INTRAPULMONARY DELIVERY OF OLIGODEOXYNUCLEOTIDES CONTAINING CYTOSINE PHOSPHODIESTER GUANINE MOTIFS (CPG-ODN) TO 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-ODN) are known for their ability to stimulate the vertebral immune system and provide protection against pathogens. We have demonstrated the effect of CpG-ODN against *Escherichia coli* and *Salmonella* infections by *in ovo* and parenteral delivery. This study's objective was to discover the efficacy of immunomodulatory effects of CpG-ODN by the intrapulmonary (IPL) route as micro droplets.

In the second chapter, we demonstrated immunoprotection of neonatal broiler chickens against a lethal challenge of *E. coli* following IPL delivery of CpG-ODN. Immunoprotection was observed 6 hours following CpG-ODN and lasted for five days post treatment. CpG-ODN treated birds showed significantly lower clinical scores and lower bacterial load in the body.

In the third chapter, we demonstrated the efficacy of IPL delivery of CpG-ODN under field conditions by developing a commercial-scale prototype nebulizer (CSPN). The CSPN was able to deliver CpG-ODN to 8,000 birds at a time. We were able to deliver CpG-ODN by the IPL route to protect neonatal broiler chicks against lethal *E. coli* septicemia at a significant level when the humidex was at or below 28 and relative humidity was 40-60% ($P<0.05$) in the CSPN. Results of this study confirmed that IPL delivery of CpG-ODN against septicemia in neonatal broiler chickens was industrially feasible and effective under different weather conditions.

In the fourth chapter, we explored the antibacterial mechanisms of CpG-ODN by IPL delivery. We observed T helper (Th)1 and Th2 type cytokine upregulation together with interleukin (IL) 1β and lipopolysaccharide-induced tumor necrosis factor (LITAF) in spleen and lungs following CpG-ODN delivery. It was also evident that the spleen and lungs had increased infiltration of antigen presenting cells (APCs) and cluster of differentiation (CD) 4+ and CD8+ T cells. Costimulatory molecule CD40 upregulation and distinct major histocompatibility class II expression in lung APCs indicated the cells were maturing and presenting antigens actively.

In the fifth chapter, we utilized the novel metabolomics analysis tool and investigated the changes in the metabolome of broiler chicks upon CpG-ODN administration. The metabolites that contributed to the distinct difference belonged to various pathways of energy metabolism. Our work on IPL delivery of CpG-ODN against *E. coli* septicemia demonstrated the potential utilization of CpG-ODN in the poultry industry as an alternative to antibiotics to prevent losses due to bacterial infections of neonatal chickens.

ACKNOWLEDGEMENTS

First, my heartfelt gratitude goes to my supervisor and mentor Dr. Susantha Gomis for providing me the opportunity to come to the beautiful country of Canada and be a part of his excellent research team. His guidance and visionary thinking helped me to build my own path in a research career. I would like to acknowledge research technician Shelly Popowich for helping me every step of the way with the development of our innovative idea. Thanks to lab manager Betty Lockerbie for her excellent support. I'm so grateful to our research associate Dr. Ahmed Khawaja (Ash) for his magnificent research mentorship and writing guidance that provoked my thoughts as a budding researcher. Thanks to our research associate Dr. Lisanework Ayalew for spending his valuable time without any hesitation, supporting various research ideas I had. My sincere gratitude goes to my colleagues, Thushari Gunawardana, Ashish Gupta, Shani Kurukulasuriya, Ruwani Karunarathna and Mengying Liu for giving their physical and emotional support for the past 6 years. Their contribution for each other's research is undoubtedly invaluable and exemplary. I would like to appreciate my advisory committee Drs. Marianna Foldvari, Phil Willson, Suresh Tikoo, Bev Kidney and Elemir Simko for being critical and constructive during my academic journey. An immense recognition goes to the RMD Engineering team; Stephen Gebhardt, Kevin Roberts and Jim Boire for their excellent support in taking our research idea to a practical application. Thanks to engineer Houman Kamali for his hard work and Dr. Carey Simonson for the inputs in making our industry application successful. I truly appreciate Dr. Neil Ambrose, Klaus Korthius, Rick Corvino, Ana Meza and staff members of the commercial poultry hatcheries in SK and BC for being a huge strength to us. I acknowledge Innovation Enterprise for their support towards our innovative idea. My gratitude goes to the funding agencies NSERC, WEDC, ALMA, Chicken farmers of Saskatchewan and Alberta Chicken Producers for their constant support. My family has been the biggest strength all these years. My gratitude to husband, Rajika Goonewardene for all the sacrifices he made to make my dream a reality. I owe my mother Pushpa and father Jayasundara Bandara, for raising me up to be this independent woman who follows her passion. Loving regards to brother Seninda and my four pawed little sister Khushi. I thank my mother and father-in-law Chintha and Jith and sister-in-law Thiyagi for their support. I'm thankful to all the friends I met in Saskatoon for accepting me and being my family away from family for the past 7 years.

DEDICATION

THIS THESIS IS DEDICATED TO MY ONE IN A MILLION HUSBAND, RAJIKA GOONEWARDENE FOR HIS LOVE, PATIENCE AND UNCONDITIONAL SUPPORT.

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^{1}H NMR	Hydrogen-1 nuclear magnetic resonance
ACU	Animal care unit
APC	Antigen presenting cell
APEC	Avian pathogenic *E. coli*
BALT	Bronchi associated lymphoid tissue
BBW	Bursa weight to body weight ratio
BCG	Bacillus Calmette Guérin
CCS	Cumulative clinical score
CD	Cluster of differentiation
CFC	Chicken Farmers of Canada
CFM	Cubic feet per minute
CFU	Colony forming units
CO_2	Carbon dioxide
CpG-ODN	Cytosine phosphodiester guanine oligodeoxynucleotides
CSPN	Commercial scale poultry nebulizer
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DNA	Deoxyribonucleic acid
ds	Double stranded
DW	Distilled water
FDR	False discovery rate
FITC	Fluoroscein isothiocyanate
FWM	First week mortality
GALT	Gut associated lymphoid tissue
GC	Germinal center
HIV	Human immunodeficiency virus
HKG	House keeping gene
Hprt 1	Hypoxanthine-guanine phosphoribosyl transferase 1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
IM	Intramuscular
IPL	Intrapulmonary
LITAF	Lipopolysaccharide-induced tumor necrosis factor-alpha factor
LPS	Lipopolysaccharide
MALT	Mucosa Associated Lymphoid Tissue
MFI	Mean Fluorecent Intensity
MHC	Major Histo Compatibility
NFκβ	Nuclear factor κ kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NO	Nitric oxide
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PCA	Principal component analysis
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PLGA	Poly (D,L-lactide-co-glycolide)
PLS-DA	Partial least Squares-discriminant analysis
PRR	Pattern recognition receptors
RH	Relative humidity
RNA	Ribonucleic acid
SEM	Standard Error of Mean
SQ	Subcutaneous
ss	Single stranded
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
Tubb1	Tubulin beta 1
VIP	Variable importance in projection
YSI	Yolk sac infection

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Worldwide, the poultry industry is choosing antibiotic-free production due to rapidly increasing customer demand resulting from public health points of view (55). Infections caused by harmful bacteria particularly during the first week of birds' lives result in severe mortalities, poor weight gain and poor flock uniformity that lead to economic losses for producers. Prophylactic and in-feed growth-promoting antibiotics have been the preventative strategy for a long time for these on-going problems. However, various public health concerns such as the emergence of antibiotic-resistant bacteria in the environment and antibiotic residues in food have raised questions regarding this practice (55,268). While the Canadian poultry industry has responsibly begun to gradually withdraw antibiotics from the system, they are keenly searching for alternatives to ensure health and wellbeing of the poultry (85). Stimulation of the immune system of poultry has been one approach explored by poultry scientists to fight against harmful bacterial infections (33,34,38). Oligodeoxynucleotides containing cytosine-phosphodiester-guanine motifs (CpG-ODN) have been proven to be immune stimulants in chickens and protect against bacterial and viral infections when delivered via parenteral routes such as intramuscular (IM) (125), subcutaneous (SQ) (126) and *in ovo* (injections into embryonating eggs at day 18 of incubation) (81,137,381). Although these routes have shown promise to a certain extent, their efficacy and practicality in the fast-paced commercial poultry industry, particularly in busy commercial broiler hatcheries, is still questionable. As a result, novel delivery systems and techniques that ensure efficient delivery of immune stimulatory CpG-ODN to neonatal broiler chickens could serve as a vital discovery in the quest to deliver antibiotic alternatives.

1.2 Poultry industry facts in Canada

Poultry is a multibillion-dollar industry in Canada that serves the most popular source of protein to the world. It includes the rearing of chickens, turkeys, ducks, quails and other bird species for meat and eggs. According to the latest information available through Agriculture and Agri Food Canada, in 2017 Canada produced chicken products worth $2.5 billion and contributed 4.1% of cash receipts to farming operations. In the same year, there were 2,836 regulated chicken producers as well as nearly 4,678 commercial poultry and egg producers that contributed to the Canadian economy. In 2017, Canada produced 1.2 billion kilograms (kg)

of chicken; of which, 60.7% were produced in Ontario and Quebec. An average chicken farm produced 421,628 kg of chicken meat. Apart from the substantial local consumption, Canada exported over 6.9 million live chicks to 28 countries including our major buyer, United States of America, and other countries including Japan, Russia, and France as well as 134.1 million kg chicken meat and edible bi-products worth over $441.1 million to 44 countries (8). As a result, it is imperative to maintain healthy poultry flocks to ensure the production of adequate poultry products to fulfill the growing consumer demand and to ensure food safety.

1.3 Bacterial infections in the poultry industry

The commercial poultry industry in general, is involved in raising birds in large quantities at high stocking densities in both broiler and layer production systems. The Canadian Poultry Code of Practice recommends a maximum of 31kg/m^2 stocking density for optimum health and welfare of broilers (448). According to European standards, Yassin *et al*. said that when daily cumulative mortality rates are too high, the farmer should reduce the number of broiler chicks the next cycle (433). However, due to the highly intensive management systems with overcrowded barn environments, the spread of infectious diseases is inevitable. Hence, minimizing mortality in a flock is crucial to gain profit in subsequent cycles. Notably, the first week of life is very important for a chick as its entire life is transforming during this period. Starting from a conditioned life in the hatchery, they transform to an independent life in a barn where they have to adjust to new feed, water, thermoregulation, competition and fight infections at the same time (408). Moreover, as their immune systems are not fully developed at hatch (86), poultry are susceptible to various infections including bacteria, viruses, and parasites. Out of these infections, bacterial infections occur worldwide in a similar pattern causing massive economic losses to the poultry industry(296).

1.3.1 First week mortality in broilers

First week mortality (FWM) is an essential indicator of the chick quality as well as welfare. The ideal first week mortality rate of a poultry flock is 1% or less (156). As FWM contributes highly to the total mortality of the operation, it is important to control it. Multiple factors contribute to an increased FWM including breeder factors such as age (younger breeders, higher chick mortality), strain, genetic line, weight of the eggs (lighter eggs caused more chick

mortality) (267), hatchery factors, feed factors and season (199,433). A study done in Taiwan reported that cumulative mortality of broiler chicks depended on the type of ventilation, flock size, distance of shipping the chicks and delivery route (54). While infectious causes contributed to 50% of FWM, dehydration, and nephropathy associated with visceral gout contributed as the other causes in layer chicks (306).

1.3.2 Bacterial infections as a cause of first week mortality

Bacterial infections are a leading cause of FWM (191). Despite the scarcity of informative studies, Kemmett *et al.* reported their findings on the significant contribution of *Escherichia coli* on the mortality of chicks within the first 48-72 hours of placement. In their study, approximately 70% of the dead chicks displayed signs of colibacillosis, and thirty different virulence profiles were identified in the *E. coli* isolates (194). In a study done on 48 flocks of layers, average FWM was found to be 1.4%. More than half of the flocks showed FWM due to infectious causes, yolk sac infections followed by septicemia being demonstrated in 94% of the flocks. *E. coli* and *Enterococus faecalis* were the most frequently isolated organisms from those infections (306). In a study done in Ethiopia 2010-2011, a prevalence of 33.1% yolk sac infections (YSI) was recorded in the FWM predominantly among chicks 3-5 days old. *E. coli* was isolated as the most prevalent bacteria from these cases followed by *Staphylococcus aureus* (17). Rai *et al.* reviewed YSI prevalence data from previous studies and reported that it is the most common cause of FWM in chicks. According to various reports they have summarized, the incidence of YSI varied between 5.1-20.7% and mortality has been up to 31.5% (328). A recent study by Karunarathna *et al*. reported the incidence of *Enterococcus* (29.71%) and *E. coli* (19.46%) isolated from broiler chicken embryos that died during incubation (191).

