days old. Therefore, we could only obtain and analyse spleen cells at this time point. CpG-ODN treatment caused a notable increase in the number of APCs (monocyte / macrophage cells) and the expression of costimulatory molecules (CD40) in spleen and lungs compared to saline treated embryos. The percentage of APCs in CpG-ODN treated embryos in spleen at 24, 48 and 72 hours post treatment were 5.88, 12.6 and 11.2 %, respectively (Figure 2-4 A, B and C). Whereas, lower APC percentages were observed in the saline treated group. A considerably higher expression of CD40 costimulatory molecules was present on APCs from CpG-ODN treated embryos compared to saline treated embryos and it was indicated by a shift in the MFI to the right (Figure 2-4). Even though there was higher expression of CD86 in CpG-ODN group compared to the saline group, no significant difference was observed at any of the time points.

A similar trend was observed in the percentage of APCs in the lungs of CpG-ODN treated embryos compared to saline treated embryos at 48 and 72 hours post *in ovo* injections. The percentage of APC in the lungs of CpG-ODN treated embryos at 48 and 72 hours post treatment were 1.73 and 1.74% whereas the percentage of APCs in saline treated birds at 48 and 72 hours were 0.03 and 0.17 % respectively (Figure 2-5 A and B). CD40 expression was significantly higher in APC in lungs of CpG-ODN treated embryos compared to saline and was indicated by a shift in the MFI to the right. Similar to the spleen, no increase in the expression of CD86 costimulatory molecule was observed in APCs in lungs.

This striking enrichment of immune cells in the CpG-ODN treated group was noticed at all three time points (24, 48 and 72 hours post CpG-ODN inoculation) in both spleen and lung.

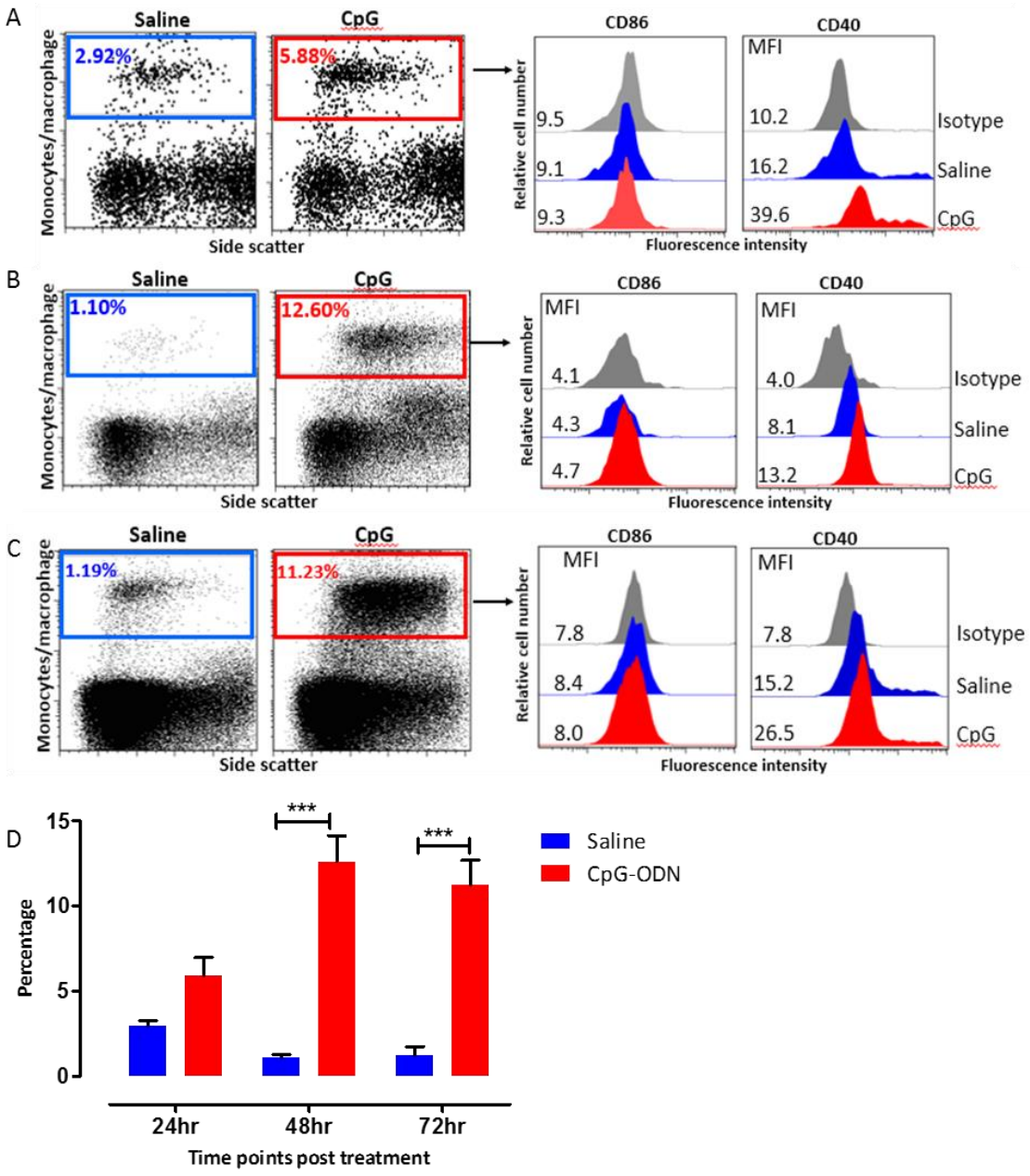

Figure 2-4: Flow cytometic analysis of spleen cells 24hr **(A)**, 48hr **(B)** and 72hr **(C)** post *in ovo* injections (day 19, 20 and 21 embryos). Monocyte/macrophage APC populations were gated based on forward and side scatter (left). Histogram panels on right indicate the level of CD86 and CD40 (costimulatory molecules found on APCs) expression on the APCs. **(D)** Bar diagram show the mean percentages of monocyte/macrophage APCs following *in ovo* treatment in spleen. vertical line and horizontal bar show the standard error of mean SEM, n=3. Two way ANOVA following Bonferroni post-test was done to compare means of APC percentages of CpG-ODN received groups with saline control. Asterisks indicate significant differeces between the groups, ***=P<0.001.

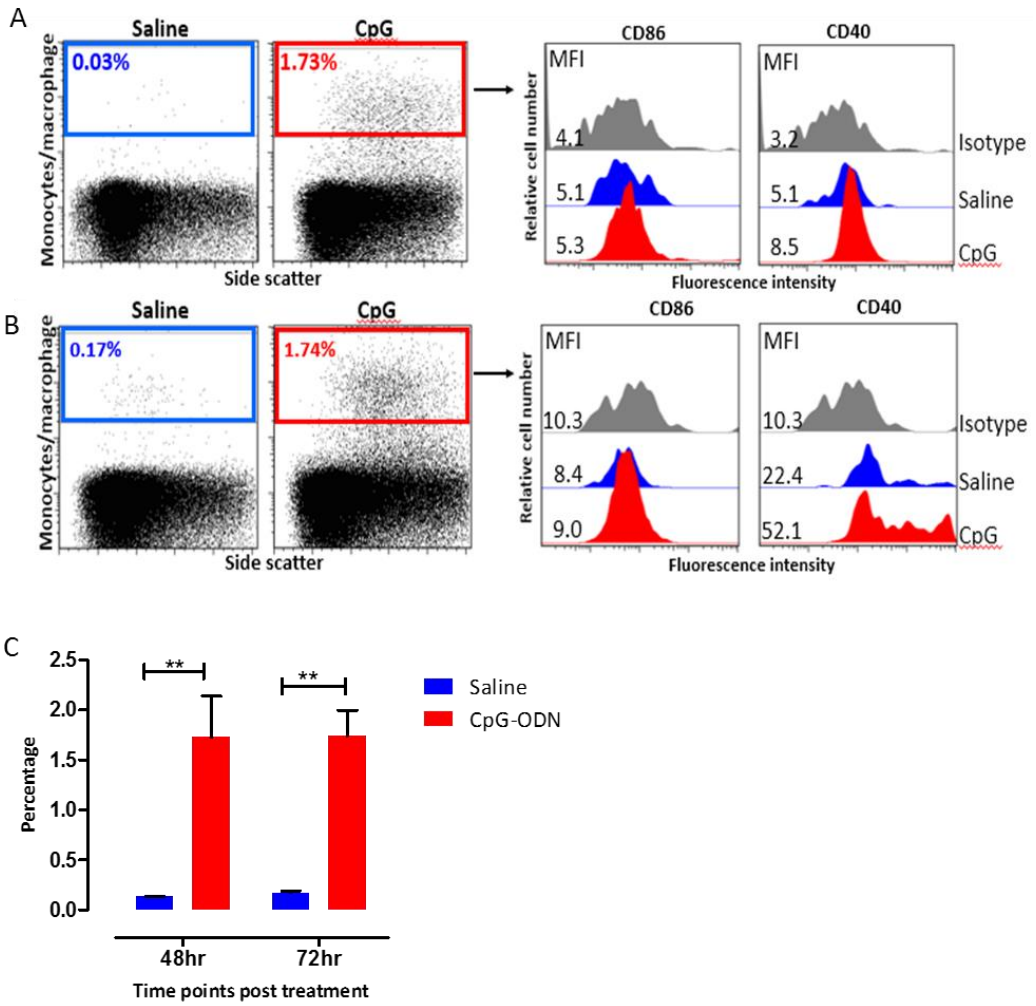

Figure 2-5: Flow cytometric analysis of lung cells. 48 **(A)** and 72 hr **(B)** post *in ovo* injections (day 20 and 21 embryos). Monocyte/macrophage APC populations are gated based on forward and side scatter (left). Histogram panels on right indicates the level of CD86 and CD40 (costimulatory molecules found on APCs) expression on the APCs. **(C)** Bar diagram show the mean percentages of monocyte/macrophage APCs following *in ovo* treatment in lung. Vertical line and horizontal bar show the standard error of mean SEM, n=3. Two way ANOVA following Bonferroni post-test was done to compare means of APC percentages of CpG-ODN received groups with saline control. Asterisks indicate significant differeces between the groups, **=P<0.01.

2.4.3.2 CD8$^+$ and CD4$^+$ T cells

CD8$^+$ and CD4$^+$ T cell populations were analysed in spleen and lung at 72 hrs post CpG-ODN injections (21 day old embryos). Both CD8$^+$ and CD4$^+$ cells were markedly increased in spleen and lung of CpG-ODN treated birds compared to the saline control. The percentage of CD4 and CD8 T cells in spleen of CpG-ODN treated embryos were 1.89% and 1.09%, respectively

compared to 0.22% $CD4^+$ T cells and 0.23% $CD8^+$ T cells in saline control (Figure 2-6A). Similarly, the percentage of CD4+ and CD8+ T cells in lungs of CpG treated embryos were 0.67% and 6.01%, respectively compared to 0.43% CD4+ T cells and 0.79% CD8+ T cells in saline control (Figure 2-6B). Interestingly, while the percentage of CD4+ T cells was significantly higher in CpG-ODN group compared to saline in the spleen (Figure 2-6C), the percentage of CD8+ T cell increase was more prominent in lungs than CD4+ T cells. (Figure 2-6D)

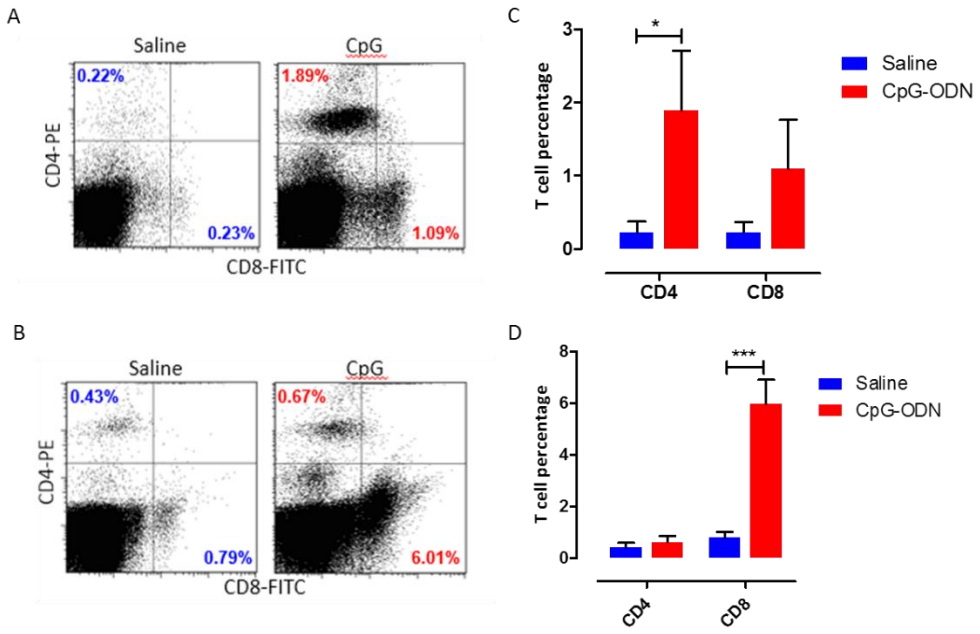

Figure 2-6: Flow cytometric analysis of spleen and lung, T cell populations in a day 21 embryo (72 hours post CpG-ODN /saline injection). (A) spleen and **(B)** lung. T lymphocytes are gated based on forward and side scatter. CD4+ and CD8+ T-cells were quantified using PE-labelled mouse anti-chicken CD4 and FITC-labelled mouse anti-chicken CD8 monoclonal antibodies. The bar diagrams **(C)** spleen and **(D)** lung show the mean percentages of CD4+ T-cells and CD8+ T-cells 72hrs following *in ovo* treatment. Vertical line and horizontal bar show the standard error of mean SEM, n=3. Two way ANOVA following Bonferroni post-test was done to compare means of T cell percentages of CpG-ODN received groups with saline control. Asterisks indicate significant differences between the groups, *= P<0.05, ***=P<0.001.

Moreover, to gain insight whether increase in number of CD4+ or CD8+ is due to increased differentiation of CD4+ or CD8+ T cells in thymus we looked at thymus cells. We found that the T cell population in thymus was remarkable, where 99% of total thymus cells were CD4+/CD8+ double positive at the time of hatch with CpG-ODN treatment. Whereas, in saline control it was only 85%. Both CD4+ and CD8+ expression levels were significantly higher with CpG-ODN than the saline control group. (Figure 2-7).