1.3.3 Economic significance of first week mortality-associated with bacterial infections

In the commercial poultry industry, FWM means a loss of income to the producer as well as to the hatchery. According to Yassin *et al.*, FWM in the Dutch poultry industry also indicates the chick quality. He also mentions that FWM acts as an indicator of the welfare status of the hatchery according to European Union guidelines (433). According to statistics, chicken producers in Canada were paid $1.57 CAD/kg for a 1.4 to 2.7 kg live weight chicken in 2018

(129). Canadians on average paid $6.24 CAD/kg for a fresh whole chicken between December 2017 to January 2018 (130). When bacterial infections such as avian pathogenic *E. coli* (APEC) result in omphalitis that leads to poorly performing birds, poor weight gains, the producer ends up losing, economically which is a significant concern (100).

1.3.4 *E. coli* infections in chickens

E. coli is commonly present in the intestinal tract and mucosal surfaces of birds. Nevertheless, only those strains containing specific virulence factors contributing to the ability to cause disease in birds are known as avian pathogenic *E. coli* (APEC). APEC cause localized and systemic infections in chickens. These infections are mostly extra intestinal with a majority being respiratory and systemic infections. As a result, APEC has been categorized as extra-intestinal pathogenic *E. coli* that share similar virulence to the *E. coli* strains that cause human urinary tract infections, sepsis and newborn meningitis (261,270). *E. coli* infections in birds are commonly referred to as colibacillosis. These infections affect birds at different stages of their lives. Fecal contamination of eggs leading to yolk sac infections during the end of the incubation period results in embryonic mortality or early chick mortality up to 3 weeks post-hatching (84,301). A study by Giovanardi *et al*. revealed that five broiler flocks having omphalitis and FWM were infected with APEC strains with genetic similarity to the bacteria isolated from the breeder flocks of the corresponding broilers. Based on the acquired time of the infection; embryonic or at hatch, mortality could start as soon as hatching or as late as 20 hours post-hatch (123).

1.3.4.1 *E. coli* morphology and colony characteristics

E. coli is the species of the genus *Escherichia* that belongs to the family *Enterobacteriaceae*. It is a gram–negative, non-acid-fast, uniform staining, non-spore-forming bacillus. Most of the *E. coli* strains have peritrichous flagella and therefore are motile. They are aerobic or non-aerobic and grow on nutrient media at 18-44 C, fermenting glucose and often producing gas. In broth cultures, *E. coli* produces diffused turbidity, and on blood agar, colonies are usually 1-3 mm. Colony morphology may vary with rough colonies which are larger with irregular margins and smooth colonies that are raised, wet looking with well-demarcated margins (301).

1.3.4.2 Serogroups and virulence factors of avian pathogenic *E. coli*

In *E. coli*, O antigen determines the serogroup and K or H antigens determine the serotypes (110). O somatic antigen comprises the antigenic portion of the lipopolysaccharide (LPS) in the cell wall. Due to the high variability of the O antigens, it's used to differentiate serogroups as an epidemiological tool (374). Immune response in poultry birds is mainly directed towards the O antigen of *E. coli*. According to serotyping the most common serotypes that have been associated with poultry infections are O1, O2, O18, O35, O36, O78, and O111 (301). Out of those O1, O2 and O78 are the most commonly isolated from cases (98,127). K1 capsular antigen is frequently associated with APEC strains, and it is associated with resistance to serum bactericidal effects (226,289). Considering the H antigen, 53 H-flagellar antigens have been serologically identified (110). Flagellum, which projects out of the cell, is rotated to provide motility. In *E. coli* and several other species, the central region of the flagellum is variable and consists of H-serotype specific epitopes (415).

Arp *et al*. in 1980 identified that piliated and motile *E. coli* were more virulent to turkey poults (22). This indicates the importance of structures that provide adhesive properties (adhesins) increasing virulence in *E. coli*. Type 1 (F1) fimbriae in APEC are identified to adhere to the upper respiratory tract and cause initial colonization (426). P fimbriae, on the other hand, are suggested to be important in colonization of bacteria in air sacs, lungs, kidney, and blood as well as pericardial fluid depicting their involvement in later stages of an infection (323). The other type of adhesin in APEC, other *E. coli* strains and *Salmonella enterica* are the curli fimbriae (289). They were identified and reported as coiled surface structures that consist of a single type of subunit called curlin (304). The function of curli fimbriae is associated with the bacterial binding to proteins of the extracellular matrix such as fibronectin, laminin, fibrinogen, and H kininogen as well as the survival in the extracellular environment (305).

Temperature sensitive hemagglutinin is a type of protein expressed by certain APEC strains, and it causes hemagglutination activity at 26 C and repression at 42 C (324). At the same time, this protein's mucinolytic ability suggests that it must be playing an important role in colonization of the tracheal mucosa (212). Another virulence determinant that plays an important role is the presence of iron acquisition systems. These are also called iron sequestering systems that assist the uptake of iron and help them to thrive under low iron conditions in the host (289).

In APEC, siderophores are one type of iron acquisition system, and aerobactin is a hydroxamate siderophore (226,279,289).

1.3.4.3 Pathophysiology of colibacillosis in neonatal chicks

In young chickens, *E. coli* mostly enters horizontally through the respiratory tract by the inhalation of faecal contaminated dust in hatchers or barn. Chicks also get infected in the hatcheries by APEC contaminated or infected eggs (302). These infections are suggested to happen by the entry of bacteria through the yolk sac. Sometimes, vertical transmission of *E. coli* is also possible from a hen with salpingitis caused by *E. coli*. During the incubation period, APEC causes yolk sac infection and embryo mortality. Post-hatch infections happen 24 to 48 hours after hatching and mortality elevates up to 10-20% for 2 to 3 weeks due to septicemia. Out of the survivors, up to 5% of the flock may be stunted, and the unaffected birds may grow normally. In the initial part of the infection, lung congestion, edematous serous membranes, and splenomegaly are apparent. A few days later, acute, fibrinoheterophilic polyserositis appears involving the pericardium, pleura, air sacs, and perihepatic tissue. These chicks usually have retained yolk sacs and yolk abscesses indicating the yolk sac entry of bacteria (252,301).

1.4 Prevention of bacterial infections in the poultry industry

Prevention of various infections that could be detrimental to poultry health is one of the significant responsibilities in managing a poultry operation. Poultry producers, veterinarians, and other stakeholders have worked together in developing protocols to prevent the introduction of infections at various stages of the production cycle.

1.4.1 Antibiotic use

For years, prophylactic antibiotics have been used in the commercial poultry industry to prevent bacterial infections such as APEC, salmonellosis and necrotic enteritis. Antibiotic growth promoters were introduced in the 1940s when feeding dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues were found to improve growth in animals (53). The earliest report of the use of growth-promoting antibiotics runs all the way to 1946 when streptothricin, sulfasuxidin, and streptomycin were used in feed (281). The Canadian poultry industry has been using different classes of prophylactic antibiotics at various stages of

commercial poultry production. In 2014, Canadian broiler farming operations used 81% of the antimicrobials for preventative purposes. Out of which, 84% was used as in feed antibiotics primarily intended to prevent necrotic enteritis caused by *Clostridium perfringens* (326).

According to the 2013 to 2015 antimicrobial use surveillance data by Agunos *et al*., prophylactic antibiotics have been delivered via *in ovo* delivery and SQ injection at commercial hatcheries and via drinking water as well as in feed delivery at the poultry barns (9). Most commonly ceftiofur (no longer in use), gentamicin and lincomycin-spectinomycin were given *in ovo* at commercial hatcheries. Once they reached the barns, enrofoxacin (no longer in use), amoxycillin, penicilin-streptomycin, oxytetracycline, sulfamethazin, sulfaquinoxaline, tetracycline, and tetracycline-neomycine were used in water to prevent or treat neonatal diseases and bacterial septicemias. As for the in-feed antibiotics, the report indicates that penicillin, sulfa-trimethoprim, tylosin, virginiamycin, bacitracin and oxytetracycline were used mainly to prevent necrotic enteritis. Ionophores such as lasalocidassalocid, maduramicin, monensin, narasin, and salinomycin were used in feed to prevent coccidiosis and nicarbasin was used as a coccidiostat in-feed (9).

When administering therapeutics to food animal species, it is paramount to comprehend that some antibiotics are commonly used between humans and animals against bacterial infections. Therefore, irresponsible use of such antibiotics in animals may have negative implications on public health. Since some of the antibiotics are essential in treating certain life-threatening human infections, it is imperative that they remain effective. The government of Canada has categorized the antibiotics used in veterinary medicine into four categories based on their importance in human medicine.

Category I antibiotics of very high importance are defined as the preferred option to treat serious human infections and no or limited alternatives are available for them. Carbapenems, third and fourth generation cephalosporin and fluoroquinolones which were popularly used in poultry as prophylactics belong in this category (446). As their first step into responsible use of antibiotics, Chicken farmers of Canada (CFC) took a bold leap and voluntarily withdrew from the use of category I antibiotics for preventative purposes since May 15, 2014 (447). Category II antibiotics of high importance are used to treat various diseases including severe infections, and alternatives are normally available for them such as category I antibiotics. A few examples are aminoglycosides, first and second-generation cephalosporin, lincosamides and trimethoprim/

sulphamethaxazole. CFC announced in May 2017 that they would eliminate category II antibiotics from preventive use by the end of 2018 (446,447). Category III antibiotics are of medium importance and generally they can be alternated by category II or I. The current goal has been set by CFC to eliminate the prophylactic use of category III antibiotics by the end of 2020 (446,447). Last is category IV, which are defined as low important and antibiotics in this category are currently not in human use. Flavophospholipol and ionophores fall in this category, and there have not been any restrictions on these yet (446).

1.4.2 Antibiotic resistance and the need for alternatives

In a survey done in 2016, it was identified that *E. coli* isolated from chicken meat in retail stores were resistant to several antibiotics. That included 53% to streptomycin, 52% to tetracycline, with 46% resistance to sulfisoxazole and 40% to ampicillin. Gentamicin resistance increased from 20% to 33% compared to 2015 according to the report (327). A very recent study conducted by Nhung *et al*. on antibiotic resistance in APEC strains isolated from several regions including Asia, Africa, the United States, Brazil, and Spain reported >70% resistance to ampicillin, amoxicillin and tetracycline. Resistance against ciprofloxacin, neomycin, and chloramphenicol were 50-70% (296). Since the public health concerns of antibiotic resistance and the resulting withdrawal of antibiotics from prophylactic uses started happening in recent years, a dire need for alternative strategies has sparked in the production animal industry. Many researchers are seeking the potential of various natural and synthetic molecules in fighting bacterial infections, weighing their efficacy and cost-effectiveness on the scale. Probiotics, prebiotics, synbiotics, organic acids, vaccinations, innate immune stimulation and improving biosecurity are at the forefront of these alternatives. Although these strategies may not have shown consistent efficiency throughout research, combinations of two or more approaches have exhibited proficiency (50,321,379).

1.4.3 Alternative strategies to antibiotics

Since the antibiotic use is diminishing due to concerns such as the emergence of antibiotic-resistant bacteria, alternative strategies have become valuable in the control of infections.

1.4.3.1 Probiotics, prebiotics and synbiotics

The Food and Agriculture Organization and the World Health Organization collectively define probiotics as “live micro-organisms that grant a health benefit to the host” (27). Several micro-organisms are being used as probiotics for poultry species such as bacterial and non-bacterial organisms. Some of the bacterial probiotics are several *Lactobacillus* species (109,280), *Bifidobacterium* (195,317), *Bacillus* (1) and *Enterococcus* (286). Yeast or fungal probiotics are added to feed such as *Saccharomyces cervisiae* (16,26) *Aspergillus oryzae* (78,361), *Candida pintolopsii* (78) and *Saccharomyces bourlardii* (332) that caused positive effects on the performance and gut health. There are also certain negative effects reported contradictory to the positive effects (319). However, there are multiple reports that depict the positive enteric health effects of probiotics against infections such as coccidiosis (73,342), and mentioning that they could be a great alternative to antibiotics in the poultry industry (238,291).