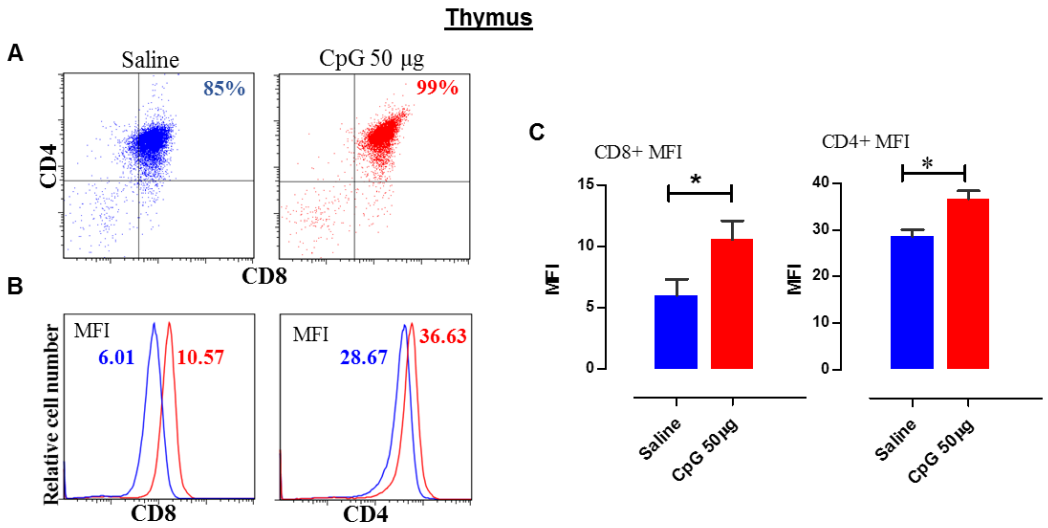

Figure 2-7: T lymphocytes in thymus. Flow cytometric analysis of thymus T cell populations of the saline control and 50 µg CpG-ODN group (n=3/group) at 72 hours post *in ovo* injections (day 21 embryo). **(A)** T lymphocytes are gated based on forward and side scatter. $CD4^+$ and $CD8^+$ T-cells were quantified using PE-labeled mouse anti-chicken CD4 and FITC-labelled mouse anti-chicken CD8 monoclonal antibodies. **(B)** Histogram panels indicate the levels of $CD4^+$ and $CD8^+$ expression on the T cells (the MFI shown in blue indicates saline treated group, and in red indicates the CpG-ODN treated group). **(C)** Bar diagrams show the MFI of $CD4^+$ and $CD8^+$ expression. Difference of MFI between the two groups were compared using Student-t test with Welch's correction for unequal variance. n=3, * = $P<0.05$.

2.5 Discussion

There is strong evidence to suggest that CpG-ODN administration modulates innate and adaptive immune responses in chickens enhancing resistance to several bacterial pathogens including *E. coli* (104-106), *Salmonella* Typhimurium (301), *Salmonella* Enteritidis (131, 202), and *Campylobacter jejuni (305).* The immunoprotective effects of CpG-ODN have also been reported in mice against *Helicobacter pylori (251)*, *Yersinia pestis* (136), influenza virus (148), and *Listeria monocytogenes* (143). These findings suggest that CpG-ODN induces protection in several species against a wide range of pathogens. However, how CpG-ODN treatment confers protection remains poorly understood. The present study was undertaken to gain a greater understanding of the mechanisms of CpG-ODN induced host responses that enhance resistance in neonatal chickens to a bacterial pathogen. In this study, we examined how CpG-ODN, delivered by *in ovo* route to18-days old embryonated eggs, protects neonatal chicks, 1-day after the hatch (four days after the CpG-ODN treatment), against a virulent strain of *E. coli.* Consistent with our

previous studies (103-106), in the present study, we found that CpG-ODN treated chicks were significantly protected against *E. coli* infection.

During a pathogenic insult, the host immune response is orchestrated by a complex network of cytokines that shape the immune process by regulating cellular communication and function. CpG ODN has been shown to elicit superior cytokine responses in chicken immune cells *in vitro* compared to other TLR ligands (lipopolysaccharide or Pam3CSK4) (303). Previous studies have suggested that the protection conferred by CpG-ODN is primarily due to an enhanced expression of cytokines and chemokines in chickens (66, 304). Therefore, we designed experiments to evaluate the effects of CpG-ODN administration on the expression profiles of cytokines involved in innate and adaptive immune responses. We examined cytokine expression kinetics of eight cytokine genes (IFNα, IL18, IFNγ, IL1β, Litaf, IL4, IL6, and IL10) in spleen and lungs of CpG-ODN treated and the saline control. In our study, cytokine expression was measured using QuantiGene Plex 2.0® assay technique, a multiplex assay that can simultaneously measure multiple cytokine genes in a single well of the assay. We found a substantial upregulation of cytokines following CpG-ODN treatment in spleen and lungs. The mRNA levels of all cytokines tested here tended to be higher in CpG-ODN treated chickens than in the control group at most time points. In agreement with a previous study (14), we observed higher expression of IFNs following CpG-ODN injections, and this increase was especially notable in the lungs of chickens. IFNs are important cytokines in shaping immune responses against pathogens. IFNα and IFNγ have been shown to act synergistically to develop an antiviral state and activate macrophages and nitric oxide secretion (274). Like Th1 cytokines (i.e.,IFNs), Th2 cytokine IL4 and regulatory cytokine IL 10 were also upregulated following CpG-ODN injections and were also more noticeable in lungs. A previous study reported that CpG-ODN induces a Th1 type of response in chickens (240). However, in our study, we observed both Th1 and Th2 types of response. This finding is also supported by the recent report that demonstrated both Th1 and Th2 types of cytokine expression in chickens in response to CpG-ODN treatment (304).

Accumulating evidence suggests that pro-inflammatory cytokines play a crucial role in promoting strong immune response against pathogens. Our findings of an enhanced expression of pro-inflammatory cytokines, IL1β, IL6, IL18, and Litaf in lungs and spleen following *in ovo* delivery of CpG-ODN suggests that CpG-ODN promotes inflammatory responses in chickens. Our finding is also supported by recent studies showing upregulation of pro-inflammatory

cytokines in chickens following CpG-ODN treatment (303, 305). IL1β is well known for its induction of IL6 (314) and in T cell proliferation (29, 95, 225). We found that among all the cytokines investigated in this study, IL1β was the most upregulated (several fold higher compared to control) cytokine following CpG-ODN administration. Protective effect of pro-inflammatory cytokines is also evident from the study that demonstrated a strong correlation between pro-inflammatory cytokine level and the resolution of *Salmonella* Typhimurium infection in chickens (26). In the present study, we observed that CpG-ODN treatment led to a more pronounced effect on the cytokine profiles in lungs compared to the spleen, which could suggest that CpG-ODN has a superior effect on the mucosal immune system. Our finding is also supported by recent studies that demonstrated enhanced expression of pro-inflammatory cytokines in response to CpG-ODN in intestinal tissues (304) and reduced intestinal colonization with *Campylobacter sp.* (305). These findings suggest that CpG-ODN treatment conditions the chicken immune system against pathogenic invasions at mucosal entry levels and support CpG-ODN as a potent mucosal adjuvant (304). Proinflammatory cytokines such as IL1β, TNFα, IL18, and IL6 are rapidly released upon TLR activation (226). IL18 can stimulate T-cell proliferation and IFNγ release in chickens (101). IL18 has also implicated in stimulating TNFα, IL1β, IL8, and IL6 (227). It is worth mentioning that IL18 and Litaf (TNFα) remained upregulated in both spleen and lungs of hatched chicks for 72 hours post-CpG-ODN treatment. Importantly, among all the cytokines tested here, Litaf remained upregulated for almost all time points studied and in both spleen and lungs. TNFα is a multifunctional cytokine that has antitumor, antiviral and antibacterial actions. TNFα induces upregulation of CD40 and other costimulatory molecules in macrophages and dendritic cells (287).

In the present study, there were substantial differences in the magnitude and kinetics of cytokine expression between CpG-ODN treated and the saline control. These differences can be ascribed to both TLR signaling and cellular composition as well as the number of cells in the organs investigated. Proinflammatory cytokines such as IL1β and TNFα orchestrate the secretion of chemokines and leukocyte cell-surface adhesion molecules, supporting the rapid recruitment of immune cells towards inflammatory area (17, 37, 323). CpG-ODN was shown to enhance cytokine gene expression in various immune cells in chickens, including bursal cells (13), macrophages (22), splenocytes (295), and thrombocytes (294). Proinflammatory cytokines such as IL1β, TNFα, IL18, and IL6 are mainly produced by macrophages when stimulated by bacterial products (315).

Therefore, we next investigated the recruitment kinetics, and activation of macrophages in the immunological niches in spleen and lungs of CpG-ODN treated and saline controls using flow cytometry. Flow cytometry data indicated a significantly enhanced enrichment of macrophages in both spleen and lungs of CpG-ODN treated embryo and chicks. The flow cytometry profile of saline controls revealed a significantly low number of macrophages in spleen and lungs. It is worth mentioning that the lungs' immunological niches had very low number of macrophages, sentinel cells that provide the first line of defense against pathogens. These data can explain why chicks are vulnerable to common bacterial infections in their neonatal life (62), as chicks' immune systems are still not fully mature at the time of hatch. Thus, the present study suggests that *in ovo* delivery of CpG-ODN enriches immunological niches in the immature immune system of the chicken embryo so that pathogenic insults can be tolerated more efficiently at hatch. Maturation of APCs such as macrophages and dendritic cells involves upregulation of costimulatory molecules such as CD40, CD80 and CD86, and the production of cytokines (137). Besides, CD40 signaling was shown to activate the APCs and facilitate $CD8^+$ T-cell priming (271) to generate protective $CD8^+$ cytotoxic T cell (CTL) immunity (201). Therefore, we investigated expression of CD86 and CD40 on macrophages in spleen and lungs of CpG-ODN treated or saline controls. Interestingly, we found that macrophages in CpG-ODN treated chicks were expressing significantly high CD40; however, CD86 was not very different than saline control. This interesting observation can be explained by the cytokine profiles in our study. In the present study, we found significantly higher expression of Litaf in CpG-ODN group in spleen and lungs for several days after treatment. A previous study has reported that TNFα induces upregulation of CD40 onto macrophages and dendritic cells (287). Furthermore, it was reported that TNFα upregulated CD40 but not CD86 in APCs such as Langerhans cells (32, 267). Thus, prolonged expression of Litaf in our study and upregulation of CD40 but not CD86 on macrophages may implicate Litaf for this observation, which corroborates previous studies on Langerhans cells (32, 267). Thus, we hypothesize that enhanced Litaf in CpG-ODN group could be the reason for increased CD40 in our study. However, further studies are needed to understand better TLR21-Litaf-CD40 axis in chickens.

We also examined the T-cell compartment in spleen and lungs of CpG-ODN treated and saline controls chicks that hatched 72 hours post-CpG-ODN treatment. Flow cytometry revealed significantly high number of both $CD4^+$ T and $CD8^+$ T cells in lungs and spleen of CpG-ODN

treated group. These data suggest that CpG-ODN promotes the enrichment of T cell immunological niches both in lymphoid (spleen) and non-lymphoid (lungs) organs in chickens. The increase in the number of immune cells in spleen and lungs can be due to either enhanced recruitment of T cells or increase thymic out (generation of T cells in the thymus) or both. Given that in thymus T cells are generated as CD4(+) and CD8(+) double positive cells and after thymic selection T cells egress thymus as either $CD4^+$ T cells or $CD8^+$ T cells. Therefore, we harvested thymus from CpG-ODN treated and saline control chicks to investigate the expression of CD4 and CD8 in thymocytes as an indicator of thymopoiesis. Interestingly, we found significantly high expression of both CD4 and CD8 in the thymocytes of CpG-ODN treated group, suggesting a potentially increased thymic output following CpG-ODN treatment but requires further studies. Above mentioned possibility is also supported by a previous study in mice, which demonstrated that CpG-ODN treatment resulted in a systemic antigen-independent expansion of both $CD4^+$ and $CD8^+$ T cell subsets and suggested that increase in T cell number was the consequence of reduced T cell death in CpG-ODN treated mice (67). Previous studies using transgenic mice reported that TNFα is constitutively expressed in the thymus (97) and promotes murine (100) and human (268) T cell development. In our study, Litaf expression pattern stands out in the cytokine profiles of spleen and lungs. Therefore, we hypothesize that CpG-ODN induced Litaf may have more significant roles in enriching immune compartments such as spleen and lungs with various sentinel immune cells; consequently, neonatal chicks become better equipped to fight against pathogenic insults.

In conclusion, the present study has demonstrated for the first time that *in ovo* delivery of CpG-ODN provides protection in neonatal chicks against *E. coli* infection not only by eliciting cytokine responses but also through enriching immunological niches in spleen and lungs. Furthermore, our data highlight the importance of Litaf in CpG-ODN induced immunoprotective mechanisms and suggest that future studies on TLR21- Litaf-mediated immune enrichment axis will help in harnessing the full therapeutic potential of CpG-ODNs against diseases.

PREFACE TO CHAPTER 3

We have shown the successful use of CpG-ODN as an immune protector against infections and as an alternative to antibiotics in neonatal chickens when given by the *in ovo* route (104, 301). In the first manuscript of this thesis, we have described the changes in the immune system of neonatal chickens following *in ovo* CpG-ODN administration. Group that received CpG-ODN showed a marked increase in APCs and higher expression of co-stimulatory molecules which suggests an effective antigen presentation. Higher T cell populations suggests the effective adaptive immune responses against pathogens. Higher levels of cytokine gene expression also suggest the stimulation of the immune system. Therefore, our data revealed that CpG-ODN not only stimulates immune cells but enriches the immunological niche, making neonatal chicks well prepared to tackle pathogenic insults.

Optimum immune stimulation of the host is needed for better protection against infectious pathogens. In order to detect a significant protection, the dose of CpG-ODN given per embryo was 50 µg or more in most studies (65, 104, 301). We investigated the possibility of achieving the same immune protection and immune stimulation with several doses of CpG-ODN lower than this amount. The next manuscript in the following chapter (Chapter 3) presents data where we have studied the *in ovo* administration of different doses of CpG-ODN, where the survivability with each dose of CpG-ODN following a lethal *E. coli* challenge was correlated with cellular changes with each dose. This manuscript deliver important data about the minimum effective dose which can provide sufficient immune protection in neonatal chickens against an *E. coli* infection.

CHAPTER 3: CpG-ODN INDUCES A DOSE-DEPENDENT ENRICHMENT OF IMMUNOLOGICAL NICHES IN SPLEEN AND LUNGS OF NEONATAL CHICKS THAT CORRELATES WITH THE PROTECTIVE IMMUNITY AGAINST *E. coli*

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This work has been submitted to *Heliyon Journal.*
"CpG-ODN induces a dose-dependent enrichment of immunological niches in spleen and lungs of neonatal chicks that correlates with the protective immunity against *E. coli".* Thushari Gunawardana, Khawaja Ashfaque Ahmed, Kalhari Goonewardene, Shelly Popowich, Shanika Kurukulasuriya, Ruwani Karunarathana, Ashish Gupta, Betty Chow-Lockerbie, Marianna Foldvari, Suresh Tikoo, Philip Willson, and Susantha Gomis

3.1 Abstract

CpG-ODNs are well known to initiate signaling pathways in cells that elicit innate immunity and protection against bacterial infections. We have previously demonstrated the successful use of CpG-ODN as an immune protector in neonatal chickens against common bacterial diseases such as *E. coli* and *Salmonella* infections. The mechanisms by which CpG-ODN alone protects against bacterial infections are not well understood. In this study, we investigated CpG-ODN mediated immune cell recruitment in the immunological niches in lymphoid (spleen) and non-lymphoid (lungs) organs using various doses of CpG-ODN; and examined whether the immunological profiles have any correlation with immunoprotection against *E. coli* infection. Eighteen-day-old embryonated eggs were injected with either 5, 10, 25, 50 µg of CpG-ODN or saline (n=~40 per group). On the day of hatch (72 hours after CpG-ODN treatment), we collected spleen and lungs (n=3 per group) and examined the recruitment of macrophages/monocytes their expression of MHCII and CD40, and the number of CD4+ and CD8+ T-cell subsets in the immunological niches in spleen and lungs using flow cytometry. We observed dose-dependent recruitment of immune cells, wherein 25 and 50 µg of CpG-ODN induced significant enrichment of immunological niches in both spleen and lungs. Four days after the CpG-ODN treatment (1-day after hatch), chicks were challenged with a virulent strain of *E. coli* (1×10^4 or 1×10^5 cfu, subcutaneously). Clinical outcome and mortality were monitored for 8 days post challenge. We found that both 25 µg and 50 µg of CpG-ODN provided significant protection compared to the saline controls against *E. coli* infection. Overall, the present study reports that CpG-ODN induced protection of neonatal chickens against pathogenic insults is not only through immune cell stimulation but also via the orchestration of immunological niches in chickens.

3.2 Introduction

Infectious diseases of neonatal poultry are common due to the immaturity of the immune system or inadequate sensitization of the immune system to antigens (77). During the first week of a bird's life high mortality associated with bacterial infections, *E. coli* septicemia in particular (118), has devastating impacts on poultry production (342). Antimicrobials are effective in controlling bacterial diseases, and thus the prophylactic use of antimicrobials is in common practice in the poultry industry (106). This prophylactic use of antibiotics in the poultry industry may lead to antibiotic residues in poultry products (46, 117) and emergence of antibiotic-resistant

strains of bacteria (108). Hence reduction of antimicrobial use is a priority of the poultry industry, and thus the use of category I antibiotics has been discontinued since 2014 (43). The poultry industry needs suitable alternatives to antibiotics for controlling diseases in neonatal chickens (15, 217).

Innate immunity is the first line of defense against infectious agents. The host needs to identify an invading pathogen to mount a rapid immune response. Cells of the innate immune system rely on a set of PRR, which can detect specific molecular structures present in pathogens known as PAMPs (171). The innate immune cells, like DC, are activated following interaction of PRR of DC with PAMP of a pathogen. Such PRR-PAMP interaction initiates cell signaling that mounts immune responses, eventually leading to the development of adaptive immunity against the invading pathogen. TLRs are the main PRR, which are important in the induction of innate immunity (3). Components of pathogens such as lipopeptides, glycerophosphatidylinositol, LPS, microbial nucleic acids (dsRNA, ssRNA, unmethylated CpG DNA) and microbial proteins (flagellin, profilin) are some of the well-known TLR ligands (PAMPs). Many potential TLR agonists have been suggested as immune modulators by different studies (205). Synthetic CpG-ODNs are recognized by TLR9 and TLR21 in mammals and avian, respectively (127, 172, 343). CpG-ODNs initiate immune responses in mice (49), fish (151), cattle and sheep (228), human (1, 213, 330), and chickens (63, 104, 301).

In quest of an alternative to antimicrobial agents against bacterial infections, our lab pioneered the use of CpG-ODN alone as an immune protective agent against *E. coli* infection in chickens . We demonstrated that CpG-ODN administered through various routes protects chickens against *E. coli* (104-106) and *Salmonella* Typhimurium infection (301). The protective effect of CpG-ODN was also reported against *Salmonella* Enteritidis infection by other studies (131, 202). It was also demonstrated that CpG-ODN formulation with nanoparticles further improved its immunoprotective action (112, 302, 304). Several studies demonstrated enhanced expression of cytokines and chemokines following CpG-ODN administration in chickens (66, 304). The previous study reported that the resolution of *Salmonella* Typhimurium infection strongly correlated with pro-inflammatory cytokine expression in chickens (26). Despite recent advances, the immunoprotective mechanism(s) of CpG-ODN alone against bacterial infections remained poorly understood.

Proinflammatory cytokines stimulate secretion of chemokines and expression of cell-surface leukocyte adhesion molecules and promote rapid recruitment of immune cells in the inflammatory area (17, 37, 323). We recently found that intrapulmonary delivery of CpG-ODN initiated the infiltration of inflammatory cells, predominantly mononuclear cells with occasional heterophils in the pulmonary parenchyma (106). We hypothesize that CpG-ODN-mediated protection may be through the regulation of immunological niches in neonatal chickens. Thus, the objective of this study was to evaluate the influx of macrophages, $CD4^+$ and $CD8^+$ T-cell subsets in the immunological niches such as spleen and lung in chickens, and investigate if immune profiles correlate with the CpG-ODN-induced protection against *E. coli* infection.

3.3 Materials and methods

3.3.1 Synthetic CpG-ODN

The sequence of CpG-ODN (class B CpG 2007) used was 5'-TCGTCGTTGTCGTTTTGTCGTT- 3'. ODNs were produced with a phosphorothioate backbone (Operon Biotechnologies, Inc. Huntsville, AL).

3.3.2 Bacteria

For the challenge, a field isolate of *E. coli* from a turkey with septicemia was used. This *E. coli* strain was serogroup O2, non-hemolytic, serum-resistant, produced aerobactin, with a K1 capsule and Type 1 pili. Aliquots of bacteria were stored at -80 C in 50% brain heart infusion broth (BHI; Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific, Inc., Montreal, Quebec). Bacteria used for challenge were cultured on Columbia sheep blood agar plates for 18-24 hours at 37 C. One colony was added to 100 mL of Luria broth in a 250 mL Erlenmeyer flask. The culture was grown at 37 C for 16 - 18 hours with shaking at 150 rpm. Stationary phase culture contained approximately 1×10^9 cfu of bacteria per mL. The cultures were further diluted in sterile saline so the concentration of bacteria required for challenge (1×10^5 or 1×10^4 cfu in 250 µL/bird) was obtained. Viable bacterial counts were determined by plating serial dilutions of the challenge culture in duplicate on Columbia sheep blood agar plates, incubating for 18-24 hours at 37 C; then counting the number of colonies.

3.3.3 Cells for flow cytometry

Cell preparation and antibody staining for flow cytometry was done as previously described with some modifications (6). Spleen and lung tissues were collected at 72 hours post *in ovo* injections from chicken embryos and processed for cell isolation. Briefly, each spleen was gently pushed through a metal strainer by manual pressure to obtain a single cell suspension with ~3mL of PBS and collected to a 15 mL centrifuge tube. For lung, each tissue was manually dissected and incubated with ~1ml of collagenase dissolved in Dulbecco's Modified Eagle Medium (DMEM) (1 mg/mL) for 30 minutes in 37 C, after incubation these tissues were filtered through a metal strainer to obtain a single cell suspension and washed twice with PBS. Spleen, lung and thymus cells were then incubated with RBC lysis buffer to lyse red blood cells. Following three washes with wash buffer (PBS containing 2% fetal bovine serum and 0.1% sodium azide), cells were stained with appropriate antibodies.

3.3.4 Animal model

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Fertilized hatching eggs were obtained from a commercial broiler breeder operation in Saskatchewan, Canada. Eggs were incubated at the ACU at the Western College of Veterinary Medicine, University of Saskatchewan. Groups of chicks were allocated randomly into animal isolation rooms at the ACU. Water and commercial broiler ration were provided *ad libitum*. Each room was ventilated with filtered, non-recirculated air at a rate of 10–12 changes/hr. Air pressure differentials and strict sanitation were maintained in this isolation facility. The birds received either 1×10^5 or 1×10^4 cfu of stationary - phase *E. coli*, in a total volume of 250 μL per bird, by subcutaneous injection in the neck, resulting in *E. coli* septicemia. In this model, *E. coli* septicemia with airsacculitis, pericarditis, or perihepatitis develops in 60%–90% of birds that are not protected by treatment intervention. Either 1×10^4 or 1×10^5 cfu of *E. coli* were given to birds in all groups to simulate field conditions since all birds in a commercial poultry barn will not be exposed to a consistent dose of *E. coli* (the number of birds that were challenged with the lower and higher dose of *E. coli* were equal in a group). Birds were evaluated three times daily at the critical stage (first 3 days post-challenge) then twice daily thereafter for 7 days post challenge. Birds were observed for clinical signs and each individual was assigned a daily clinical score: 0 = normal; 0.5 = slightly

abnormal appearance, slow to move; 1= depressed, reluctant to move; 1.5 = reluctant to move, may take a drink and peck some; 2 = unable to stand or reach food or water; and 3 = found dead. Birds that received a clinical score of 2 were humanely euthanatized by cervical dislocation. Chicks that were found dead or euthanatized were necropsied immediately. On day 7 post *E. coli* challenge, the remaining birds were euthanatized by cervical dislocation. Bacterial swabs were taken from the air sacs and cultured on Columbia sheep blood agar using a typical method of inoculation and streaking on four quadrants of the plate of medium. A semi-quantitative estimate of *E. coli* isolation was conducted on Columbia sheep blood agar. Growth on these plates were recorded on a scale from 0 to 4+, where 0 = no growth; 1+ = growth of bacteria on the area 1; 2+ = growth of the bacteria on areas 1 and 2; 3+ = growth of bacteria on areas 1, 2, and 3; and 4+ = growth of bacteria on areas 1, 2, 3, and 4.

3.1 Experimental design

3.1.1 Delivery of CpG-ODN by the *in ovo* route

Embryonated eggs which had been incubated for 18 days, received either 50, 25, 10 or 5 µg of CpG-ODN diluted in sterile pyrogen-free saline, in a total of 100 µL/egg or 100 µL of sterile saline (n=~40/group). Injections were administered by the *in ovo* route into the amniotic cavity through the air cell side of the egg using a 22 gauge, 1 inch hypodermic needle. The volume of the injection and the length of the needle were selected to simulate the standard *in ovo* injection technology used in the poultry industry. Following *in ovo* injection, the injection sites of eggs were covered with melted paraffin applied with a wooden applicator and transferred to the hatcher until hatch.

3.1.2 Tissue sample collection for flow cytometry

Three embryos from each group were humanely euthanized at 72 hours post *in ovo* injections by cervical dislocation and necropsied for tissue collection. Spleen and lung tissues were collected in to 1.5 mL micro centrifuge tubes.

3.1.3 *E. coli* challenge

Four days after *in ovo* injections (day-1 post-hatch), either 1×10^4 or 1×10^5 cfu of a virulent strain of *E. coli* was inoculated subcutaneously in the neck in all remaining birds [bird numbers per group; CpG-ODN 5 µg: n = 37; CpG-ODN 10 µg: n=33; CpG-ODN 25 µg: n= 34; CpG-ODN 50 µg: n=30, saline: n=35]. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for 8 days following challenge with *E. coli*.

3.1.4 Flow cytometry

The cell populations of spleen and lungs collected at 72 hours post *in ovo* injections from each group (n=3), were stained for the presence of APC, cellular markers and $CD4^+$ and $CD8^+$ T-cell subsets. For detecting APCs, CD40 and MHCII molecule one set of spleen and lung cells (~5 x 10^5 cells) were incubated with mouse anti-chicken CD40 primary antibody at 4 C for 30 min followed by three washing steps and incubated with goat anti-mouse IgG-PerCP/Cy5.5 secondary antibody at 4 C for 30 min. After three washes, cells were stained with mouse anti-chicken monocyte/macrophage KUL01 (PE) and mouse anti-chicken MHCII (AF 488) antibody together at 4 C for 30, subsequently washed three times and suspended in ~300 µL flow cytometric buffer for the analysis.

Another set of spleen and lungs (~$5x10^5$ cells) were incubated with anti-chicken CD8 (FITC) and CD4 (PE) together at 4 C for 30 minutes to determine $CD4^+$ and $CD8^+$ T cells. Following three washes these cells were also suspended in ~300 µL flow cytometric buffer in flow tubes and processed for flow cytometric analysis. Flow cytometry data were acquired by Epics XL (Beckman Coulter) and FACS Caliber (BD Bioscience), and data was analyzed with FlowJo software (Tree Star).

3.2 Statistical analysis

Prism 5.0, GraphPad Software Inc., San Diego, CA was used to analyse and graph survival trends, CCS, bacterial percentages and cell populations from flow cytometry analysis, with a significance level of $P<0.05$. The survival patterns and median survival times were compared using the log-rank test and chi-square statistic. The reduction of relative risk of mortality of groups of birds were calculated using Microsoft excel. The clinical score for each bird was summed over the 8-day observation period to calculate the CCS and the significance of differences among groups

were tested with the use of Kruskal-Wallis nonparametric analysis of variance. For tetsing significant differences of the means of immune cell numbers and their maturation marker expression between groups, ANOVA testing was done. Dunnett's test was used as a *post hoc* test following ANOVA to assess for significant differences between each treatment group compared to the saline control group. For testing difference of $CD4^+$ and $CD8^+$ expression between groups, we used a two way ANOVA followed with Bonferroni post test and Student-t test with Welch's correction for unequal variance was used, with a significant difference of $P<0.05$.

3.3 Results

3.3.1 Flow cytometry

Flow cytometry analysis of spleen and lungs at 72 hours post *in ovo* injections (on the day of hatch) showed a significant influence of CpG-ODN on immune cell components of birds. Results demonstrated a dose-dependent influence of CpG-ODN affecting cell population percentages and cell maturation marker expression levels.