According to Food and Agriculture Organization, prebiotics are defined as non-digestible food ingredients that beneficially affect the host. They selectively stimulate the growth as well as the activity of bacteria in the colon, and thereby improves host health (320). They are supplying a substrate for beneficial microorganisms in the gastrointestinal tract. Although the previous definition has focused only on few carbohydrates, researchers have redefined prebiotics including various oligosaccharides containing varying carbon lengths and collectively call them non-digestible oligosaccharides. Different molecules such as fructooligosaccharides, galactooligosaccharides, mannanoligosaccharides, inulin and isomaltooligosaccharide are among the non-digestible oligosaccharides that have beneficial properties as prebiotics (103,340). Reports suggest that certain non-digestible oligosaccharides like fructoseoligosaccharide, inulin type fructans and mannanoligosaccharides have the ability to modulate gastrointestinal microbiota by increasing the *Lactobacillus* population while reducing harmful pathogens such as *E. coli* and *C. perfringens* (196,322).

Synbiotics are made by synergistically combining probiotics and prebiotics in order to provide beneficial effects to the host by promoting the establishment of microbial feed supplements in the gastrointestinal tract (20). Studies have shown the beneficial effects of synbiotics on the reduction of harmful bacteria such as *Campylobacter jejuni* (25) and *Salmonella* Typhimurium (339). Markazi *et al.* recently reported that drinking water synbiotic supplementation helps to influence a healthy microbiota and an effective immune response in the

intestines of laying hens during a *Salmonella* challenge (263). Micciche *et al.*'s most recent review also compiled study reports of synbiotics effect on improving gut health and weight gain other than reducing harmful effects of *Salmonella* (272). Markowiak and Slizewska published a review in 2018 compiling a majority of the studies that showed beneficial effects of synbiotic use in poultry birds and other production animals rather than using probiotics or prebiotics alone (264). Depending on the probiotic and prebiotic combination, the immunomodulatory and microbiome modification effects in the host can vary, as observed via *in ovo* delivery (92). An interesting study revealed that synbiotic treatment during heat stress could act as an effective management tool to minimize detrimental effects particularly in hot regions of the world (277).

1.4.3.2 Vaccines against bacterial infections

Vaccination is a common strategy used in the control of a majority of viral infections. However, several vaccines have been developed to prevent diseases caused by bacterial pathogens such as *Salmonella, E. coli, Campylobacter, Pasteurella multocida* (fowlpox), *Haemophilus paragallinarum* (infectious coryza), *Ornithobacterium rhinotracheale* and *Bordetella avium* in poultry birds (83,105,124). Although several vaccine candidates such as whole organism and subunit vaccines have been experimentally tested, the protective efficacies of these commercial vaccines against salmonellosis and colibacillosis have been minimally studied since their development, particularly regarding the mechanisms of immune protection.

Poultry researchers are using different strategies from generating autogenous vaccines to modified live, inactivated and subunit vaccines in order to help producers prevent *E. coli* infections (121). There are currently two commercial vaccines available in the market against colibacillosis. Nobilis® *E. coli* (MSD Animal Health, Summit, NJ, USA) is an inactivated subunit vaccine consisting of fimbrial antigen F11 and a flagellar toxin (131,278). Gregersen *et al*. conducted a study to test the effect of breeder vaccination on broiler mortality and found no significant impact or benefit of this vaccine against colibacillosis in the progeny. However, he found a significant effect of this vaccine in the breeders against *E. coli* infection (131).

Introduction of the live attenuated commercial vaccine Poulvac® *E. coli*, (Zoetis, Florham Park, NJ, USA) has tempted poultry producers to begin using it due to the reports of its protective effect (118). The current vaccine against colibacillosis in the market comprises of live attenuated *E. coli* EC34195 serotype O78 with deleted *aroA* gene as the active substance (227).

Researchers say that the members of *Enterobacteriaceae* family require *aroA* genes for the biosynthesis of aromatic amino acids to acquire the full expression of virulence. Thus the deletion of the gene and adding the aromatic amino acids would result in the sufficient growth of the bacteria on minimal media producing safe live vaccine candidates (162). Studies revealed that coarse spray vaccination of day-old chicks with this vaccine could protect birds against homologous and heterologous *E. coli* challenge (105,227,348). At the same time, it was identified safe (278). Sadeghi *et al.* reported that this vaccine was protective against *E. coli* O78 as well as an untypable acute field isolate and the birds did not develop any lesions (347). Further, it is been elucidated that the immune response induced by the vaccine is dominated by APC in the early stage that diminish by the end of 7 days. Cell mediated immunity managed by CD 4+TCRVβ1+ on the mucosal surfaces producing immunoglobulin (Ig) A and CD8 cells that prevent tissue invasion of bacteria are hypothesized as the protective mechanisms of the vaccine-induced immune response (105).

In Japan, a group reported that only the deletion of *aroA* gene is not sufficient enough to attenuate full virulence and the need of extra genes to delete. They reported the mutation of *crp* gene of O78 in APEC as the development of a live vaccine candidate (288) and mentioned a vaccine containing this bacterial strain (Gall N tect CBL) had been marketed in Japan since 2012. Their studies indicated that with the use of this vaccine, survival rate and egg laying rate improved in egg layers (401). A recombinant multi-antigen vaccine comprising of a combination of common Extraintestinal Pathogenic *E. coli* surface proteins has been recently reported to produce significant levels of IgY against specific antigens and impose immune responses favorable for bacterial killing. It has reduced bacterial growth of multiple APEC serotypes, reduced bacterial load in organs and reduced lesions (406). Designing of a more sophisticated bacterial ghost vaccine of APEC O78:K80 was reported by a group of Iranian scientists. They demonstrated increased IgA, IgY and reduced air sac lesions against homologous challenge with the use of this vaccine (93).

1.4.3.3 Other strategies

Other than the above methods, poultry producers use a variety of strategies to prevent bacterial infections in farms. Biosecurity is a vital measure that is typically underrated in the field. Keeping strict biosecurity in terms of segregation, control of traffic, cleaning and

disinfection helps to prevent a large proportion of harmful bacteria and viruses entering poultry barns (359). Apart from good management practices, there are many alternative approaches proposed and being explored by researchers around the world to overcome bacterial infections in poultry birds. While immune modulatory agents will be discussed separately, it is worth brushing over the other strategies here.

Bacteriophages and their lysins have been studies for a long time for their antibacterial effects as potential solutions. These viruses that specifically parasitize on bacteria are currently being recognized for their ability to prevent infections by pathogens like *E. coli, Salmonella,* and *Campylobacter* (57). Huff *et al.* demonstrated that the administration of two bacteriophages as an aerosol spray as well as an IM injection reduced mortality significantly against an *E. coli* infection (167). However, due to the obligatory parasitic nature of phages and their strict dependence on specific bacterial species, many precautions have to be taken during the phage preparation process. Despite the challenges, phage therapy has lots of potential due to their natural presence, minimal harm and the ability to adjust to its host bacterium's mutations (341).

Several plant extracts have been identified with antimicrobial properties and applied in various studies in poultry as alternatives to antibiotics (57). Phytobiotics or phytogenic compounds are divided into several categories such as herbs, spices, phenols, essential oils, alkaloids, and lectins. Adding these to animal diets have been identified to improve the quality of the animals' lives as well as the food derived from them (424). Essential oils such as oregano, rosemary and lavender oil used in broiler trials have shown efficient antimicrobial activity (6,266,432)

1.5 Immune modulation as an alternative to antibiotics

Various immune modulatory strategies are hot topics as alternatives to antibiotics in food animal production. Antibacterial vaccines are part of this category although; they are not in a position to control all bacterial infections due to their specificity to different bacterial agents. Nevertheless, the essence of immune modulation is about modulating the host immune system or immune functions using the action of immune modulatory molecules such as toll like receptor (TLR) ligands and agonists, hyper immune antibodies, bacteriophages, probiotics, herbs and essential oils (239). Out of the agents, TLR ligands and agonists have been widely studied as strategies to control infections owing to their potent immunogenicity as stand-alone immune

stimulants as well as vaccine adjuvants. As opposed to vaccines, the other benefit of these TLR agonists is that they target the host rather than the pathogen so, it is improbable that their repeated use would revert to any virulence or give rise to resistance (273).

TLRs are a cluster of pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs) that comprise the invading pathogenic organisms such as cell wall components like LPS, peptidoglycans, bacterial deoxyribonucleic acid (DNA) and double stranded viral ribonucleic acid (RNA) (189). Synthetic counterparts of CpG-ODNs, polyinosinic:polycytidylic acid and other molecules are promising analogs that are mostly being used in research as TLR agonists (439). Recognition of specific PAMPs by the TLRs result in the activation of signaling cascades that leads to the expression of various innate immune responses such as the expression of pro-inflammatory cytokine genes, reactive oxygen intermediates and nitrogen intermediates (386). Such immunomodulatory properties have been acknowledged to use in vaccine production as antigens, adjuvants as well as direct immune stimulators to prevent or fight pathogenic infections (10,33).

Since we discussed about the use of immunomodulatory molecules as alternatives to antibiotics, it would be useful to review the organization of the avian immune system in order to understand their mechanisms of action.

1.6 Overview of the avian immune system

Despite several anatomical differences, functions of the avian immune system are based on the same immunological principles of the mammalian immune system. Most of the immunological findings have been done in the domestic chicken (80). In chickens, the immune system is made of a network of organized immune organs, scattered lymphatic tissues, immune cells and chemical mediators (310). Central or primary lymphoid organs of epithelial origin in the embryo such as the thymus and bursa of Fabricius and peripheral or secondary lymphoid organs of mesenchymal origin such as the spleen compose the network of lymphoid organs. Just like other vertebrates, the avian immune system consists of a humoral arm and a cell mediated arm. In terms of differences, avian humoral immune system is led by antibody-producing B lymphocytes that are generated by the primary immune organ bursa of Fabricius. The cellular arm is mostly under the control of the T lymphocytes and macrophages (360).

In contrast to mammals, avian thymus could be divided into 12 separate lobes (108). Unlike mammals, avian species do not have well-organized lymph nodes although there are many scattered lymphatic nodules. When it comes to the mucosa associated lymphatic tissues (MALT), chickens show several differences. Their gut associated lymphoid tissue (GALT) consists of Payer's patches as well as cecal tonsils (108).

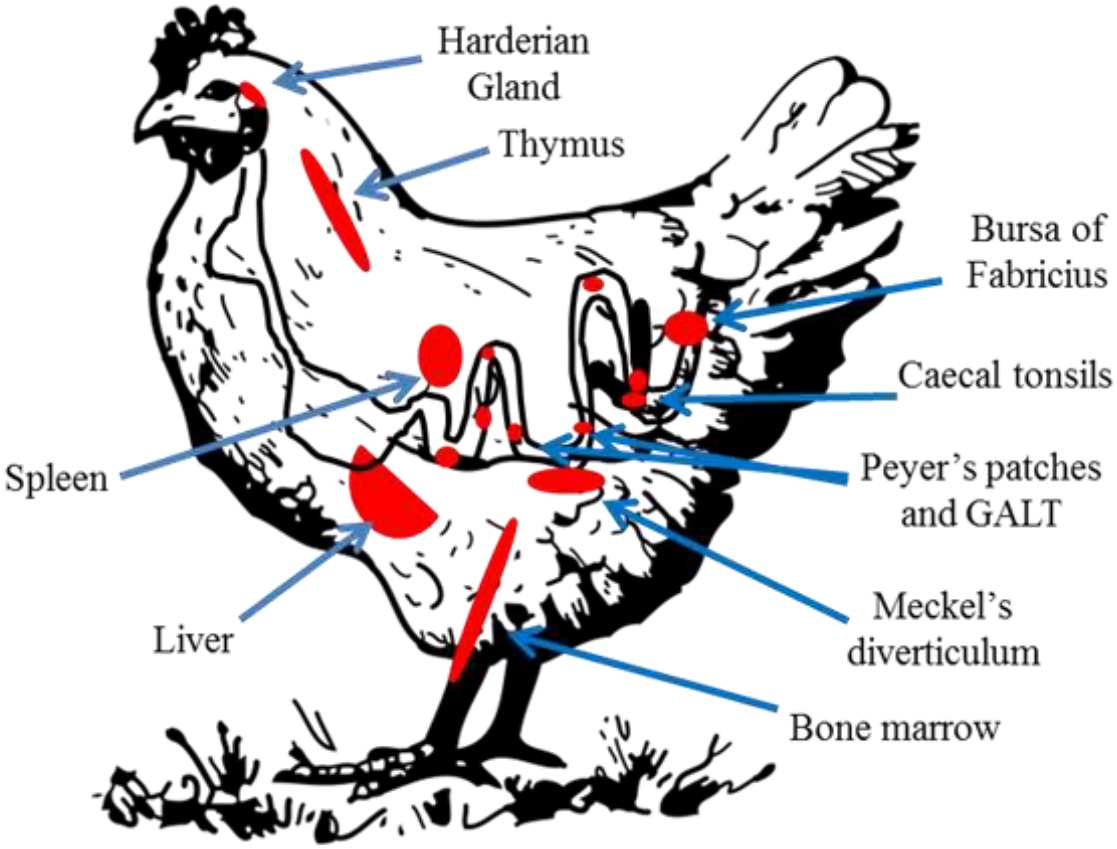

Figure 1.1 Distribution of immune organs in the chicken. Adapted from Structure of Avian Immune System, Dr. Fares El-Khayat, LinkedIn Slide Share: https://www.slideshare.net/ssuser662c3a/structure-of-avian-immune-system-dr-fares-elkhayat and the picture of chicken by https://creativecommons.org/licenses/by-sa/3.0/

1.6.1 Primary lymphoid organs

Primary lymphoid organs are the sites that progenitor cells develop into mature lymphocytes (200). In the avian immune system, two primary lymphoid organs contribute to the immune functions; bursa of Fabricius and thymus.