3.3.1.1 Antigen presenting cells

CpG-ODN showed a strong effect on MHCII expressing APC cell population (monocyte/macrophages) 72 hours post *in ovo* injections. The same pattern was observed in both spleen and lung where the APC percentage increased with CpG-ODN dose. The highest APC percentage was seen with 50 µg of CpG-ODN and lowest with the saline control. However, both groups that received 25 µg and 50 µg of CpG-ODN per bird showed significant increase of APCs compared to the saline control group (~5 times higher in spleen and ~4 times higher in lung). Whereas, CpG-ODN doses that we tested lower than 25 µg (5 µg and 10 µg) did not show a significant effect on APC population compared to the control group in spleen (Figure 3-1A, Figure 3-1B and Figure 3-1C) and lungs (Figure 3-1D, Figure 3-1E and Figure 3-1F). APCs (monocyte/macrophages) were further analysed for the expression of maturation markers (CD40 costimulatory molecule). The CD40 signaling is well known to activate APCs and facilitate T cell priming (271) to generate protective $CD8^+$ cytotoxic T cell (CTL) immunity (201). The mean fluorescence intensity (MFI) of CD40 expression on APCs, with each CpG-ODN dose tested are shown in histograms. The MFI increased in a dose-dependednt manner and was maximum with the 50µg dose in both spleen (Figure 3-1B) and lung (Figure 3-1E).

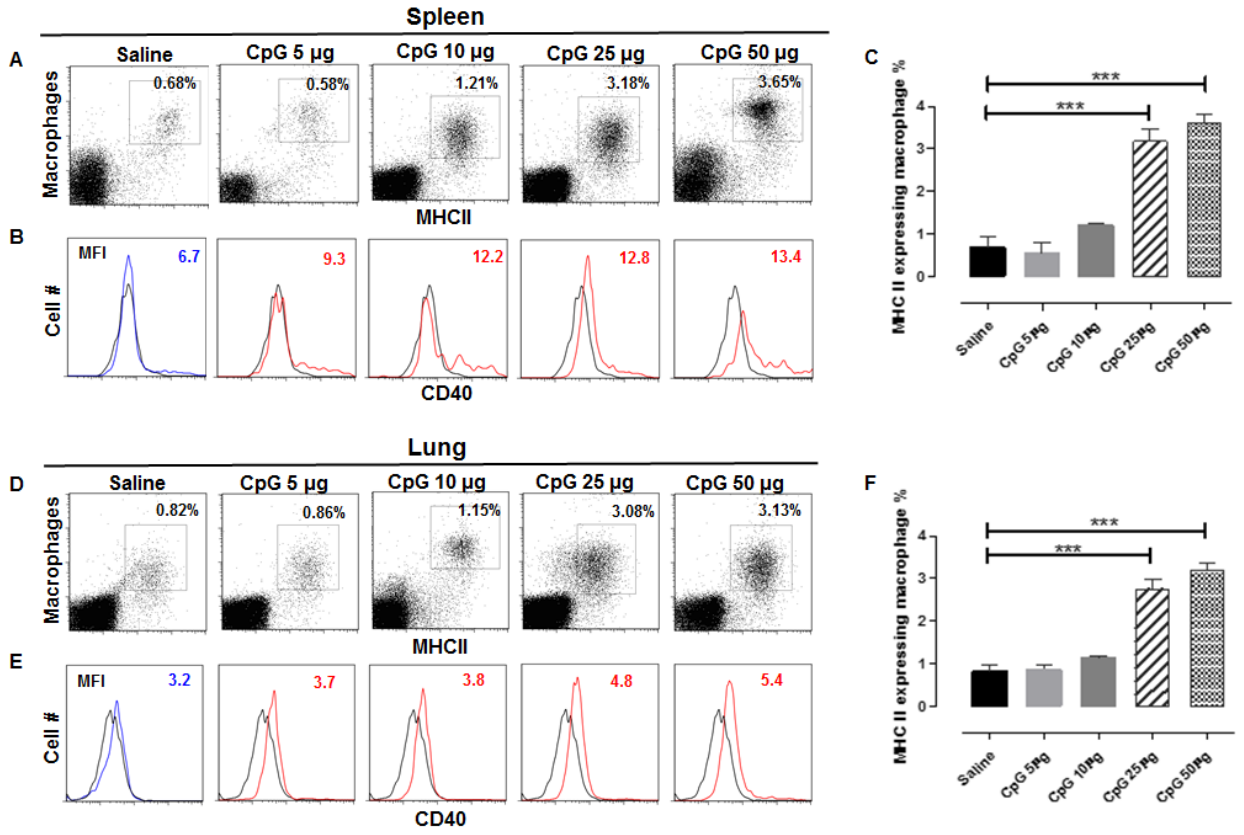

Figure 3-1: Antigen presenting cells of spleen and lung. Flow cytometric analysis of spleen (**A and B)** and lung **(D and E)** cells at 72 hours post *in ovo* injections (day 21 embryo) (n=3-4/group). Monocyte/macrophage APC populations with MHCII expression were gated. APCs were quantified using PE-labelled mouse anti-chicken monocyte/macrophage and AF488-labeled mouse MHCII monoclonal antibodies. Histogram panels (B, spleen and E, lung) below indicates the level of CD40 expression on the APCs (the MFI is indicated). Black histogram = isotype control; blue = saline, and red = CpG-ODN treated. Bar diagrams on right show the means of total percentage of $MHCII^{+}$APC in spleen **(C)** and lung **(F)** (vertical line and horizontal bar show the standard error of mean SEM), n=3. Dunnett's test following ANOVA testing was used to test for significant differences between CpG-ODN doses and the saline control group. Asterisks indicate groups that were significantly different from control group, ($P<0.05$) (MFI – mean fluorescence intensity).

3.3.1.2 $CD4^{+}$ and $CD8^{+}$ cells

$CD4^{+}$ cells (T helper cells) and $CD8^{+}$ (cytotoxic T cells) are the main two types of T lymphocytes which play important roles in both humoral and cell mediate immunity. We evaluated the effect of CpG-ODN on $CD4^{+}$ and $CD8^{+}$ cell populations in spleen and lung in chickens 72 hours post-CpG-ODN *in ovo* injections. Both $CD4^{+}$ and $CD8^{+}$ cell populations increased after CpG-ODN administration in a dose-dependent manner in spleen (Figure 3-2A) and lungs (Figure 3-2B). Significant increase in $CD4^{+}$ and $CD8^{+}$ T cells (Figure 3-2C, spleen; Figure 3-2D, lungs)

(as well as total number of T lymphocytes ($CD4^+$ and $CD8^+$ T combined) in the spleen (Figure 3-2E) were detected in the group that received 50 µg CpG-ODN and both groups that received 25 µg and 50 µg in the lungs (Figure 3-2F).

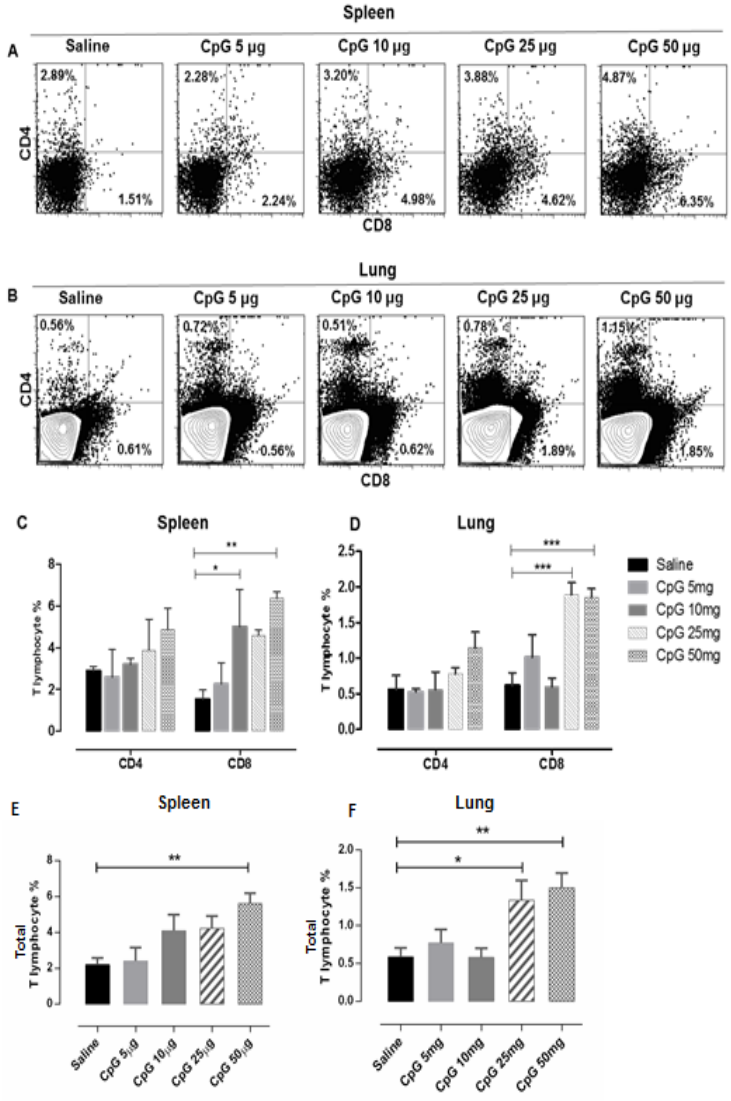

Figure 3-2: T lymphocytes in spleen and lung. Flow cytometric analysis of spleen and lung T cell populations of each group (n=3) at 72 hours post *in ovo* injections (day-1 hatch). $CD4^+$ and $CD8^+$ T-cells were quantified in spleen **(A)** and lungs **(B)** using PE-labelled mouse anti-chicken CD4 and FITC-labelled mouse anti-chicken CD8 monoclonal antibodies. Bar diagrams show $CD4^+$ and $CD8^+$ T cell number in spleen **(C)** and lungs **(D)** and the total number of $CD4^+$ T-cells and $CD8^+$ T-cells combined **(E)** spleen and lungs **(F)**. Two way ANOVA following Bonferroni post test was done when $CD4^+$ and $CD8^+$ cells were compared in CpG-ODN recived groups with saline control. Dunnett's test following ANOVA testing was used to test for significant differences of total T cells between different CpG-ODN doses and the saline control group. Vertical lines and horzontal bars show the standard error of mean-SEM. Asterisks indicate groups that were significantly different from the control group, *=P<0.05, **=P<0.01 and ***=P<0.001.

3.3.2 *E. coli* challenge

Survival following *E. coli* challenge was significantly higher in groups of birds that received 25 or 50 µg of CpG-ODN compared to the saline control group (P=0.03 and 0.04 respectively). (Figure 3-3A). Moreover, the reduction in the relative risk of mortality following *E. coli* challenge was 48.5% and 47.5% respectively in these groups, compared to the saline control group indicating that incrdasing the dose beyond 25 µg did not improve survival. This study revealed that the level of protection following *E. coli* challenge is similar in birds that received 25 and 50 µg of CpG-ODN (Figure 3-3B). Birds that received 10 µg of CpG-ODN tended to have higher survival than the control but was not significant (P=0.59). The survival of birds that received 5µg of CpG-ODN was similar to the saline control group (Figure 3-3A). The data of groups that received either 1×10^4 and 1×10^5 cfu of *E. coli* were combined for clarity of presentation and analysis.

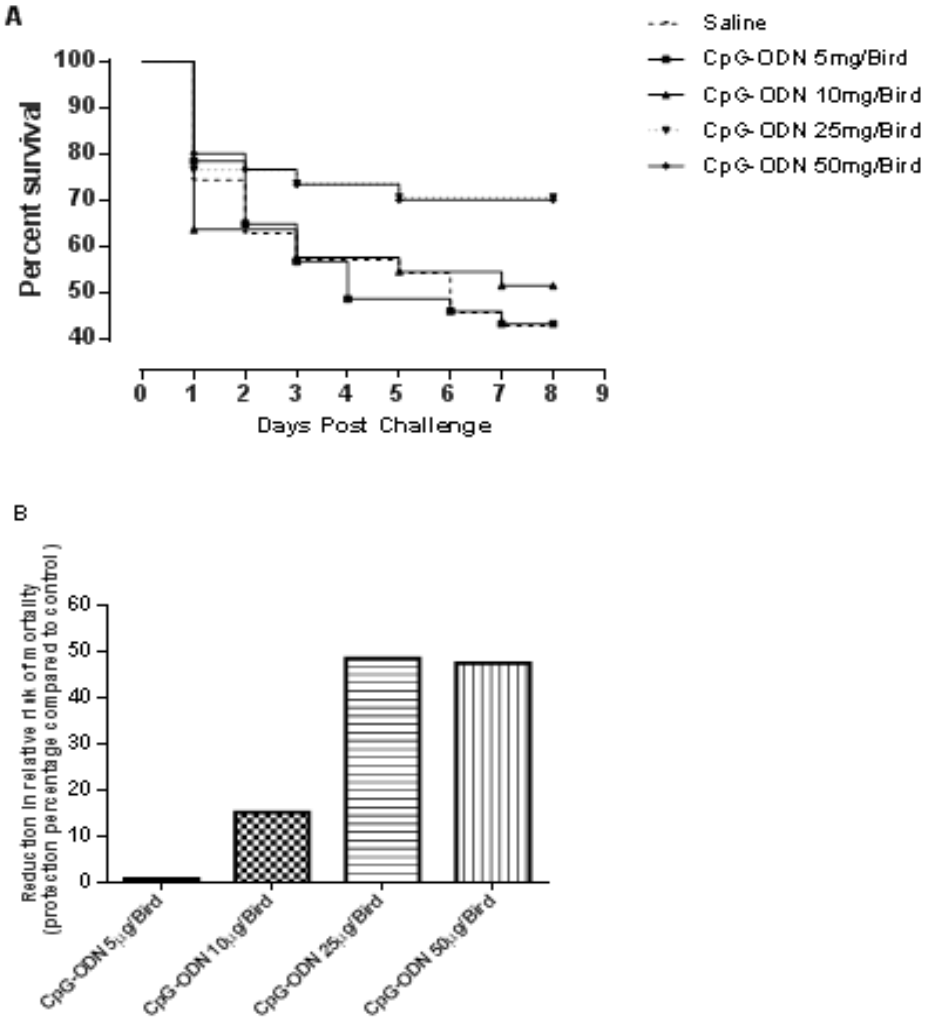

Figure 3-3: Survival pattern and reduction of relative risk of mortality following *E. coli* challenge.(A). Survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day 18 of incubation were injected with either 5 (—■—), 10 (—▼—), 25 (···▼···), 50 µg (—●—) of CpG-ODN or sterile saline (--●--) by the *in ovo* route and then challenged with either 1×10^5 or 1×10^4 cfu of *E. coli* three days later, at the day of hatch. Survival graph was plotted based on the event of a death in birds following challenge for each day post challenge in each group. **(B)**. The reduction of relative risk of mortality compared to the saline control group following *E. coli* challenge.

The CCS for each bird was calculated by summing the daily scores throughout the 7 day observation period post *E. coli* challenge. A significant difference of CCSs were not detected between groups. However, a pattern of increasing CCSs were seen. The lowest CpG-ODN doses and saline control. The lowest CCSs were seen in birds that received 50 µg of CpG-ODN while the highest CCSs were detected from the saline control group (Figure 3-4 A). Moreover, birds that

received CpG-ODN had lower amount of bacteria isolated from air sac swabs compared to birds that received saline (Figure 3-4B). Birds that died or were euthanatized either had airsacculitis or pericarditis or a combination of airsacculitis together with pericarditis or polyserositis.

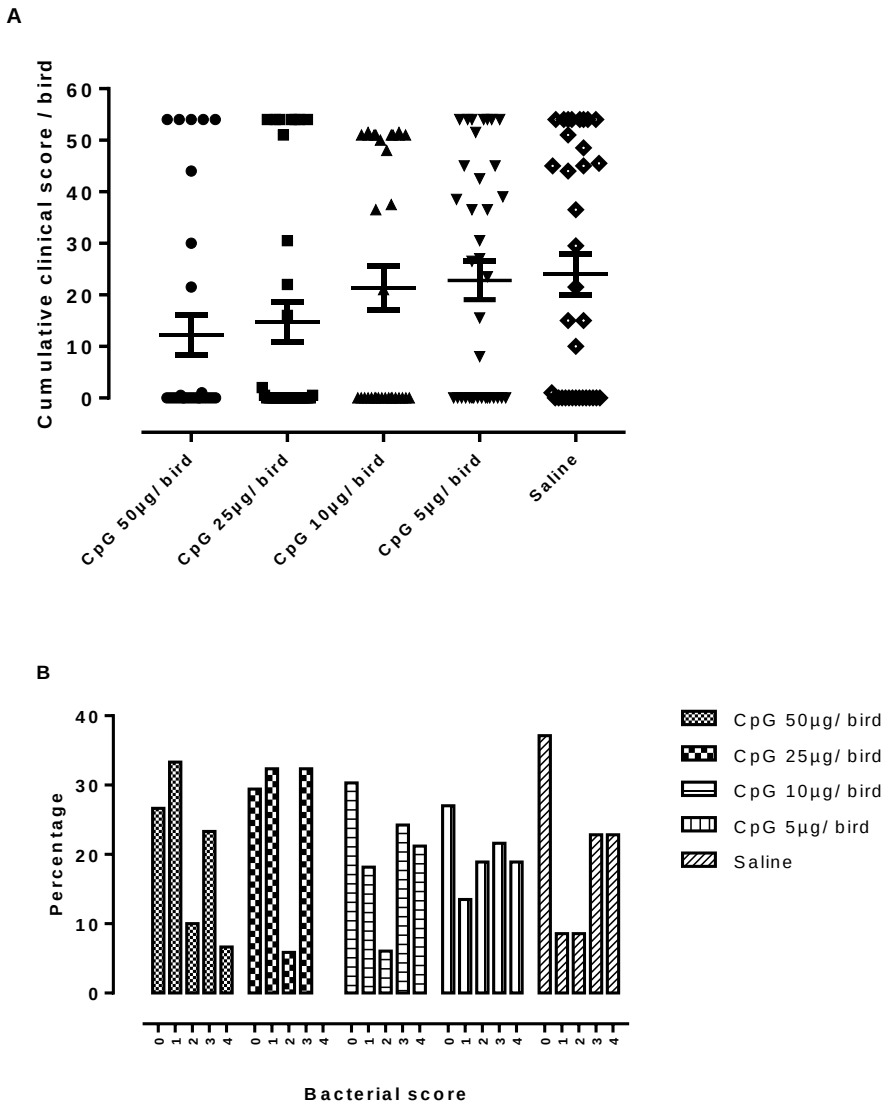

Figure 3-4: CCS and bacterial growth following *E. coli* challenge.
(A) CCS of broiler chickens following *E. coli* challenge. Each data point represents the CCS of one bird. (bar = mean with SEM). **(B)** Bar graph show the percentage of birds in each treatment group that had each classification of bacterial growth. Bacterial swabs were taken from air sacs. Groups that received 50 and 25 µg of CpG-ODN had fewer birds that showed higher levels (3+ and 4+) of bacterial growth compared to other groups.

3.4 Discussion

While many preventive strategies are being implemented to minimize infectious diseases in poultry, they may not be sufficient to avert all pathogenic insults. Immune-based methods that stimulate the innate immunity against a broad range of pathogens may provide a promising solution to disease problems in the poultry industry. CpG-ODN, a TLR agonist, is well known as an immune protective agent against bacterial (105, 301), viral (65, 310) and protozoal (63) infections in chickens. Furthermore, several studies have shown that CpG-ODN is effective as an immunostimulant in both mature and neonatal chickens against *E. coli* infections (104-106, 112). We have previously shown that CpG-ODN delivered through *in ovo* route, a most desirable and economical method of drug delivery in the chicken industry (258), can be effective in protecting neonatal chickens against bacterial diseases such as those caused by *E. coli* (104, 112) and *Salmonella* Typhimurium (301). The immunoprotective effect of CpG-ODN was also reported by other studies against *Salmonella* Enteritidis (131, 202) and *Campylobacter sp.* (304). The formulation of CpG-ODN with nanoparticles further improves the immune protective effect (302, 304). Previous studies suggested that enhanced expression of cytokines and chemokines (66, 304) and increased cellular functions, such as the increase in heterophil degranulation and oxidative burst (132), play important roles in CpG-ODN-induced protection in chickens. We have recently reported an enhanced infiltration of inflammatory cells in the pulmonary parenchyma following an intrapulmonary delivery of CpG-ODN in chickens (106). Despite recent progress, the manner that CpG-ODN alone confers immunoprotection against bacterial infection remains poorly understood.

The present study was undertaken to investigate CpG-ODN-mediated immune cell recruitment in the immunological niches in lymphoid (spleen) and non-lymphoid (lungs) organs in chickens, and examine further if the immunological profiles correlate with protection against *E. coli*. infection in chicks. In this study, we administered various doses of CpG-ODN through in ovo route (18-days old embryonated eggs), and harvested spleen and lungs at hatch to investigate CpG-ODN dose effect on the immunological profiles. One day after hatch (four days after the CpG-ODN treatment) chicks were challenged with a virulent strain of *E. coli* to test protection from disease. Here, we examined the recruitment of macrophages/monocytes, $CD4^+$ and $CD8^+$ T cell subsets in the immunological niches in spleen and lungs. APCs, such as macrophages/monocytes constitute essential components in the immune system, which process

and present antigens, and initiate activation of effector immune cells. Maturation of APCs such as macrophages and dendritic cells involves increased expression of costimulatory molecules such as CD80, CD86, and CD40 (137). CD40 signaling on APC results in APC licensing (full activation) that facilitates $CD8^+$ T cell priming (271) to orchestrate protective $CD8^+$ cytotoxic T cell (CTL) immunity (201). Therefore, we examined the number of macrophages/monocytes present in spleen and lungs as well as the expression of CD40 on these APCs in CpG-ODN treated or saline controls.

Our results showed a CpG-ODN-dose-dependent increase in the number of macrophages/monocytes in the immunological niches in both spleen and lungs. Moreover, we also observed an increase in CD40 expression with different doses of CpG-ODN. CD40 signaling is important for B cell proliferation, differentiation, T cell proliferation, monocyte and dendritic cell growth and cytokine production (169). These data suggest that CpG-ODN administration not only enriches immune compartments with sentinal cells such as macrophages/monocytes that play important roles fighting pathogens (31) , but also activates them for proper maturation leading to the expression of a costimulatory molecule, CD40, which is well known for its role in the orchestration of immunity against pathogens (7). These findings seen with CpG-ODN such as immune cell activation and maturation were comparable to results of many earlier studies in other species (119, 214, 259, 293). The significant increase we observed in MHCII-expressing APCs with 25 and 50 µg of *in ovo* CpG-ODN in both lung and spleen could suggest enhanced antigen presentation capability for an effective and rapid pathogen clearance in neonatal chicks. Also, we observed a dose-dependent increase in $CD4^+$ and $CD8^+$ T cell populations, which play important role in humoral and cell-mediated immunity, in spleen and lungs following CpG-ODN administration. These findings suggest that the immunological niches in CpG-ODN-treated chicks are well equipped with mature APCs with the ability to activate effector immune cells such as $CD4^+$ and $CD8^+$ T lymphocytes.

We next investigated whether the levels of enrichment of immunological niches in spleen and lungs by different doses of CpG-ODN have any correlation with immunoprotection against *E. coli* infection. Therefore, we have assessed the clinical protection of neonatal chickens treated with various doses of CpG-ODN by challenging them with a virulent strain of *E. coli*. Here we noted that the protection level of chicks that received 10 µg or below CpG-ODN was not different from the chicks that received saline. Consistent with our previous studies (103-106), chicks that received

25 µg and 50 µg of CpG-ODN were siginificantly protected compared to the saline controls. This protection correlates with the changes we detected in immune cell compartments in chickens at the time of hatch by different doses of CpG-ODN. We found that 10 µg, but not 5 µg, CpG-ODN dose resulted in the increase of immune cells both in spleen and lungs. However, both the 5 µg and 10 µg doses were not significantly different than the saline controls in immune profiles and protection. In contrast, chicks that received 25 µg and 50 µg of CpG-ODN demonstrated significantly enhanced MHCII-expressing APCs, CD40 expression on these APCs and T cell populations that correlated with their ability to resist *E. coli* infection. Overall, our data revealed a previously unrecognized phenomenon that CpG-ODN not only stimulates immune cells but remarkably enriche the immunological niche, making neonatal chicks well prepared to tackle pathogenic insults.

PREFACE TO CHAPTER 4

We have discussed the immune modulation and an effective minimum dose of CpG-ODN that can induce protective immunity in neonatal chickens when given by *in ovo* route, in the previous manuscripts in this thesis (chapters 2 and 3). However, as the PRR that identify CpG-ODN (identified as TLR21 in chickens) is located intracellularly, localized in the ER of resting cells (41), injected CpG-ODN that reach cells may be destructed along the way. In addition, the route of *in ovo* delivery itself can affect for the loss of CpG-ODN reaching target cells due to numerous barriers and nucleases. This directed us in considering methods to improve cellular delivery of CpG-ODN.

Improved cellular delivery of a bioactive substance can be achieved by many proposed drug delivery systems. The field of nanoparticle based drug delivery has become very popular over the last few decades (256). Lipid based drug delivery systems (333) and carbon nanotube based delivery systems (34) have been tested and proved as effective CpG DNA delivery vehicles. Lipid based systems are well known for their ability to encapsulate and protect CpG-ODN, enhance immune cell targeting and facilitate intracellular uptake (333). Whereas carbon nanotubes are distinguished as agents that can facilitate and enhance cellular uptake of CpG-ODN (34).

The following manuscript in this thesis (chapter 4) present data on two types of lipid and carbon nanotube based CpG-ODN formulations given by the *in ovo* route to broiler chickens. We have studied and compared the survivability, bacterial loads, and clinical outcome of these neonatal chickens following an *E. coli* challenge within different groups receiving different formulations. Furthermore, this manuscript provide data of the safety aspects of these formulations when given to chicken embryos

CHAPTER 4: PROTECTION OF NEONATAL BROILER CHICKENS FOLLOWING *IN OVO* DELIVERY OF CpG-ODN FORMULATED WITH CARBON NANOTUBES OR LIPOSOMES

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This work is published in *Avian Diseases*, 2014. 59 (1): 31-37.
"Protection of neonatal broiler chickens following in ovo delivery of oligodeoxynucleotides containing CpG motifs (CpG-ODN) formulated with carbon nanotubes or liposomes." Gunawardana, Thushari, Marianna Foldvari, Tara Zachar, Shelly Popowich, Betty Chow-Lockerbie, Marina Vaneva Ivanova, Suresh Tikoo, Shanika Kurukulasuriya, Philip Willson, and Susantha Gomis*.

4.1 Abstract

Unformulated ODN containing CpG motifs are well known agents that stimulate the innate immune system of vertebrate species against bacterial, viral and protozoan infections. We have previously shown significant protection against *E. coli* or *Salmonella* Typhimurium infections in neonatal broiler chickens with *in ovo* delivery of unformulated CpG-ODN. In the present study we studied the immunoprotective effects of CpG-ODN, formulated with two types of CNTs or two types of lipid-surfactant (LSC) delivery systems against *E. coli* septicemia and their safety in neonatal broilers. Embryonated eggs which had been incubated for 18 days, received either 50 µg of CNT-CpG-ODN, 50 µg of LSC-CpG-ODN, 50 µg of unformulated CpG-ODN or saline. Four days after exposure to CpG-ODN (day-1 post-hatch), 1×10^4 or 1×10^5 cfu of a virulent strain of *E. coli* was inoculated subcutaneously in the neck. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for eight days following challenge with *E. coli*. Birds that received CpG-ODN formulations had a significantly higher survival rate of 60% to 80% following *E. coli* infection. Whereas, survival rates of birds that received saline was 20% to 30%. Bacterial loads and clinical scores were low in groups treated with CNT- or LSC-CpG-ODN compared to the groups receiving CpG-ODN or saline. Moreover, any adverse effects were not observed in any organs or growth rate of birds that received these formulations. We report for the first time that CpG-ODN formulated with CNT and LSC have been demonstrated to have an immunoprotective effect against an *E. coli* infection in neonatal broiler chickens following *in ovo* delivery.

4.2 Introduction

Immune-based methods are gaining much attention as a means of providing protection against diseases in animals, mainly food animal species. Neonatal chickens are highly susceptible to infections due to their immature immune systems until several weeks post hatch (77) and many other stressors, such as handling and transportation. *E. coli* is one of the main pathogens that causes a variety of disease syndromes in poultry including yolk-sac infection, omphalitis, respiratory tract infection, septicemia, and cellulitis (107). Although vaccines are effective in stimulating the immune system for improved disease protection, they may not be available for all disease agents and are not effective when an immediate response is required. The use of antibiotics is controversial because residues may persist and it can lead to the emergence of antibiotic-resistant

microbes. This may result in food allergies and soil or water contamination via fecal excretion of antibiotics in poultry manure applied to the land. Thus, there is pressure to restrict the use of antibiotics in agriculture. As a result, category 1 antibiotic use was withdrawn from poultry farming in 2014 (43). Hence, these trends point to a need for the development of alternative strategies for infectious disease control (9).