In the embryo, hematopoietic stem cells derived from embryonic yolk sac and liver migrate to the thymus and bursa anlages and develop into immunologically competent T and B lymphocytes (147). Once immunologically mature, these cells enter the circulation and colonize

the peripheral lymphoid organs (303). The bursa of Fabricius is identified as the avian functional equivalent of bone marrow in mammals. The bursa of Fabricius or the cloacal bursa is a sac-like structure outgrown from the cloacal epithelium that attaches to the proctodeal region of the cloaca by a duct. This organ was named after Hieronymous Fabricius of Aquapendent, professor of surgery at the University of Padua from 1565-1613 who pioneered the discovery its function (80). Cloacal bursa is the home where bursal-derived lymphocytes or B lymphocytes develop and contribute to humoral or antibody mediated immune responses. The discovery of the cloacal bursae has given rise to the understanding of the humoral and cell mediated division of the avian immune system and provided the foundation to study the immunological concepts of the T and B lymphocyte lineages (80,108,360).

The thymus is the other primary lymphoid organ that controls the cellular arm of the immune system. It derives from the epithelium of the pharyngeal pouches at an early embryonic stage. Chicken thymus contains multiple lobes (7-12), and it is located on either side of the neck sometimes extending up to the thoracic cavity (360). The thymus reaches 6-12 mm maximum size by 3-4 months of age before it starts to go through physiological involution. Anatomically, each lobe is encapsulated with a fibrous capsule, and septae originating from it divides the thymic parenchyma into lobules. The parenchyma is composed of a central medulla and a lobulated cortex in the periphery. Hematopoietic cells arrive via the epithelial anlage of the thymus, enter the medulla during T cell maturation and exit the thymus through medullary post-capillaries (303).

1.6.2 Secondary lymphoid organs

Several important secondary or peripheral lymphoid organs can be identified in the chicken. They are the spleen, MALT such as the Harderian gland related to the eye, nasal, bronchus and genital associated lymphoid tissues, GALT that comprises of several tonsils in the gut, Peyer's patches, cecal tonsils, Meckels's diverticulum, as well as the skin and pineal gland accompanied lymphoid tissues (303). There are many solitary lymphoid aggregates scattered around the body which is unique to avian species (345). Immunologically mature lymphocytes get into the circulation and settle in the secondary lymphoid organs. T and B lymphocytes inhabit different compartments in these organs. These areas are named as T cell and B cell dependent zones. The anatomy of the peripheral lymphoid organs has been much explored in the avian

spleen. Compared to other peripheral lymphoid organs, avian spleen's T cell-dominant zone is well defined and known as the peri-arteriolar lymphatic sheath, surrounding the splenic central artery. Germinal centers (GC) and periellipsoid lymphocyte sheaths are the B cell rich compartments (180,303).

1.6.2.1 Avian bronchi associated lymphoid tissue

In the chicken respiratory system, organized lymphoid structures can be found in the bronchial mucosa. These lymphoid aggregates are named as bronchi associated lymphoid tissue (BALT) due to their similarity to Peyer's patches and other GALT. In chickens, they are located at the junctions of the primary and secondary bronchi, confined to the most caudal secondary bronchi as well as at the ostia to the air sacs. Development of the BALT depends on the age and exposure to the environment. Mature BALT is lined by a lymphoepithelium, known as follicle associated epithelium, with ciliated and non-ciliated epithelial cells that harbor lymphocytes. Follicle associated epithelium covers lymphoid aggregates which contain different immune cells at various ages. At 6-8 weeks age, GCs are present in the nodules containing plasma cells, macrophages, and heterophils in considerable numbers. When GCs are not present, B cells are limited to the periphery surrounding aggregates of T lymphocytes in the middle of the nodule (99,336).

In day-old birds, there are very few infiltrating lymphocytes seen in the primary bronchus. The first week of life is when the primary and secondary bronchi get infiltrated with CD45+ leucocytes. Within 3-4 weeks, lymphoid nodules containing specific B lymphocytes or plasma cells start to appear. At the same time, CD4+ and CD8+ T lymphocytes start to localize in a unique pattern. Birds 2-4 week old usually have developed GCs (236,336).

1.6.2.2 Avian lung and air sac associated phagocytic system

Unlike the well-known alveolar macrophages in the mammalian lung, comparatively little is known about the similar cell type in avian lung and air sacs. They are referred to as avian respiratory macrophages or phagocytes or free avian respiratory macrophages by different authors (35,61). They are present in lesser numbers compared to that of the mammalian lungs. Free avian respiratory macrophages are strategically distributed on the surfaces of the atria and the infundibulae where fresh air enters into the gas exchange area so that particles can be

effectively removed (259). In the air sacs, heterophils have been identified as the most abundant phagocyte followed by macrophages and few lymphocytes. These macrophages migrate to the respiratory surface upon inflammatory stimulation. They get activated and elicit phagocytic activity to remove pathogens from the respiratory system (336,396).

1.7 Cytosine phosphodiester guanine oligodeoxynucleotides (CpG-ODN)

CpG-ODNs are immune stimulatory synthetic counterparts of naturally occurring CpG motifs in bacterial DNA, a PAMP that can trigger the innate immune responses in vertebrate and some invertebrate hosts (209).

1.7.1 History of CpG evolution – Bacterial extracts to synthetic CpG-ODN

It was originally observed that patients with malignant tumors would experience spontaneous concomitant remission if they developed concurrent bacterial infections (422). Dr. William B. Coley (1891-1936), who was a pioneering surgeon, recognized this phenomenon as a possible therapeutic option to cure his cancer patients. He voluntarily induced erysipelas using a broth of *Streptococcus pyogenes* in a patient with sarcoma in the neck region. Although the bacteremia caused high fever in the patient, the tumor shrank and completely disappeared for seven years (422). Due to the difficulty in inducing and controlling erysipelas, he later switched to using heat-killed *Sterptococci* and incorporated *Serratia marcescens* to improve his therapy. This gave rise to the famous "Coley's toxins" which comprised of gram-positive, heat-killed *S. pyogenes* and gram-negative, heat-killed *S. marcescens* (67,290).

Afterward, Mycobacterium bacillus Calmette-Guérin (BCG) has been used as the local treatment of bladder cancer (283). Furthermore, Complete Freund's Adjuvant which is an emulsion of killed bacteria is currently known as the gold standard of vaccine adjuvants (216). Researchers found out that the antitumor property of BCG was connected to the DNA in the bacteria (394). The DNA from invertebrates but not from vertebrates or plants could inhibit tumor growth, increase natural killer (NK) cell activity and induce interferon (IFN) alpha/ beta and gamma (α, β and γ) in mouse spleen cells and human peripheral blood lymphocytes. These activities were related to the presence of self-complementary palindromes of ODNs containing at least one CpG dinucleotides more commonly present in bacterial genome (215,395).

1.7.1.1 Bacterial vs higher organism CpG motifs

Although bacteria, viruses and vertebrates all carry DNA, there are structural changes that cause a significant difference between each other. First, the bacterial DNA has a higher frequency of CpG dinucleotides in their genome, about 16-fold compared to about four-fold in the vertebrate DNA. In vertebrates, the bases flanking CpG are not random. Most commonly, the base prior to a CpG is C and the base after a CpG is a G. Further, vertebrate or eukaryotic DNA is methylated at about 80% of the cytosines compared to bacterial DNA which is not methylated (215,412). These differences in the bacterial genome make them different from the vertebrate DNA.

Apart from the vertebrate and bacterial CpG DNA comparison, invertebrate DNA in some insects, crustaceans (shrimp and crabs) and mollusks have been identified differently from bacterial DNA due to their methylation state (337). In recent studies, synthetic CpG-ODN has been determined to stimulate the innate and humoral immune systems in invertebrates, particularly in crustacean and mollusk families (376,377).

1.7.1.2 Synthetic CpG-ODNs

Synthetic oligodeoxynucleotides containing CpG motifs are similar to those found in bacterial DNA. Synthetic CpG ODNs are made with unmethylated cytosine and guanine dinucleotides bound with a modified nuclease resistant phosphorothioate backbone, where one of the oxygen atoms of the phosphodiester linkages is replaced with sulfur (215). Yamamoto *et al.* were the first to demonstrate that synthetically developed DNA containing unique palindromic sequences similar to those in the DNA fraction of bacteria got immune stimulatory properties (429). According to Klinman and Krieg however, palindromes are not required, but maintenance of the CpG motif was necessary for the immune stimulation (211). The optimum specific unmethylated DNA sequences in mice are usually flanked by two 5’ purines and two 3’ pyrimidines (purine-purine-C-G-pyrimidine-pyrimidine) (211) out of which the best motif to activate mouse or rabbit cells has been identified as GACGTT (333). Such CpG ODN has been able to activate macrophages, dendritic cells (DC), NK cells and B cells in mouse models (29,145,211).

1.7.2 Classes of CpG-ODN

Depending on the nature of the backbone and the sequence motifs, variations in immunostimulatory effects could be identified such as on the type of activating cell types or the cytokine profile. As a matter of that fact, we cannot consider all CpG-ODNs as one agent with uniform immunomodulatory properties (207).

1.7.2.1 Class-A/ type D: CpG-ODN

Class-A CpG-ODNs are closer to bacterial CpG motifs by structure. They contain phosphorothioate bonded poly G tails in the 5' and 3' ends and a modified phosphodiester bonded palindromic CpG motif in the middle. Their phosphodiester backbone particularly induces NK cell activation in human and murine cells and IFN-α secretion from human plasmacytoid DC precursors. Further, the poly G tail enhances the uptake by cells and activation of the above effects (29,223).

1.7.2.2 Class-B/ type K: CpG-ODN

Class B or type K CpG-ODN contains a fully modified phosphorothioate backbone without a poly G tail. This class of CpG has an improved ability to stimulate B cells and better stability due to the backbone. It's relatively weak in NK cell stimulation and tumor necrosis factor (TNF)-α production compared to class-A CpG-ODNs (367,411).

1.7.2.3 Class-C CpG-ODN

Class-C CpG-ODNs structurally contain a phosphorothioate backbone with one or more CpG motifs in 5' end and a palindrome in the 3' end (187). They upregulate and enhance proliferation of B cells and NK cell mediated cytotoxicity very effectively as well as plasmacytoid dendritic cell (pDC) mediated secretion of IFN-α midway compared to class-A and class-B (411).

1.7.2.4 Class-P CpG-ODN

The novel class-P CpG-ODNs have two palindromic regions that give the ability to create higher ordered structures/ multimeric units such as concatemers. They stimulate B cells and pDCs (89). The class I interferon producing ability of the class-P CpG-ODN is significantly high

compared to the class-C and *in vivo*, their cytokine production is greater. The discoverers of the class-P CpG-ODN mention that it can be broken down to monomers and dimers while preserving the strong immune stimulatory effects (352).

Table 1.1 Comparison of CpG-ODN types (Reprinted with permission from the publisher. Courtesy to Bode et al., 2011, CpG DNA as a vaccine adjuvant, Expert Review of Vaccines)

ODN type	Representative sequence	Structural characteristics	Immune effects
D also referred to as A-class	GGTGCATCGATGCAGGGGGG	-Mixed phosphodiester/ phosphorothioate backbone -Single CpG motif -CpG flanking region forms a palindrome -Poly G tail at 3′ end	-Induces strong pDC IFN-α secretion -APC maturation
K also referred to as B-class	TCC**ATGGA**CG**TTCCT**GAGCGTT	-Phosphorothioate backbone -Multiple CpG motifs -5′ motif most stimulatory	-Induces strong B-cell activation pDC maturation -Preferentially supports the production of TNF-α and IL-6
C	TCGTCGTTCGAACGACGTTGAT	-Phosphorothioate backbone -Multiple CpG motifs -TCG dimer at 5′ end -CpG motif imbedded in a central palindrome	-Induces B-cell and pDC proliferation and differentiation -Induces production of IL-6 and IFN-α
P	TCGTCGACGATCGGCGCGCGCCG	-Phosphorothioate backbone -Two palindromes -Multiple CpG motifs	-Stimulates pDC and B cells -Strong IFN-α secretion

1.7.3 CpG-ODN mechanism of action

As discussed earlier, unmethylated CpG motifs in bacterial DNA act as PAMPs and warn the immune system about the entry of pathogens. Both bacterial DNA and synthetic CpG-ODN were found to generally stimulate B cells, NK cells, pDCs, and macrophages/monocytes although the specific bases flanking the CpG regions as well as the type of backbone may change the degree of stimulation (215). Studies conducted using TLR9 deficient (TLR9 $^{(-/-)}$) mice confirmed that in mice, bacterial CpG motifs are explicitly recognized by TLR9, and the recognition leads to innate immune responses such as splenocyte proliferation, B cell stimulation and the production of inflammatory cytokines; TNFα, IL6 and IL12. It was also proposed that TLR9 was an intracellular receptor (157). Early studies mostly done on isolated cultured cells revealed that humans express TLR9 only in pDC and B cells whereas mice have a broader range of cells expressing TLR9 including myeloid dendritic cells and monocytes/ macrophages (216). Later studies reported the presence of TLR9 in human and mouse lung epithelial cells and pulmonary macrophages (355). The same group revealed the expression of TLR9 in a variety of cells in the lungs of horses (356), pigs, dogs and cattle (357). On the other hand in chickens, CpG-ODN recognition is supposed to occur through TLR21 which is identified as a functional homolog of the mammalian TLR9 (47). According to experts, the CpG-ODN mediated immune modulation happens in two stages; an early stage of innate immune activation then extends to an enhanced adaptive immune response.

1.7.3.1 Cellular recognition and uptake

It is still unclear how exactly CpG-ODN reaches the intracellular compartment in order to bind with intracellular TLR9. Several groups have explored and reported various mechanisms of cellular CpG-ODN uptake. An early study reported that the cellular entry of phosphorothioate modified ODNs is concentration dependent. At high extracellular concentrations (>1μM), they enter a cell by "fluid phase endocytosis" (pinocytosis) whereas when ODN concentrations are <1μM, "receptor-mediated endocytosis" (clathrin dependant) kicks in. This study further explained that phosphorothioate modified ODNs are specifically binding to cell membrane proteins for intracellular trafficking. When imaged in a cell line, the ODNs were visualized in clathrin-coated pits on the cell surface and eventually within the endosomes, freely in the cytoplasm and in the nucleus thereafter symbolizing endocytosis (40). Later, another study

pointed out phosphatidylinositol 3-kinases must be playing a role in the internalization of CpG-ODN. This group also defied the previous conclusion by Chu *et al.* regarding the involvement of DNA dependent protein kinase-mediated CpG signalling (174). A recent *in vivo* study showed that DEC-205, a multilectin receptor on DC, B & T lymphocytes and thymic epithelial cells are specifically involved in the uptake and internalization of class B CpG-ODNs (51,229). A study performed using wild-derived mice reported that mannose receptor-1 is the component involved in uptake and intracellular trafficking of CpG-ODN in peritoneal macrophages. They also discuss that, serum proteins and other surface proteins could bind with CpG-ODN and assist entering the intracellular compartment through the mannose receptor (285).

1.7.3.2 Activation of immune pathways

Once internalized, CpG ODN then interacts with TLR9 within the endocytic vesicle. Formation and maturation of the endocytic vesicles with CpG-ODN and TLR9 co-localization are important for initiating the TLR mediated intracellular signaling (10). Just like all functionally characterized TLRs, TLR9 signaling also progresses through a common pathway that involves myeloid differentiation marker 88 which is an adaptor protein enrolled to the Toll/IL-1R homologous region of the TLR9 upon CpG-ODN binding. This recruitment activates the IL1 receptor-activated kinase, Tumor Necrosis Factor receptor-associated factor 6 and TGFβ-activated kinase 1 (145,388). Then this cascade gets involved with pathways such as mitogen-activated protein kinases: p38, c-JUN NH_2-terminal kinase, extracellular receptor kinase and nuclear factor kappa light-chain-enhancer of activated B cells (NF-κB) inducing kinase (387,438). This signaling cascade results in the activation of transcription factors such as NF-κB and activating protein-1, which end up in the elevation of cytokine/ chemokine gene expressions (Figure 1.2) (145).

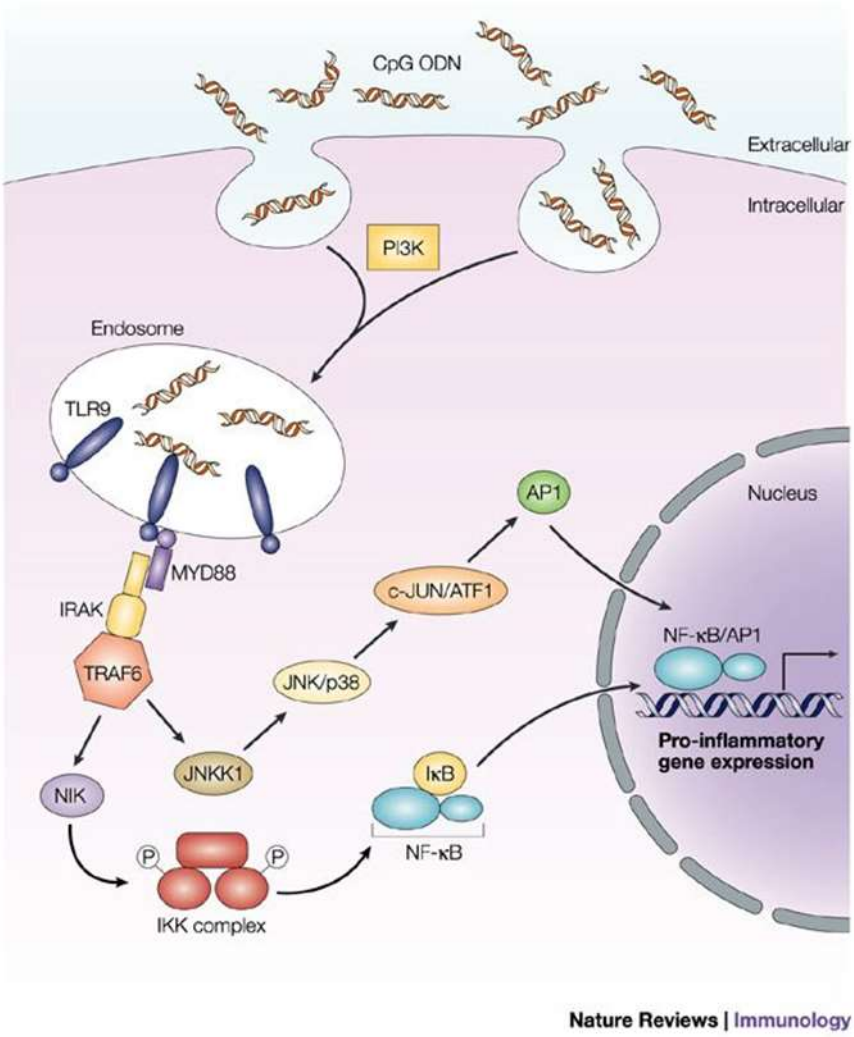

Figure 1.2 CpG DNA-TLR9 cell signaling. (Reprinted with permission from the publisher. Courtesy to Dennis M. Klinman, 2004, Immunotherapeutic uses of CpG oligodeoxynucleotides, Nature Reviews Immunology).

1.7.3.3 Cellular responses

Cytokine gene expression levels change within about 30 minutes following CpG-ODN *in vivo* administration. A study done in mice reported a bi-modal pattern of gene upregulation with the use of ingenuity pathway analysis. In the first 3 hours, peaking of cytokine genes related to immune response (IL1α, IL1β and TNF), cell signaling and cell movement were observed. Then it declined and five days following treatment genes related to cell proliferation and repair peaked (201). CpG-ODN, particularly class B ODNs, stimulates B lymphocytes at a higher degree to secrete IL6 and IL10. IL6 is important for B cells to carry on the secretion of IgM (436).

Further, CpG-ODN stimulation of B cells leads to the expression of co-stimulatory molecules such as MHC class II, CD80, CD86 and in human B cells CD40 and CD54 (222). Plasmacytoid DCs are the next cell type that highly expresses TLR9 and are greatly responsive to CpG-ODN stimulation. Studies show that both human and murine pDCs directly get activated by CpG-ODN to secrete cytokines IL1, IL6, IL12, IL18, TNFα (42,369) and IFNα (150) indicating a T helper cell (Th) 1 biased immune response that provides superb antiviral immunity (223). They noted increased surface expression of MHCII, intracellular adhesion molecule 1 and co-stimulatory molecules CD40, CD54, CD80 and CD86 (150) leading to maturation, enhanced antigen presenting ability and resistance to IL4 induced apoptosis (215).

Bacterial DNA and CpG-ODN both induce macrophages to secrete TNFα, IL12 and produce nitric oxide (NO) (402). Class-A CpG-ODNs are found to activate human and murine (NK) cells potently. Early studies report that bacterial DNA induces NK cells both directly (211) and indirectly in mice. In the indirect path, monocyte/ macrophage stimulation with cytokine (IL12, TNFα and type I interferons) induced IFNγ production with an enhanced killing activity of NK cells (56). Ballas *et al.* reported that highly purified human NK cells did not activate directly by CpG-ODN probably due to the absence of TLR9. They confirm in their study that CpG-ODN activates DC and that in turn activates NK cells (28). Similarly, CpG-ODN indirectly causes the maturation, differentiation, and expansion of T lymphocytes (211). Th1 type cytokines secreted by both B cells and APC (DC) prime naïve T lymphocytes to Th1 cells that produce IFNγ (378). Use of CpG-ODN before a peptide vaccination indicated an increased number and survival of CD8+ T lymphocytes (307). In the first human trial done with the administration of CpG-ODN with a T cell peptide antigen, they have shown a rapid expansion of antigen-specific CD8+ T lymphocytes (370). They hypothesized that the Th1 cytokines must be contributing to the increased CD8+ T cell activity by stimulating pDC to mature (42,307). Figure 1.3 (218) has summarized a majority of these innate and adaptive cellular changes upon CpG-ODN stimulation.