The vertebral innate immune system identifies PAMPs by PRR, inducing immune responses which prevent infections (211). Non-vertebral DNA which contains comparatively higher amounts of unmethylated CpG dinucleotides than vertebral DNA acts as a PAMP. These unmethylated CpG dinucleotides (CpG motifs), are recognized as the molecular pattern that contributes to the immunostimulatory activity of non-vertebral DNA (5). TLR9 in mammals, which is a PRR, recognize these unmethylated CpG motifs and initiates immunostimulatory pathways (5). The context of the unmethylated CpG dinucleotides including specific flanking sequences are responsible for the extent of immune stimulation (171). Synthetic preparations of oligodeoxyneucleotides containing CpG-ODNs simulating non-vertebral DNA, have shown similar immunostimulatory activity (341). This discovery has shown an important preventive immunotherapeutic treatment option which stimulates the host's immune system. Studies have revealed the immunostimulatory action of CpG-ODNs in many vertebral species including mice (49) fish (151) cattle and sheep (228), and chickens (105, 301). Other than the immunostimulatory action, CpG-ODNs are being used as vaccine adjuvants due to the ability to activate antigen presenting cells and B cells (162). CpG-ODNs are also used for cancer therapy due to natural killer cell and cytotoxic T cell activation (162). Furthermore, CpG-ODNs are being used for anti-allergic treatments due to the ability to promote Th1-type immune response and thus suppress Th2 type allergic responses (162). The immunoprotective effect of CpG-ODN against several crucial diseases such as *E. coli* (103-105) and *Salmonella* Typhimurium (301) bacterial infections, protozoan diseases caused by *Eimeria* (63) and viral diseases such as infectious bursal disease (324) and infectious bronchitis (65) have been demonstrated in chickens.

There have been several drawbacks to using CpG-ODN as immunostimulatory molecules such as cost, large doses needed for an effective response and intra-cellular location of CpG-ODN recognizing PRR which is tough to reach. Drug delivery systems have been considered in order to overcome these challenges. Many studies have proven that lipid based and carbon nanotube associated delivery systems are able improve the immunostimulatory effect and the uptake of CpG-

ODN in many species, including mice (20),(72),(333), and pigs (10). However, the use of CpG-ODN delivery and formulation systems in neonatal chickens have not being investigated in detail. The therapeutic value of CpG-ODN can be increased by extending the bioavailability and duration of action . Unfortunately, CpG-ODN used *in vivo* is rapidly eliminated from the circulation due to adsorption onto serum proteins and degradation by serum nucleases (197). A possible technique for protecting CpG-ODNs from degradation while increasing uptake by cells of the immune system involves liposome encapsulation (333) or fusion with biphasic lipid vesicles (10). Formulating CpG-ODN with polyphosphazene polymers showed enhanced protection of chickens from am *E. coli* infection (302).

CNTs exist in two types, single-wall (SWNT) and multi-wall (MWNT). CNTs well studied and recognised as multipurpose carriers for drug delivery and diagnostic applications. The organic functionalization of CNTs can significantly improve their solubility and biocompatibility profile; as a result, their manipulation and integration into biological systems has become possible. Functionalised CNTs currently hold strong promise as novel systems for the delivery of drugs, antigens and genes (33). Use of CNTs to enhance uptake of CpG-ODN and increase antiglioma immunity in mice have been reported (20). To our knowledge, CNTs have not been used for formulation of CpG-ODN and delivery in chicken embryos. The goals of this study were to determine the safety and immunoprotective effects of two types of CNTs and two types of LSCs in delivery systems formulated with CpG-ODN as immunostimulants in neonatal broiler chickens.

4.3 Materials and methods

4.3.1 Bacteria

A field isolate of *E. coli* from a turkey with septicemia was used as the challenge strain. This *E. coli* was serogroup O2, nonhemolytic, serum-resistant, produced aerobactin, a K1 capsule, and Type 1 pili. Aliquots of bacteria were stored at -70 ^{0}C in 50% brain heart infusion broth (BHI; Difco, Detroit, MI) supplemented with 25% (w/v) glycerol (VWR Scientific, Inc., Montreal, Quebec). Bacteria used for the challenge were cultured on Columbia sheep blood agar plates for 18-24 hours at 37^{0}C. One colony was added to 100 mL of Luria broth in a 250 mL Erlenmeyer flask. The culture was grown at 37 C for 16 - 18 hours while shaking at 150 rpm. Stationary phase culture contained approximately 1×10^9 cfu of bacteria per mL. The cultures were further diluted

in sterile saline so the concentration of bacteria required for the challenge (1×10^5 or 1×10^4 cfu/bird) was obtained. Viable bacterial counts were determined by plating serial dilutions of the diluted culture in duplicate on Columbia sheep blood agar plates, incubating for 18-24 hours at 37^0C; then counting the number of colonies.

4.3.2 Animal model

This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and followed the Canadian Council on Animal Care guidelines for humane animal use. Hatching eggs were obtained from a commercial broiler breeder operation in Saskatchewan, Canada. Eggs were incubated at the ACU at the Western College of Veterinary Medicine, University of Saskatchewan. Groups of chicks were allocated randomly into animal isolation rooms at the ACU, University of Saskatchewan. Water and commercial broiler ration were provided *ad libitum*. Each room was ventilated with filtered, non-recirculated air at a rate of 10–12 changes/hr. Air pressure differentials and strict sanitation were maintained in this isolation facility. The birds received either 1×10^5 or 1×10^4 cfu of stationary - phase *E. coli*, with a total volume of 250 μL per bird, by subcutaneous injection in the neck and resulting in *E. coli* septicemia. In this model, *E. coli* septicemia with airsacculitis, pericarditis, or perihepatitis develops in 60%–90% of birds that are not protected by treatment intervention. Two doses of *E. coli* were given to groups of birds to simulate field conditions since all birds in a commercial poultry barn will not be exposed to a consistent dose of *E. coli*. Birds were evaluated three times daily at the critical stage then twice thereafter for seven days post challenge. Birds were observed for clinical signs, and each individual was assigned a daily clinical score as following,: 0 = normal; 0.5 = slow to move and slight abnormal appearance; 1= depressed and reluctant to move; 1.5 = reluctant to move, may take a drink and peck some feed; 2 = unable to stand or reach food or water; and 3 = found dead. Birds with a clinical score of two were euthanatized by cervical dislocation. Chicks that were found dead or euthanatized were necropsied immediately. On day seven post *E. coli* challenge, all remaining birds were euthanatized by cervical dislocation. Then, bacterial swabs were taken from the air sacs and cultured on Columbia sheep blood agar using a typical method of inoculation and streaking on four quadrants of the plate of medium. A semi-quantitative estimate of *E. coli* isolation was conducted on Columbia sheep blood agar. Growth on these plates were recorded on a scale from 0 to 4+, where 0 = no growth; 1+ = growth of bacteria on the area 1; 2+

= growth of the bacteria on areas 1 and 2; 3+ = growth of bacteria on areas 1, 2, and 3; and 4+ = growth of bacteria on areas 1, 2, 3, and 4. (24).

4.3.3 Synthetic CpG-ODN and CpG-ODN formulation

The sequence of CpG ODN (CpG 2007) used was 5'-TCGTCGTTGTCGTTTTGTCGTT-3'.and non CpG-ODN was 5'-TGCTGCTTGTGCTTTTGTGCTT-3'.ODNs were produced with a phosphorothioate backbone (Operon Biotechnologies, Inc. Huntsville, AL). CpG-ODN formulations were prepared either with SWNTs or LSCs at the School of Pharmacy, University of Waterloo, Canada. Synthetic CpG-ODNs were diluted in sterile, pyrogen-free saline or formulation substrates and administered in a 100 μL volume by the *in ovo* route into the amniotic cavity through the air cell of the egg using a 22 gauge, 1 inch needle. The volume of the injection and the length of the needle were selected to simulate the *in ovo* injection technology used in the poultry industry.

4.3.3.1 CNT formulations

SWNT #1 CpG-ODN was prepared using 0.1 mg/mL HiPco SWNTs (Unidym, Inc., Sunnyvale, CA) and 0.5 mg/mL CpG-ODN aqueous solution. SWNT #2 CpG-ODN contained 0.1 mg/mL Puretubes SWNTs (NanoIntegris, Inc., Menlo Park, CA) and 0.5 mg/mL CpG-ODN aqueous solution. Both CNT formulations were prepared in glass vials and bath sonicated for 5 hours at temperatures not exceeding 40 C. Final doses contained 10 μg SWNT and 50 μg CpG-ODN per 100 μL.

4.3.3.2 Lipid surfactant formulations

LSC-CpG-ODN (LSC # 1-CpG-ODN) formulations were made up of two phases: an aqueous phase (AP;1.1 mg/mL gemini surfactant 12-7NCH_3-12 [synthesized in house] and 0.5mg/mL CpG-ODN dissolved in sterile water for injection (WfI) [Fisher Scientific, Toronto, ON, Canada] and a lipid phase (LP; soya phosphatidylcholine, 200 mg/mL (Phospholipon 90H, Nattermann Phospholipids GmbH, Koln, Germany], cholesterol, 20 mg/mL[Croda, Vaughan, ON, Canada], propylene glycol, 400 mg/mL [Spectrum Chemicals, Gardena, CA]). In a glass vial, all of the LP ingredients were weighed out and melted in a 70 C water bath until clear. The premixed, prewarmed (to 40 C) AP was added to the LP and incorporated by intermittent vortexing/heating

until a homogenous formulation formed. This final mixture was then sonicated in a cup-horn Sonicator 4000 (Misonix, Qsonica, LLC, Newtown, CT) on amplitude 11 for 30 min.

Gemini surfactant-CpG-ODN complexes (LSC # 2-CpG-ODN) were prepared by dissolving 0.5 mg/mL CpG-ODN in 1.25 mg/mL gemini surfactant 12-3-12 (synthesized in house) in WfI. The mixture was bath sonicated for 3 hours at temperatures not exceeding 40 C.

4.3.4 Experimental design

4.3.4.1 Delivery of CpG-ODN formulations by the *in ovo* route

The objective of this experiment was to enhance the immunoprotective effects of CpG-ODN by formulating with SWNTs or LSC in neonatal birds. Eighteen day old embryonated eggs, divided to four groups were injected with either 50 µg of CpG-ODN formulated with SWNT (SWNT # 1 CpG-ODN), LSC-CpG-ODN (LSC # 1- CpG), unformulated CpG-ODN or saline in a total volume of 100 µL/embryo by the *in ovo* route to the amniotic cavity . Forty embryonating eggs were randomly allocated into each of the four groups. After hatching, the groups of birds were inoculated on day 1 of age (which was approximately 24 h following hatch) with 1×10^5 or 1×10^4 cfu of stationary phase *E. coli* by the subcutaneous route in the neck. Half of each group (n=20) were inoculated with $1x10^5$ cfu of *E. coli* and the remaining birds (n=20) were inoculated with $1x10^4$ cfu of *E. coli*. Birds were examined daily for eight days for clinical signs following *E. coli* challenge. Bacterial swabs from air sacs were collected from all dead and euthanized birds during the clinical evaluation period, and at the termination of the experiment and cultured.

The second part of the objective was to study the immunoprotective effect of CpG-ODN formulated with two other delivery systems: SWNT (SWNT # 2 CpG-ODN) and LSC # 2-CpG-ODN. *In ovo* delivery of formulations, *E. coli* challenge experiments, ensuing clinical evaluation, mortality and bacterial isolations were performed as stated above. In this trial the group sizes were SWNT #2-CpG-ODN: n = 33; LSC # 2-CpG-ODN: n = 40; CpG-ODN: n = 26; and saline n = 40. A group of 25 birds was maintained with no *E. coli* challenge for each experiment as the viability control of the broiler chicks.

4.3.4.2 Safety of CpG-ODN formulated with CNT or LSC

The third objective of this project was to study the safety and toxicity of these CpG-ODN formulations in neonatal broiler chickens until 9 or 42 days post-hatch while being conducted in two separate experiments. In the first experiment, four groups of birds (n=18 for each group) were injected with SWNT#1-CpG-ODN , LSC # 1-CpG-ODN, CpG-ODN or saline by the *in ovo* route as described above, but were not challenged with *E. coli*. Birds were examined daily for any clinical signs and mortality.For histopathological examination, tissue sections were collected from three embryonated eggs or birds that were randomly selected and euthanized from each group at six different time points. Tissues harvested were, the gastrointestinal tract (crop, esophagus, proventriculus, gizzard, duodenum, jejunum, ileum, and ceca), kidneys, lungs, liver, muscle, trachea and lymphoid organs (spleen, bursa, and thymus) at 19, 20 and 21 days of incubation and at 3, 6 and 9 days post-hatch. Upon collection, tissue sections were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5μm thickness and stained with hematoxylin and eosin.

In the second experiment, 10 groups of birds (n= 35 for each group) were injected with SWNT#1-CpG-ODN ,SWNT #1-non CpG-ODN, LSC # 1-CpG-ODN, LSC # 1-non CpG-ODN, SWNT#2-CpG-ODN ,SWNT #2-non CpG-ODN, LSC # 2-CpG-ODN, LSC # 2-non CpG-ODN , CpG-ODN or saline by the *in ovo* route as described earlier. These birds were not challenged with *E. coli* and were observed daily for any clinical signs or mortality up to 42 days post hatch. Tissue sections were collected as in the first experiment from the above mentioned organs at the day of hatch and at days 7, 14, 28 and 42 post-hatch for histopathology. At each time point, three birds were randomly selected from each group and euthanized for sample collection. Tissue sections were processed for histopathology as above. Bursal weight to body weight ratio (BBW) is a good indicator of growth of birds and the reflection of the immune function of growing birds (19). At day 42 post-hatch, following euthanasia, BBW was obtained in addition to tissue sample collection for histopathology.

4.4 Statistical analysis

Survival and other data were analyzed with the use of Prism (Prism 5.0, GraphPad Software Inc., San Diego, CA) and Statistix7 (Analytical Software, Tallahassee, FL) with a significance level of $P<0.05$. The survival patterns and median survival times were compared using the log-

rank test and chi-square statistic. The relative risk of mortality for control subjects was calculated using proportional hazards regression. The clinical score for each bird was summed over the 8-day observation period and the significance of differences among groups and comparison of BBW between groups were tested with the use of Kruskal-Wallis nonparametric analysis of variance. The homogeneity of distribution of bacterial scores was tested using chi-square statistics.

4.5 Results

4.5.1 Delivery of CpG-ODN formulations by the *in ovo* route

No mortality was reported from the unchallenged viability control birds. The groups of birds that received CpG-ODN either alone or in any formulation experienced about half of the relative risk of mortality as the birds that received saline (0.48; P=0.0020 in the first trial and 0.51; P=0.0044 in the second trial). This immunomodulation occurred in groups challenged with either 1×10^4 or 1×10^5 cfu of *E. coli*, and the data were combined for clarity of presentation and analysis because there was no significant difference in group mortality between low- and high-challenge doses.

Birds in the first trial that received either CpG-ODN formulated with SWNT no. 1 or LSC no. 1 (>75% survival) experienced one third (32%, P<0.001) the relative risk of mortality following a lethal dose of *E. coli* compared to CpG-ODN alone and saline control groups (30% survival;(Figure 4-1A). The groups that received formulations with no CpG-ODN were not protected against *E. coli* challenge (P>0.05). The CCS was significantly lower in birds that received CpG-ODN formulated with SWNT no. 1 or LSC no. 1 compared to birds that received either CpG-ODN alone or saline (P < 0.05; Figure 4-1B). Moreover, birds that received CpG-ODN formulated with either SWNT no. 1 or LSC no. 1 had a significantly greater proportion of lower bacterial loads compared to birds that received CpG-ODN alone or saline (P<0.005; Figure 4-1C). Birds that died or were euthanatized either had airsacculitis or pericarditis or a combination of airsacculitis together with pericarditis or polyserositis.