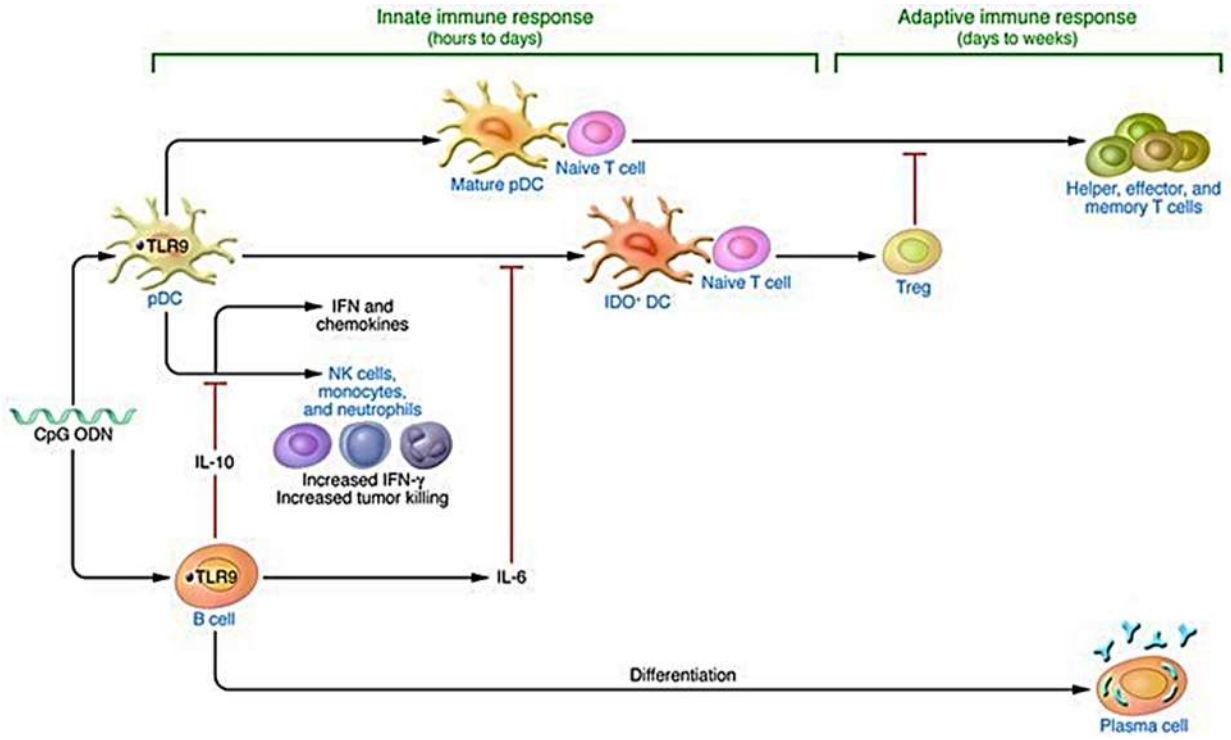

Figure 1.3 Activation of innate and adaptive immunity by TLR9 activation (Reprinted with permission from the publisher. Courtesy to Arthur M. Krieg, 2007, Development of TLR9 agonists for cancer therapy, Journal of Clinical Investigations)

1.7.4 CpG-ODN safety

Now that CpG-ODNs are used as an immunotherapeutic agent in human and animal clinical trials, its safety aspect is a vital parameter to assess. CpG-ODN has been used in more than 500 subjects in phase I and phase II clinical trials without any serious harmful effects (207). Early human pre-clinical trials using CpG-ODN reported the development of local inflammatory reactions (swelling, erythema, pain, heat, pruritus) and flu-like symptoms in patients that received CpG-ODN containing vaccines (217). When class B CpG-ODN was administered to healthy adults as well as cancer patients, there have been no major adverse reactions noticed (220,244).

However, one safety concern that has been backed by several studies is the potential development of autoimmune disease or inflammation. Studies showed that administration of bacterial DNA to lupus-prone mice developed anti-DNA antibodies (122) by the production of polyclonal B lymphocytes, IL6, and the persistence of self-reactive lymphocytes through inhibited apoptotic death (206,245). However, *in vivo* experiments were carried out to test this hypothesis by repeatedly injecting CpG-ODN to normal and lupus predisposed mice. Although

the number of anti-DNA IgG-secreting B cells increased 2-3 fold, the titer of anti-single stranded DNA antibodies was insufficient to induce or deteriorate systemic autoimmunity providing evidence that DNA vaccines such as CpG-ODN wouldn't initiate or accelerate systemic autoimmunity (282).

Still, when a CpG-ODN containing Hepatitis B vaccine; Heplisav was tested at phase 3 clinical trial, further trials were halted due to the development of Wegener's granulomatosis; an autoimmune vasculitis condition in one of the subjects suspecting CpG-ODN to cause the autoimmunity (82). In a review by Scheiermann and Klinman, they discussed summarizing several clinical studies how human immunodeficiency virus (HIV)-infected individuals and cancer patients showed more frequent and serious adverse effects to CpG-ODN adjuvant vaccines and therapies. It is hard to interpret whether CpG-ODN or the patients' clinical conditions predisposed to those adverse effects (354). The other challenge is that CpG-ODN could potentially cause deleterious effects in certain organ-specific autoimmune conditions that involve Th1 response. Experimental development of allergic encephalomyelitis, autoimmune myocarditis with chlamydia and autoimmune arthritis were evident with CpG-ODN administration in animal models (207).

The other concern for CpG-ODN is that it might promote toxic shock. Klinman discussed few studies in his review that CpG-ODN co-administered with LPS and D-galactosamine cause toxic shock due to the overproduction of TNF. However, his idea was that these studies were deliberately administering higher doses and frequencies of CpG-ODN until the development of adverse effects instead of immunotherapeutic use (207). There is a recent report of class A CpG-ODN 1585 being able to rescue mice from LPS induced endotoxin shock by activating platelet-activating factor acetylehydrolase (431). Liu *et al.* conducted a safety profile study for B class CpG-ODN 684 in rats since its being used as an adjuvant for hepatitis B and rabies vaccines and reported it to be devoid of any harmful effects (247).

Due to the popularity in using CpG-ODN as a vaccine adjuvant in veterinary animals, several studies have been conducted to evaluate its safety in these animal species. Ioannou *et al.* reviewed the safety of CpG-ODN in veterinary species by going through different experiments mainly done by their group (171). Administration of 50 and 200mg of CpG-ODN in cattle did not cause a harmful tissue reaction compared to other adjuvants, so it was identified safe to use in large quantities (91). They also found that CpG-ODN can be used to reduce the toxicity in

conventional adjuvants without compromising the immune response by adding CpG-ODN to a reduced amount of mineral oil adjuvant (169). Regarding the safety in chickens, Gomis *et al.* reported for the first time that IM and SQ administration of CpG-ODN developed mild to severe cellular infiltrations which completely resolved in the matter of few days (126).

1.7.5 CpG-ODN applications

Due to its immunostimulatory potential and relatively safe profile, CpG-ODNs are used in a variety of applications such as protective immunity, as vaccine adjuvants to enhance immune responses, cancer immunotherapy and allergy therapy (Figure 1.4).

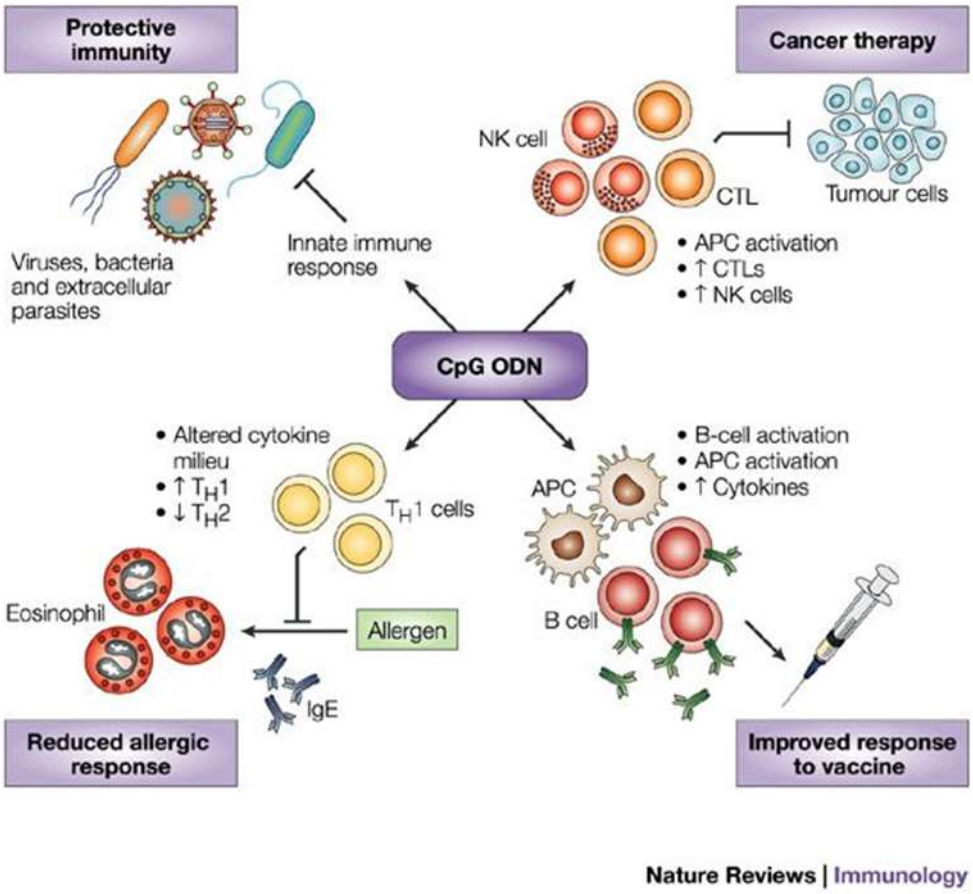

Figure 1.4 Potential applications of CpG-ODN (Reprinted with permission from the publisher. Courtesy to Dennis M. Klinman, 2004, Immunotherapeutic uses of CpG oligodeoxynucleotides, Nature Reviews Immunology)

1.7.5.1 For protective immunity as an innate immune stimulant

CpG-ODN has been identified to stimulate the innate immune systems of mammals (222), birds (63) as well as fish (233) and protect them non-specifically against a multitude of infectious organisms including viral pathogens (23,81,188,335), bacteria (126,208,233) and parasites (44,444). Even protection against Ebola and Anthrax has been reported in mice (210). In mouse models that studied immunoprotection against *Listeria monocytogenes*, this protection has been attributed to pDC that get stimulated by CpG-ODN and the secretion of IL12 and type I IFNs that induce an antiviral and antibacterial state. A new subset of DC was activated by CpG-ODN stimulation in the spleens of mice that secreted IFNγ,and unlike the conventional pDC played a role in the protective efficacy against *Listeria* (173,221). Other cells that are involved in this immune modulation are B cells, macrophages, and NK cells. B cells are directly induced by CpG DNA through TLR9 and produce polyreactive antibodies that help to eliminate pathogens (95,198). A very recent study discovered a distinct subset of pDC in human blood, bone marrow, and tonsils which get stimulated by CpG-ODN but do not produce type I IFN. However, they strongly stimulated B cell activation, antibody production, T cell proliferation and regulatory T cells formation providing the immune stimulatory effect (442).

Studies done in non-human primates revealed that CpG-ODN treatment could even protect immunocompromised hosts from lethal infections. Rhesus macaques infected with simian immunodeficiency virus were able to withstand a leishmanial infection upon D type CpG-ODN treatment (407). Similarly, in pregnant mice, CpG-ODN improved resistance to Listeria infection and reduced placental transfer of pathogens to the fetuses (176). Although there is ample evidence of CpG-ODN's prophylactic nature against infections, it is debatable whether it can protect post-exposure to pathogens. A murine study showed that mice infected with the hepatitis B virus were able to control virus replication in the liver within 24 hours of CpG-ODN delivery in an indirect IFNα/β dependent manner (175). During leishmania infection in mice as well as macaques, CpG-ODN treatment imposed protection and improved survival through innate immune stimulatory mechanisms (407,444).

1.7.5.2 As a vaccine adjuvant

TLR9 activation by CpG-ODN enhances the humoral and cell mediated immunity for many vaccine antigens. The synergy between TLR9 and the B cell receptor helps in stimulating

antigen-specific B cells and thereby cause CpG-ODN to become a strong adjuvant (94). Further on, this synergy has been found to inhibit B cell apoptosis (437) and improve IgG class switch (152). Young-Ha Kim *et al.* published their group's work on the kinetics analysis of CpG-ODN induced mouse B cell growth and Ig production. They mention that CpG induced B cell growth and Ig production is dose-dependent and an optimal dose of 10nM was necessary for that (198). It was identified that giving CpG-ODN directly conjugated to the antigen using alum or lipid emulsions, induced higher adjuvant properties (79,140). Since early experiments, it was understood that CpG-ODN could induce both humoral (antigen-specific Ig) and cytotoxic T lymphocytes (CTL) immune responses when administered with peptide antigens (246). In a very recent study, Alipour *et al.* demonstrated that CpG-ODN and chitosan adjuvants on the HIV-1-Tat-based vaccine induced higher IgG levels, IFNγ producing T lymphocytes and the highest Tat-specific CTL (13). There is also evidence that CpG adjuvant induces prolonged immune responses. The licensed human anthrax vaccine containing CpG-ODN, induced antibodies that lasted over a year and a pool of high-affinity memory B cells that could produce protective antibodies through *de-novo* synthesis after challenge (399). With the development of co-delivery of CpG-ODN with vaccine antigens, different strategies were examined to improve the presentation of both antigen and CpG-ODN to the same APCs.