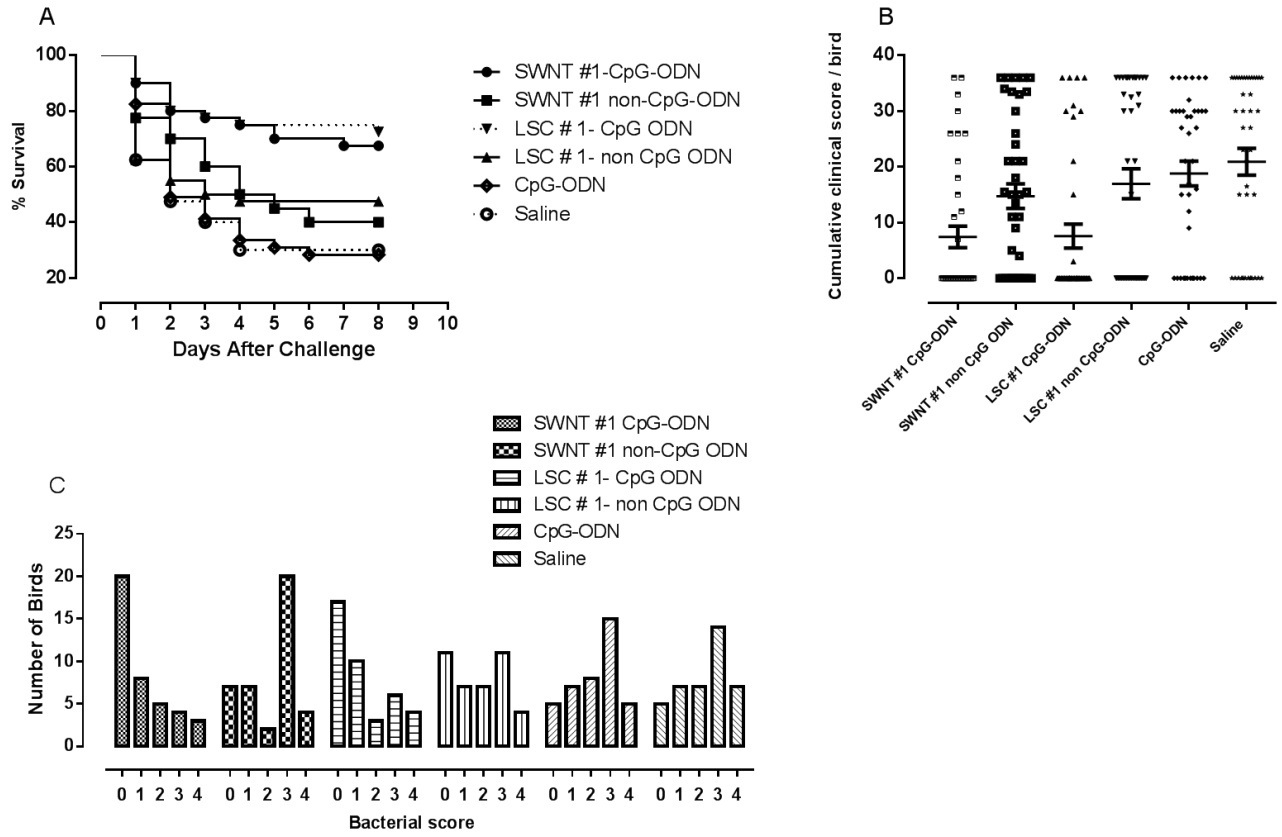

Figure 4-1: Experiment 1 results (survival percentages, CCS and bacterial scores). (A) Survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day 18 of incubation were injected with CpG-ODN or non CpG-ODN formulated with SWNT # 1 or LSC # 1, CpG-ODN alone or saline by the *in ovo* route (n = 40 per group). 50% of birds in each group (n = 20) were SC inoculated with 1×10^4 *E. coli* and the remaining 50% of birds (n = 20) were SC inoculated with 1×10^5 cfu of *E. coli* at 1 day post-hatch which was approximately 24 hours following hatch. Groups of birds that received SWNT # 1 CpG-ODN or LSC # 1-CpG-ODN were significantly protected against *E. coli* infection compared to the control group ($P<0.0001$). **(B)** CCS of broiler chickens following *E. coli* challenge. CCS of neonatal broiler chickens following challenge with 1×10^4 (n = 20) or 1×10^5 (n = 20) cfu of *E. coli*. Groups of birds that received 50 µg CpG-ODN formulated as SWNT # 1-CpG-ODN, or LSC #1-CpG-ODN had a significantly lower CCS ($P<0.05$) compared to the unformulated CpG-ODN or saline groups. (Bar = median). **(C)** Bars show the number of birds in each treatment group that had each classification of bacterial growth. Bacterial swabs were taken from air sacs. Groups that received formulated CpG-ODN had fewer birds that showed higher levels of bacterial growth.

In the second trial, egg hatch was incomplete at the time of *E. coli* challenge; hence some groups had a reduced number of birds (SWNT no. 2 CpG-ODN, n = 33; SWNT no. 2 non-CpG-ODN, n = 32; LSC no. 2 CpG-ODN, n = 40; LSC no. 2 non-CpG-ODN, n = 36; CpG-ODN, n =26; and saline, n=40). Birds that received CpG-ODN formulated with SWNT no. 2 or LSC no. 2

experienced significantly lower risk of mortality (57%, P=0.0149) compared to the birds that received CpG-ODN alone or saline (Figure 4-2A). The groups that received formulations with no CpG-ODN were not protected against *E. coli* challenge (P>0.05). The CCS was significantly different among groups (P=0.016), and there were two sets of groups between which the CCS was significantly different (P< 0.05), as shown in (Figure 4-2B) (SWNT no. 2 CpG-ODN or LSC no. 2 CpG-ODN vs. CpG-ODN or saline). Additionally, birds that received various treatments had significantly different bacterial loads (overall chi-square = 30.94; P=0.002), and more of the birds that received CpG-ODN alone or saline had the higher levels of bacteria (Figure 4-2C)

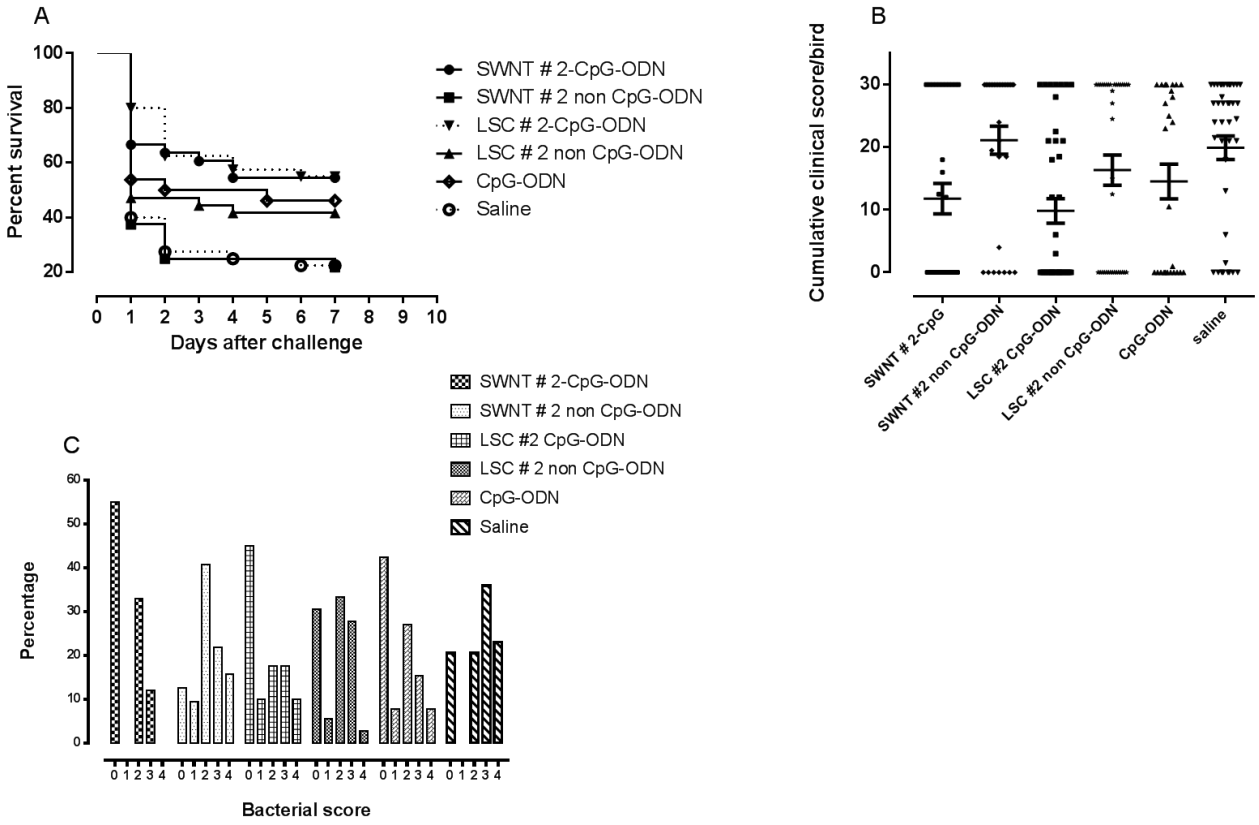

Figure 4-2: Experiment 2 results (survival percentages, CCS and bacterial scores). (A) Survival of broiler chickens following *E. coli* challenge. Groups of broiler chicken embryos at day 18 of incubation were injected with CpG-ODN and non CpG-ODN formulated with SWNT # 2 or LSC # 2, CpG-ODN alone or saline by the *in ovo* route (n = ~40 per group). Groups of birds that received - SWNT # 2 CpG-ODN or LSC # 2- CpG-ODN were significantly protected against *E. coli* infection compared to the saline control group and control SWNT #2 non CpG-ODN group ($P<0.0001$). The group that received unformulated CpG-ODN tended to have increased survival, but survival was not statistically significant ($P=0.0573$). **(B)** CCS of broiler chickens following *E. coli* challenge**.** CCS of neonatal broiler chickens following challenge with 1×10^4 (n = 20) or 1×10^5 (n = 20) cfu of *E. coli.* Groups of birds that received 50 µg CpG-ODN formulated as SWNT # 2-CpG-ODN, or LSC # 2 CpG-ODN had a significantly lower CCS compared to the unformulated CpG-ODN or saline group. [Bird Numbers per group; SWNT #2-CpG-ODN: n = 33; SWNT #2 non CpG-ODN: n = 32; LSC # 2-CpG-ODN: n = 40; LSC # 2 non CpG-ODN: n = 36; CpG-ODN: n = 26; and Saline n = 40] (Bar = median). **(C)** Heavy bacterial growth was observed more frequently in lesions from birds in the control group than in the groups treated with SWNT # 2-CpG-ODN, LSC # 2 CpG-ODN or unformulated CpG-ODN.

4.5.2 Safety of CpG-ODN formulated with CNT or LSC

No mortality or clinical signs were detected in any of the embryos or birds following administration of CpG-ODN formulations throughout day 9 post-hatch in the first safety experiment. No histopathological lesions were diagnosed in any of the organs at any of the time

points of embryos or birds throughout day 9 post-hatch in groups that received any CpG-ODN formulation, CpG-ODN or saline.

In the second safety experiment, no histopathological lesions were observed at any of the time points throughout day 42 post-hatch in groups that received CpG-ODN formulations, formulation controls (SWNT # 1 non-CpG-ODN, LSC non-CpG-ODN, SWNT # 2 non-CpG-ODN and surfactant non-CpG-ODN), CpG-ODN or saline. The expected mortality or euthanasia of birds due to leg problems (*i.e* vulgar and varus deformity), sudden death syndrome, ascites and right ventricular failure was not significantly different among groups ($P>0.05$). There was no significant difference of the BBW ratio among groups treated with CpG-ODN formulations and control groups ($P>0.05$, Figure 4-3).

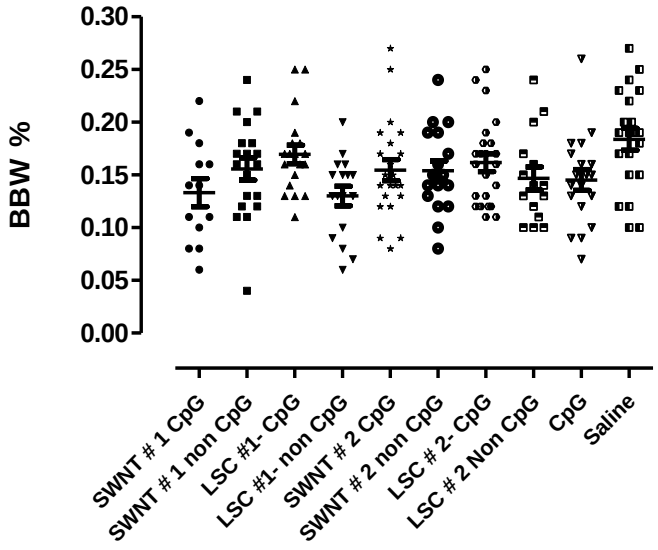

Figure 4-3: BBW of birds at 42 days of age. There was no significant difference between the BBW in groups treated with CpG-ODN formulations or control groups (bar = median).