1.7.5.3 Applications in cancer immunotherapy

As discussed earlier the discovery of bacterial DNA's immune stimulatory effects initiated when Dr. William Coley injected a bacterial lysate intra-tumorally and caused regression of incurable tumors. With the introduction of more stable CpG-ODNs with potent immunostimulatory and antitumor effects, researchers started to conduct more human clinical cancer research using the molecule (5). TLR9 stimulation by CpG-ODN results in predominantly a Th1 type immune response where pDC and B cells secrete cytokines such as IFNα as well as TNF-related-apoptosis-inducing-ligand which could induce tumor cell death. IFNα could cause stimulation of NK cells and cause indirect tumor killing (218). Another hypothesis tested in mice is the IFNγ mediated lymphocyte stimulation and enhanced immunosurveillance to prevent tumor development which has been identified by treating with CpG-ODN (215). Although small tumors were successfully controlled with CpG monotherapy, for large tumors CpG often had to be combined with other anti-tumor strategies like chemotherapy (5,217). In Krieg's review

where he summarized the results of several studies, he hypothesized that in these combined therapies, Th1 response induced by the activation of pDC resulted in cytotoxic T lymphocytes that were capable of controlling the tumor (217). Class B CpG 7909 (PF3512676) is one of the most extensively studied ODN in both pre-clinical and clinical trials. It was able to develop cancer-specific CD8+ cytotoxic T lymphocytes against a variety of cancers like breast cancer, melanoma, and sarcoma (190,287).

Hanagata recently defined cancer immunotherapy in his review as a therapeutic method that utilizes cytotoxic T lymphocytes that recognize tumor associated antigen peptides specifically expressed on tumor cells and thereby specifically identify and kill only the tumor cells after differentiating between normal and tumor cells (148). In early pre-clinical studies where the development of cancer vaccines to eliminate and prevent metastasis of cancers, it was identified that the co-delivery of multiple tumor antigens with TLR9 agonists would enhance the tumor-specific immune response (363). When Shirota and Klinman studied the effect of directly conjugated CpG-ODN with whole apoptotic cancer cells, they noticed that a substantial expansion of tumor-specific cytotoxic T cells that reduced the size of the tumors as well as prevented the metastasis (362). However, compared to systemic administration, local delivery of CpG-ODN into the cancer was much more promising. In the clinical trial of Non-Hodgkin's lymphoma, the patients received CpG-ODN with rituximab or local radiotherapy had a better response with CD8+ T lymphocytes infiltrating the tumor (112). Although murine pre-clinical studies that used CpG-ODN for cancer therapy showed promising results, there is always inconsistency when it comes to clinical trials. One reason could be the broad range of cells expressing TLR9 in mice compared to only pDC and B cells in humans (5).

1.7.5.4 Application in allergy therapy

Allergic diseases such as asthma develop mainly due to Th2 type immune responses. Allergens captured by pDC secrete Th2 cytokines IL4 and IL5 that predominantly promote B cells to differentiate into IgE producing plasma cells. IgE would bind with high affinity Fc receptors on mast cells or basophils. Binding of the allergens with the IgE result in the degranulation of mast cells or basophils; releasing pro-inflammatory and vasoactive chemicals such as histamines, prostaglandins, leukotrienes and cytokines (215). Continuous exposure to allergens through the airway can lead to lung inflammation, injury and airway obstruction (183).

Although not completely proven, the "Hygiene Hypothesis" could be playing a role in allergy where children are not exposed to microbes and allergens due to increased hygienic practices like heavy antibiotic use and vaccinations during childhood. Otherwise, exposure to microbial stimuli would induce Th1 type immune responses (IL12 and IFNγ) that could diminish Th2 (151,215). CpG-ODN could mimic that microbial exposure (151). Two specific strategies based on CpG-ODN therapy has been described for allergy. First is vaccination and second is immunomodulation. Vaccination induces an allergen/ antigen specific immune response that stays for a long duration whereas immunomodulation induces a non-specific innate immune response that usually lasts for a shorter duration (163).

In their earlier studies to test CpG-ODN's immunomodulatory ability to prevent allergic asthma, Krieg's team was able to show that mice exposed to CpG-ODN at the time of initial allergen exposure (schistosome eggs) were protected against eosinophilic airway disease through a Th1 type immune response (205). In fact in a much recent study, the researchers reported that intranasal treatment of mice with CpG-ODN reduced the upper and lower airway inflammation and injury caused by allergic rhinitis and asthma syndrome (237). There are several reports of the immunomodulatory efficacy of CpG-ODN on inhibiting airway hyper-reactivity, eosinophilic inflammation, mucus production and airway remodeling upon chronic exposure to ovalbumin and aspergillus (31,60). Another important allergy condition that is seeking help is atopic dermatitis. Inoue and Aramaki reported about transdermal use of CpG-ODN in a mouse model of human atopic dermatitis and how the Th2 response transformed to Th1 type upon treatment. They said that serum IgE levels decreased with increasing IgG2a levels together with diminished infiltration of inflammatory mast cells. The skin lesions reduced and they also identified T regulatory cells in the skin (168). Similar work was done by another group by topical application of a novel sequence of CpG-ODN and they were able to identify a similar reduction of Th2 responses and upregulation of Th1 responses (197). Nano formulations are being experimented as a means to improve the delivery of CpG-ODN and to facilitate targeted delivery. A recent pilot study was done on dogs with atopic dermatitis by subcutaneously administering CpG-ODN bound to gelatin nano particles. Clinically, pruritus and lesions improved in 8 weeks and serum IL4 levels decreased in a majority of dogs indicating the successful delivery of the formulation (413). According to the extensive research done and being done on the anti-allergy properties of CpG-ODN there is positivity for the future of allergy therapy.

1.7.6 CpG-ODN applications in veterinary species

A study conducted by Rankin *et al.*, 2001 is one of the first to describe immune stimulatory CpG-ODN's for different animal species. That *in vitro* study utilized cat, dog, horse, pig, sheep and chicken peripheral blood to isolate peripheral blood mononuclear cells (PBMC) while splenocytes from mice and cotton rats and popliteal lymph node cells from New Zealand white rabbits were used. They identified that three GTCGTT motifs (2006, 2007, 2014, 2135, 2143) were the strongest and constant immunuostimulatory ODNs in all species. The ODN 2142 containing one GTCGTT, GACGTT, TGCGTT each was stimulatory in all species except sheep and mice. The optimal immunostimulatory motif known for mouse cells; GACGTT was also immunostiulatory in rabbit cells but not for any other species indicating its efficacy only in inbred strains of mice and rabbits. This part of the experiment explained that CpG-ODN recognition is highly conserved among species. Next, this group showed for the first time that CpG-ODN could be used as an adjuvant by formulating with HBV-1 tgB vaccine. The CpG-ODN formulated vaccine gave rise to significant levels of neutralizing antibodies in a dose dependant manner (333).

In contrast to previous experimental results of using CpG-ODN as a successful adjuvant, more recently Parameswaran *et al*., reported that the inclusion of CpG-ODN in a malignant catarrhal fever vaccine alone or combined with emulsigen did not improve the magnitude or duration of immune response in Holstein Friesian calves (308). The authors commented that earlier studies of using CpG-ODN as an adjuvant with emulsigen in bovine herpes virus vaccine prolonged the immune response as well as increased IFNγ production (169,170). In previous studies where CpG-ODN was used with a well-established adjuvant and antigens such as *Mycobacterium bovis* or foot and mouth disease virus, the immune responses have been augmented (338,420). There are studies that identified CpG-ODN's innate immune stimulation beneficial for vaccination outcomes in cattle as well as sheep. One study proved that administering CpG-ODN to newborn lambs was able to reduce herpes virus-1 shedding through inducing an antiviral state (298). Nichani *et al*., were the first to report the in vivo immunostimulatory effect of CpG-ODN in ruminant species. They found CpG-ODN 2007 to increase the animals' body temperatures transiently, increased circulating neutrophils and elevate haptoglobin levels. In sheep it increased the antiviral 2'5'-A Synthetase, but not in cattle. Again,

it was found that formulating CpG-ODN 2007 with 30% emulsigen further increased the immunostimulatory properties (299). In another study done by the same group, CpG-ODN 2007 administration in sheep expressed elevated levels of antiviral 2'5'- A Synthetase, with a peak level of 4 days post treatment and more sustained levels of the enzyme was observed with repeated injection. *In vivo*, they could not detect the correlation of IFNα or IFNγ levels with the enzyme level but *in vitro* it correlated. Furthermore, there have been a lot of cellular recruitment at the CpG-ODN injection site; majority of them being CD172+ myeloid cells, many of them expressed IFNα and some, IFNγ (297).

Another interesting study was done on sheep to understand the difference in cytokine secretion upon TLR signaling in neonatal animals. CpG-ODN treatment caused a very high IL12 secretion from mesenteric lymph node and spleen in neonatal lambs compared to adults. The IL12 secretion in the mesenteric lymph node reduced after 20 days but in the spleen it remained a long time. What's interesting is that the neonatal lambs had a higher proportion of CD14+CD11b+ cells that produced higher levels of IL15 and that increased the production of IL12 through an amplifying feedback loop via CD40 (104). In a study done by Booth *et al.* they found that in sheep intestinal Peyer's patches, TLR9 stimulation is differently regulated compared to other lymphoid organs. Upon stimulation with CpG-ODN, Peyer's patches cells exhibited poor cytokine secretions compared to lymph node cells, and PBMC. Peyer's patches cells secreted IL10 spontaneously; mainly by a newly identified CD21 (+) B regulatory cells type. This IL10 regulated the innate immune responses in the Payer's patches against CpG-ODN stimulation (43).

Van der Stede *et al* conducted and. reported the *in vivo* experiments in pigs concluding that CpG-ODN is a suitable adjuvant for farm animal species for the first time (405). Distinct classes of CpG-ODN have shown different gene expression levels in pigs. A class CpG-ODN 8954 stimulated production of significant but transient levels of IFNγ, IL12, IL6, IL4 and TNFα mRNA. C class CpG-ODN (2429) induced significant levels of IFNγ, IFNα, IL12 and IFNγ inducible protein 10. B class CpG-ODN also induced significantly higher levels of IFNγ inducible protein 10 (74). Hu *et al.* explored the breed differences in CpG-ODN mediated immune response using Dapulian and Landrace pig breeds. Their observation was that PBMC of Dapulian pigs had more TLR9 mRNA compared to Landrace pigs and that contributed to a higher level of immune response (high IFNa, IL8, IL12 and chemokines CXCL9 and CXCL13

mRNA) in the Dapulian pigs' PBMC upon stimulation with CpG-ODN. They correlated this difference to Dapulian pigs' higher disease resistance trait (164). Studies also indicated that CpG-ODN can be an effective vaccine adjuvant for pigs through a Streptococcal vaccine; that induced significantly high levels of antigen specific antibodies, lymphocytic proliferation, IFNγ, IL6 and MHC II (243) as wells as formulating in an attenuated pseudorabies virus vaccine to deliver in aged pigs. The latter induced a Th1 response in the aged pigs indicating that CpG-ODN can be used to overcome age associated immune depression in animals (276).

CpG-ODN formulated with gelatin nano particles has been successfully administered to horses by inhalation to control recurrent airway obstruction due to equine asthma by Klier *et al*. and the studies have proceeded to phase I/II clinical trials. They mention that gelatin nano particles are biodegradable and immunologically inert in the body systems acting as a vehicle to deliver CpG-ODN to target cells. This therapy induced a Th1 response reducing the allergen induced Th2 response as well as anti-inflammatory IL10. Researchers have seen clinical improvements in the nasal discharges, breathing rate, neutrophil percentage and partial oxygen pressure (143–145,337).

CpG-ODN's immunomodulatory effect has been found beneficial in treating immunodeficiency caused by infectious diseases like *Rhodococcus equi* pneumonia. Class B CpG-ODN (2135 and 2142) and class C CpG (2395) have been applied *in vitro* on PBMC taken from 14-56 days old healthy foals or their mothers. Only the class B CpG-ODNs have been able to upregulate IFNγ, IL6 and IL12P40 mRNA expression at a greater degree indicating their potential in using in *R. equi* pneumonia (248). A study by Lopez *et al*. confirmed for the first time that formulation of a killed vaccine with CpG-ODN could enhance antigen specific antibody responses to the equine influenza vaccine (249).

1.7.7 CpG-ODN applications in chickens

Initially, Vleugels *et al.* reported that CpG-ODNs have an immunostimulatory effect in birds and showed CpG-ODN treated birds developed a consistently higher humoral immune response (410). When CpG-ODN was only used in *in vitro* studies and laboratory animal models, Gomis *et al.* conducted *in vivo* experiments to explore its immunomodulatory effects against infectious pathogens in chickens. They delivered CpG-ODN 2007 via IM or SQ routes and tested its efficacy in controlling an *E. coli* infection in a cellulitis model. They found SQ or IM

injection of CpG-ODN could significantly improve survivability, reduce clinical signs and reduce bacterial load. SQ injection in the caudal abdomen was identified to improve survivability better than IM delivery and overall, the protection lasted up to 3 days following CpG-ODN delivery (126). Thereafter this group explored the efficacy of *in ovo* delivery of CpG-ODN in eggs with day 18 old embryos and found that the chicks could be protected against *E. coli* and *Salmonella* infections for up to 6 days post treatment (125,382). Innate immune modulatory effect of CpG-ODN has been further improved by formulating them with liposome, carbon nano tube and D, L-lactide-co-glycolide (PLGA) nano particles (38,137).