4.6 Discussion

Immune-stimulatory measures that provide protection against infectious diseases have gained great attention at present. They are well known to stimulate the host's immune system and prepare the host to fight pathogenic microbes. Disease prevention by prophylactic measures like immune stimulation is favoured compared to the use of therapeutic agents like antibiotics which may lead to concerns about residues in edible products, particularly in the food-animal industry. Over the years, the use of antibiotics in the food-animal industry has resulted in the emergence of

resistant strains of bacteria and the possibility of drug residues in meat products. For these reasons, novel immune-stimulants are proposed and investigated as alternative, desirable ways to prevent disease in these species. CpG-ODN has been used as an immunostimulant in many species, including chickens (49, 105, 151, 228, 264). Previously, we have demonstrated that CpG-ODN is an effective immunostimulant in chickens and appeared to have its effectiveness for six days (105) following administration. Moreover, we were able to increase the effectiveness of CpG-ODN by formulating with polyphosphazenes (302). In the present study; we demonstrated a significant health promoting effect of CpG-ODN formulated with CNT or LSC. CpG-ODN formulations significantly increase survival, reduce clinical signs and decrease the bacterial load in birds challenged with *E. coli.*

E. coli or *Salmonella* septicemia is common in the neonatal period of broiler chickens and causes significant mortality and production losses (301). Furthermore, if broiler flocks experience mortality greater than 2% in the first week of life, the overall performance of the flock becomes very poor due to chronic infections, poor weight gain and uneven birds in the flock (160). It is essential to maintain good health during the first week of broiler chicken growth as it will provide better performance and profitability at the time of production (77). Currently, in the broiler chicken industry, birds can reach a 2 kilogram body weight by 30-35 days post-hatch (253). Thus, *in ovo* delivery of CpG-ODN formulations either with CNT or lipid formulations (LSC) may provide effective immunostimulation and may be a better alternative to antibiotics. Likewise, *in ovo* delivery of vaccines is common practice in the broiler chicken industry. Hence, the delivery of CpG-ODN formulations is practically feasible. We have previously demonstrated that *in ovo* delivery of unformulated CpG-ODN are effective against *E. coli* (105) or *Salmonella* Typhimurium (301) septicemia in neonatal broilers, and here we demonstrate a significant potentiation of CpG-ODN with CNT or lipid delivery systems in chicken embryos. Moreover, CpG-ODN formulations significantly reduce the bacterial load in birds infected with *E. coli,* and suggest that CpG-ODNs may be able to reduce the bacterial load in birds in commercial production. We cannot use our animal model of *E. coli* to validate this possibility since it causes very high (between 60-80%) mortality following challenge.

Even though the potential of using CNTs in drug delivery are widely demonstrated, studies have also shown that these particles can potentially cause adverse effects because of their small size and extreme aspect ratio (89). However, controversy surrounds the interpretation ascribed to

CNT toxicity data. The toxicity reported has found to be related to many properties of the CNT material, such as their structure (SWNT vs. MWNT), length and aspect ratio, surface area, degree of aggregation, extent of oxidation, surface topology, bound functional group(s), and method of manufacturing which can leave catalyst residues and result in impurities. Toxicity of CNTs is also related to their concentration and the dose to which cells or organisms are exposed (90). Broiler chicken embryos are a well suited model to study toxicity of CNT hence we are able to study embryo mortality, hatchability, and clinical signs during a life span of the broiler chicken. In addition, all organs can be studied for histopathology to identify any morphological changes. We did not see embryo mortality, decreased hatchability, clinical signs or any evidence of alteration of the morphology of any internal organs that we studied during day 18 of incubation throughout day 42 post-hatch. The bursa of Fabricius is an organ which reflects the immune system and overall health of a chicken. Therefore, bursal size is a biological indicator of how well the health of a flock is managed and protected from disease. Therefore the BBW is a good indicator of the health status of broiler chickens. The BBW ratio was not significantly different among groups of birds treated with CpG-ODN formulations or control groups hence, CpG-ODN formulated with CNT or LSC did not affect the growth of birds and their immune system. Furthermore, lipid based delivery systems, have previously proven to be safe and effective for delivering CpG ODN in many species of animals (10, 11).

In conclusion, CpG-ODN formulated with CNT or lipid surfactant vesicles was observed to synergistically enhance the immunoprotective efficiency against *E. coli* infections in neonatal broiler chickens. A large-scale field study will eventually determine the utility of CpG-ODN in the broiler chicken industry. It is very encouraging to note that CpG-ODN formulations with CNT did not cause adverse reactions in chicken embryos or neonatal broiler chickens. To our knowledge, this is the first time that *in ovo* delivery of CpG-ODN formulated with CNT or lipid-surfactant delivery systems have shown protection against a lethal bacterial infection in broiler chickens.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

The global poultry production was 111,000 thousand metric tons in 2015 and world poultry production is projected to increase by 24% over the next decades, reaching 131,255 thousand metric tons in 2025 (246). Canada produced chicken products worth $2.5 billion in 2016 (4). Although global poultry production increases at a significant rate there are few diseases causing significant impact on its growth. Among diseases of the broiler chicken industry, yolk sac infections due to bacterial infections in neonatal broiler chickens is a major problem in any poultry producing country. Immaturity of the immune system and stressors associated with handling and transport at the time of hatch can make neonatal broiler chickens susceptible to bacterial pathogens that could originate from the enviroment. The poultry industry has been using antibiotics as preventive measure to reduce these bacterial infections in neonatal chickens but this practice has been reducing in many countries due to emergence of antimicrobial resistant bacteria. In 2014, the Canadian poultry industry voluntarily withdrew the use of category I antibiotics (43). Because of these reasons, alternatives to antibiotics and immunotherapeutic agents were received a great interest. Among them, CpG-ODN or synthetic CpG motifs have demonstrated immunostimulatory properties against patogens. CpG-ODN has shown its effective immune stimulation in chickens against many bacterial (104, 105, 301), viral (65, 310) and protozoal (63) infections. However, the mechanism of CpG-ODN induced immunoprotection remains poorly understood in chickens. The objective of this study was to investigate immune mechanisms at cellular and cytokine gene expression levels following *in ovo* delivery of CpG-ODN as an immune stimulator in broiler chicken embryos. We have demonstrated cytokine gene expression and T-cell activation following *in ovo* CpG-ODN administration and effect of CpG-ODN on its dose against *E. coli* infection. We have also demonstrated formulation of CpG-ODN with CNTs and and liposomes to enhance immunoprotive efficacy of CpG-ODN against bacterial septicemia.

The innate immune system plays an important role in identifying and fighting pathogens leading to effective adaptive immunity that protect the host against infection (145). In the second chapter, we demonstrated increased expression of key immunomodulatory cytokines and their role associated with CpG-ODN induced immunoprotection in neonatal chickens against bacterial infections. We selected the spleen and lung as organs of interest to assess cytokine gene expression and cellular changes. Cytokines play a significant role in inflammation and immune modulation. We showed that all cytokines that we evaluated were upregulated than the control group at most

time points in both organs. Not only Th1 (IFNs) cytokines but IL4, a Th2 cytokine and IL10 which is a regulatory cytokine was upregulated upon CpG-ODN administration. Although we have previously shown that CpG-ODN promote Th1 type immunity in chickens (240), our current study showed both Th1 and Th2 type immune responses following CpG-ODN administration as shown in a recent study in chickens agaist *Campylobacter* colonization in the intestine (304). The data from our study can also be used in designing CpG-ODN as a vaccine adjuvant for poultry vaccinest. Furthermore, the upregulation of pro-inflammatory cytokines (IL1β, IL6, IL18, and Litaf) in both spleen and lung shows that CpG-ODN induce an proinflammatory response, which may contribute to fight against bacterial infections.We also noted a distinct CpG-ODN induced pro-inflammatory cytokine gene upregulation in lung compared to the spleen, suggesting enhanced mucosal immunity at the time of hatch in chickens. These findings are also supported by recent studies that show CpG-ODN induce pro-inflammatory cytokine gene upregulation (304) as well as their contribution to supress *Campylobacter* colonization in the gut (305) in chickens. The conditioning of mucosal surfaces by CpG-ODN to face pathogens more effectively are proven by these findings.

Cytokines are well known as immune mediators, acting on immune cells and promoting induction of other cytokines (154). They are also modulators of recruiting immune cells to inflammatory sites (81) and causing upregulation of costimulatory molecules on APCs, resulting in optimal interaction of APCs with naive CD4+ and CD8+ T cells (137). Our flow cytometry data showed a significantly enhanced enrichment of macrophages in both spleen and lungs of CpG-ODN treated embryo and chicks, and these macrophages in CpG-ODN treated chicks were expressing significantly higher CD40; whereas, CD86 was not very different from saline control. The changes in APCs of CpG-ODN treated chickens can be explained by their cytokine profiles. We specifically noted the upregulation of Litaf gene expression throughout the experiment, a cytokine that have shown to contribute to the upregulation of CD40 (267, 287). Moreover, it has been shown that CD40 expressing APCs are required to efficiently activate, cause maturation and proliferation of T cells in mice (111). Our findings also show that CpG-ODN promote the enrichment of T-cell immunological niches in both spleen and lung in chicken. We also evaluated the thymic T cell population and observed high expression of both CD4+ and CD8+ in the thymocytes following CpG-ODN treatment, suggesting a potentially increased thymic output. Thus, we suggest that the increase of T cells in both spleen and lung following CpG-ODN

treatment may be due to enhanced recruitment of T cells and increase thymic output. Interestingly, previous studies have shown that TNFα factor is constitutively synthesized in the thymus (97) and promotes T cell development (268). Thus, CpG-ODN induced upregulation of Litaf gene can be acknowledged for playing a significant role in enriching the immune compartment in chickens. However, further detailed studies are needed for a proper understanding of CpG-ODN induced Litaf action in chickens. All these immunological changes can be explained as a cascade of events interconnected and priming the immune system of chickens at the time of hatch, which also explains the protection against infections shown in previous studies in neonatal chickens.

We recently report that intrapulmonary delivery of CpG-ODN in chickens resulted in enhanced infiltration of inflammatory cells in the pulmonary parenchyma (106). In the third chapter of this thesis, we investigated immune cell recruitment at the time of hatch, following different doses of CpG-ODN (5, 10, 25, 50 µg of CpG-ODN per embryo) by *in ovo* delivery in chickens and further examined the correlation of their immunological profiles with the protection against *E. coli* infection in chicks. We selected spleen, the main secondary lymphoid organ, which represent systemic changes, and lung, which represent a local mucosal change and one of the most common sites for pathogen encounter. Our results showed a CpG-ODN-dose-dependent increase in the number of macrophages/monocytes in the immunological niches in both spleen and lungs. Moreover, we observed an increase in CD40 expression with different doses of CpG-ODN. CD40 is recognised as an important molecule involved in many immune functions such as, B cell proliferation, differentiation, T cell proliferation, monocyte and dendritic cell growth and cytokine production (7, 31, 169). Moreover, we observed a significant increase in MHCII-expressing APCs with 25 and 50 µg of *in ovo* CpG-ODN in both lung and spleen, which suggest enhanced antigen presentation ability resulting in effective and rapid pathogen clearance. These findings suggest that the immunological niches in CpG-ODN treated chicks are well equipped with mature APCs with the ability to activate effector immune cells such as CD4+ and CD8+ T lymphocytes. We also report a dose-dependent increase in CD4+ and CD8+ T cell populations, which play important role in humoral and cell-mediated immunity following CpG-ODN treatment. In order to evaluate the correlation of immunological changes with immune-protection against *E. coli* infection, we assessed the survival (protection level) following a lethal *E. coli* challenge. We found significant survival levels in birds that received 25 and 50 µg of CpG-ODN compared to the saline control, but not birds that received 10 µg or less CpG-ODN. The changes in immune profiles correlated

with survival data, where 25 µg and 50 µg of CpG-ODN demonstrated significantly higher MHCII-expression on APCs. CD40 expression on APCs and T cell populations correlated with their ability to resist *E. coli* infection at the time of hatch. Birds that received 10 µg or less CpG-ODN showed enhanced immune profiles, but were not significantly different from the saline control. Our findings revealed that CpG-ODN not only stimulates immune cells but also remarkably enriches the immunological niche, making neonatal chicks well prepared to tackle pathogenic insults at the time of hatch.

Although *in ovo* delivery CpG-ODN showed immunoprotective effects on neonatal chickens against bacterial infections, there are few optimizations to be maid prior to use in poultry hatcheries. Among them, the dose of CpG-ODN is important since amount of CpG-ODN reaching the chicken embryo is limited due to protective substances such as nucleases of the amniotic fluid surrounding the embryo. Although CpG-ODN reaches the embryo through the mouth, it is difficult to estimate amount of CpG-ODN needed to stimulate intracellular receptors such as TLR21 of APCs and immune cells of mucosal surfaces of lungs and systemic organs such as the spleen. Nanoparticle based delivery systems are often considered as ways to overcome these challenges. A successful CpG-ODN delivering system will condense and encapsulate the ODN, while protecting it from neucleases (325) and facilitate cellular uptake by acting as a nano-carrier (349). We have evaluated the possibility of using two types of *in ovo* CpG-ODN delivery systems in broiler chickens. The fourth chapter of this thesis reveal data on potentiation of CpG-ODN immune stimulatory action when formulated with either a lipid based delivery system or CNT based delivery system. Survival of birds were significantly higher with CpG-ODN formulations used, compared to CpG-ODN alone and saline groups following an *E. coli* challenge. Moreover, these formulations did not cause any adverse effects on birds throughout the trial period and until they reached their processing age (42 days of age). These data revealed that the lipid surfactant and CNT delivery systems that we used can safely enhance the immune protective effect of CpG-ODN against an *E. coli* infection in neonatal broiler chickens.

Findings of our research shows that *in ovo* CpG-ODN not only protect chickens from neonatal bacterial infections but also possible to enhance the effectiveness of poultry vaccines that given in neonatal life of broiler chickens. Several vaccines are given to chickens at day 18 of incubation or at the time of hatch at hatcheries. Developing a desired immunity in neonatal chickens against vaccine antigens can be a challenge due to their immaturity of the immune system

or due to lack of sensitization of immune system with antigens at hatch. We have shown a maturation and enrichment of immune compartments following *in ovo* delivery of CpG-ODN treatment and this can be used as beneficial prerqisite of neonatal vaccine delivery. Therefore, CpG-ODN treated chicken immune systems will be more receptive to vaccine antigens, resulting in rapid immune responses and immunological memory. This will be particularly be beneficial for vaccines against diseases like necrotic enteritis, where single vaccinations at day one are non-protective in broiler chickens, (221) and quicker, effective immune responses are desired. We also highlight the role of CpG-ODN induced Litaf in modulating many aspects of immune functions in our study. However, further studies are needed for better understanding and confirmation of the TLR21-Litaf-mediated immune enrichment in chickens. This can be done as in other laboratory animals although knockout chicken models are rare but not impossible (272). A Litaf gene knockout chicken model may be an ideal model to study the role of CpG-ODN induced Litaf in maturation and enhancement of immune cells. Exploring the expression levels of TLR21 gene or TLR21 receptor itself following CpG-ODN treatment will also confirm the involvement of this TLR in chicken immune system.

In conclusion, we reveal that *in ovo* delivery of CpG-ODN provides protection in neonatal chicks against *E. coli* infection by eliciting cytokine responses, as well as enriching immunological niches in spleen and lungs. Finally, our findings on optimization of CpG-ODN and enrichment of immune cells and cytokines in differenet immune compartments and its correlation with protection of neonatal broiler chickens against *E. coli* infection provides importance of dose of CpG-ODN when CpG-ODN is used under field conditions. Furthermore, we have demonstrated formulation systems such as CNTs or liposomes to potentiate the immune stimulation of CpG-ODN and shown their safety in the poultry industry as an alternative to antibiotic. Although we have demonstrated utility of CpG-ODNs under laboratory conditions it is necessary to conduct field trials in order to validate our observations under field conditions.

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