1.7.7.1 CpG-ODN immunomodulatory mechanisms in chickens

Although in mammalian species CpG-ODN binds to TLR9, an orthologue PRR was not discovered in chickens until about 2009. Brownlie *et al.* reported TLR21 acts as a functional homologue in birds to TLR9 in mammals in recognizing CpG-ODNs (47). Then, Keestra *et al.* reported that expression of TLR21 in HEK293 cells induced NFκB pathway upon stimulation with unmethylated CpG-ODN. It was also localized in the same intracellular compartment as human TLR9 and it was functional in the endolysosomes. Further on, TLR21 was found absent in humans but had homologs in fish and frogs and it was found similar to mouse TLR13 (192). It is been identified that class B CpG-ODN preferentially stimulate chicken B cells (419). *In vivo* administration of class B CpG-ODN 2007 upregulated MHCII, IFNγ and IL10 (371). In a recent study, researchers showed that *in ovo* delivery of CpG-ODN transiently upregulated IFNα, IFNβ and IL6 with more significant upregulation of IFNγ and IL10 in the spleens (350). Patel *et al.* reported that CpG-ODN predominantly induces a Th1 type immune response with increased expression of IL1β, IL6, IL8, IL18, IL10 and IFNγ cytokine genes in the spleen and IL10 and IFNα in the bursa. Due to the expression of IL10 but not IL4, it is identified as a Th1 biased response (312). Upon CpG-ODN stimulation, recruitment of immune cells can be seen in sites of administration and in other lymphoid and non-lymphoid tissues. *In ovo* delivery of CpG-ODN resulted in increased macrophages, CD8+ and CD4+ T lymphocytes recruitment into the respiratory tracts of neonatal chicks together with upregulated IFNγ mRNA expression. This cytokine and cellular response was able to reduce infectious bronchitis virus load and associated mortality in chicks (81). Furthermore, CpG-ODN stimulated turkey and chicken monocytes to synthesize NO which was vital for microbial killing. It was found that the monocyte stimulation

was CpG dinucleotide dependant. They found that turkey monocytes were less sensitive to CpG-ODN. Overall, the optimum ODN for NO production was GTCGTT (153).

1.7.7.2 CpG-ODN as a poultry vaccine adjuvant

Apart from using as a direct innate immune stimulant, researchers have incorporated CpG-ODN in to poultry vaccines against virus infections such as avian influenza (14,114), Newcastle disease (242,443), Marek's disease (37,39,311), infectious bursal disease (383,418), infectious laryngotracheitis (2,392) and infectious bronchitis (75,77,81) in order to boost the immune response to vaccine antigens (172). Fu *et al.* compared the adjuvant activity of different CpG-ODNs designed based on the CpG-ODN 2006 sequence and identified upregulation of Th1 cytokines (IL6, IL12, and IFNγ) and TLR21 mRNA in the upper respiratory tract shortly after intranasal vaccination with inactivated avian influenza antigen plus CpG-ODN. Avian influenza specific IgA levels were high in respiratory lavage as well as IgG in serum (113). In avian influenza vaccine formulations, more recently CpG-ODN has been identified to induce significantly high local and systemic high affinity IgY responses that reduced virus shedding when formulated with biodegradable poly lactic-co-glycolic acid (PLGA) nano particles. Furthermore, some of these formulations were successfully delivered mucosal (nasal and ocular) and resulted in the secretion of IgA and IgG in lachrymal secretions (14,365,366). Marek's disease vaccine has used CpG-ODN as an adjuvant successfully in the recent years. In an initial study CpG-ODN was administered prophylactically and it was able to delay Marek's disease onset plus decrease virus load in the spleens (311). Then the same group formulated CpG-ODN with PLGA nano particles and reported that prolonged release of CpG-ODN by the nano particles induced innate responses (38). Finally, they administered PLGA formulated CpG-ODN *in ovo* to day 18 embryos and found that it could reduce tumor incidence accompanied by IL1β and IL18 mediated immune response (37). In a very recent attempt to adjuvant *in ovo* herpes virus of turkey vaccine with encapsulated CpG-ODN, the authors found that tumor incidence was reduced although non-significantly and the virus load in feathers were reduced as well. Interestingly, they reported an inversely proportional relationship between IFNγ and IL10 mRNA expression with the tumor incidence and virus load (39).

CpG-ODN used in Newcastle disease vaccine research has shown promising results particularly as a strong mucosal vaccine adjuvant (87,139). When the vaccine was administered

together with CpG-ODN, vaccine specific IgG levels increased with more lymphocytic proliferation. Further, the combination protected chickens from a lethal Newcastle disease virus challenge (242). Intranasal delivery of Newcastle disease vaccine with CpG-ODN was identified to increase systemic IgG levels and lymphocytes as well as IgA levels in intestinal washings and feces (443).

Due to the focus of our work on intrapulmonary (IPL) delivery of aerosolized CpG-ODN micro-droplets, it is important to understand the structure and function of the avian respiratory system.

1.8 Overview of the avian respiratory system

The respiratory system of birds is considered the most efficient out of all the vertebrates thanks to the existence of both lungs and air sacs (258). In birds, respiratory system not only conducts gas exchange but also is involved in thermoregulation and phonation. The beginning of the respiratory system marks at the nares and that continues as a passage to the larynx. The trachea extends from larynx in varying lengths depending on the bird species and divides into two extra pulmonary primary bronchi where each enters into a lung. Trachea in birds has complete tracheal cartilage rings and could be very hard to compress in birds like ducks and geese (101). Upon entry into the lung, the primary bronchus extends to the caudal margin and opens into the abdominal air sac. The primary bronchi divide into four sets of secondary bronchi. The primary bronchi and the initial parts of the secondary bronchi are lined with a ciliated mucosa (336).

Air sacs are thin walled, poorly vascularized large sacs and they occupy a large volume available in the coelomic cavity. They act as bellows and depending on the change in air pressure in them, air movement occurs unidirectional. Air sac epithelium is squamous and cuboidal with few ciliated columnar epithelial cells. Most birds including chickens have nine air sacs. Three types of cranial air sacs; two cervical, one clavicular and two cranial thoracic arise from the first set of secondary bronchi. The caudal air sacs; two caudal thoracic and two abdominal air sacs arise from the second and third set of secondary bronchi and the continuation of the primary bronchus (101,336).

During inspiration, air enters through the lungs and travel to the air sacs. Half of the inspired air travel through the paleopulmonic parabronchi and the other half travel through the

neopulmonic parabronchi and directly into the caudal thoracic and abdominal air sacs. The air that is traversed through neopulmonic parabronchi reduces only a small amount of oxygen partial pressure compared to the air that goes through a much larger area of paleopulmonic parabronchi. During expiration, some of the air from caudal air sacs travel again through neopulmonic parabronchi and a major proportion go through paleopulmonic parabronchi. The air in the cranial air sacs exits through the secondary bronchi and the primary bronchi, keeping the air movement in caudo-cranial direction. The air taken in during one cycle goes through the parabronchi several times making this a very efficient air exchange system (101).

A large number of parabronchi arise from the secondary bronchi interconnecting mediodorsal and lateroventral secondary bronchi with medioventral bronchi. Parabronchial wall consists of air exchange tissue. When inhaled air traverse through parabronchi, they enter the gas exchange tissue through atria, infundibula and then the network of air capillaries that are closely intertwined with a network of blood capillaries that form the most efficient gas exchange surface. The blood gas barrier consists of an endothelium, a thin basal lamina and very thin squamous epithelial cells, that is essentially 56-67% thinner than that of a mammal and 15% greater in surface area. Although efficient in gas exchange, this structural adaptation makes them susceptible to pulmonary injury (258,336).

The arrangement of air sacs and the direction of air flow during inspiration and expiration are illustrated in figures 1.5 and 1.6 (251).

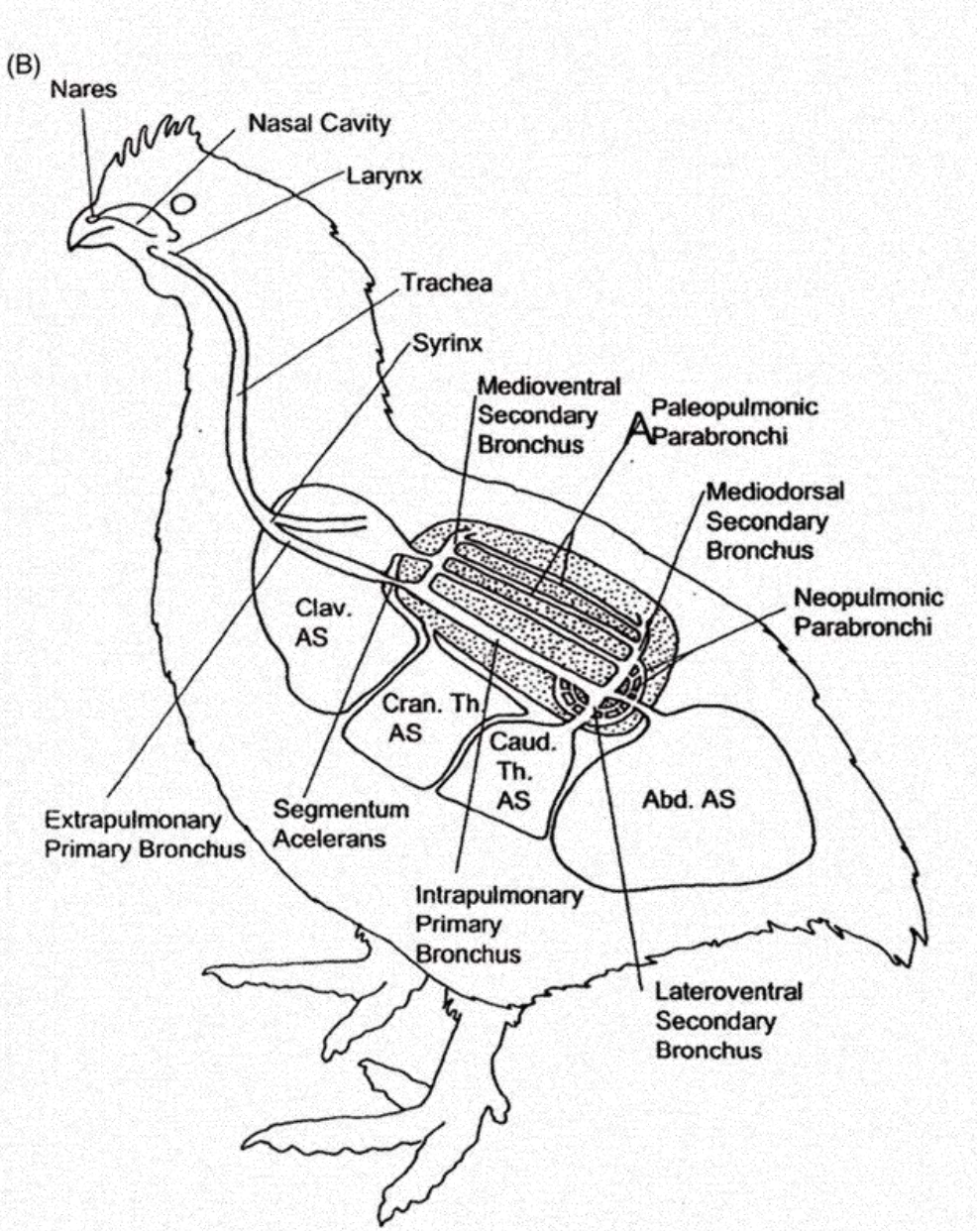

Figure 1.5 Organization of the respiratory system in the chicken Clav. AS=Clavicular air sac, Cran. Th. AS=Cranial thoracic air sac, Caud. Th. AS=Caudal thoracic air sac, Abd. AS=Abdominal air sac. (Reprinted with permission from the publisher. Courtesy to M. R. Fredde, 1998, Relationship of Structure and Function of the Avian Respiratory System to Disease Susceptibility, Poultry Science)

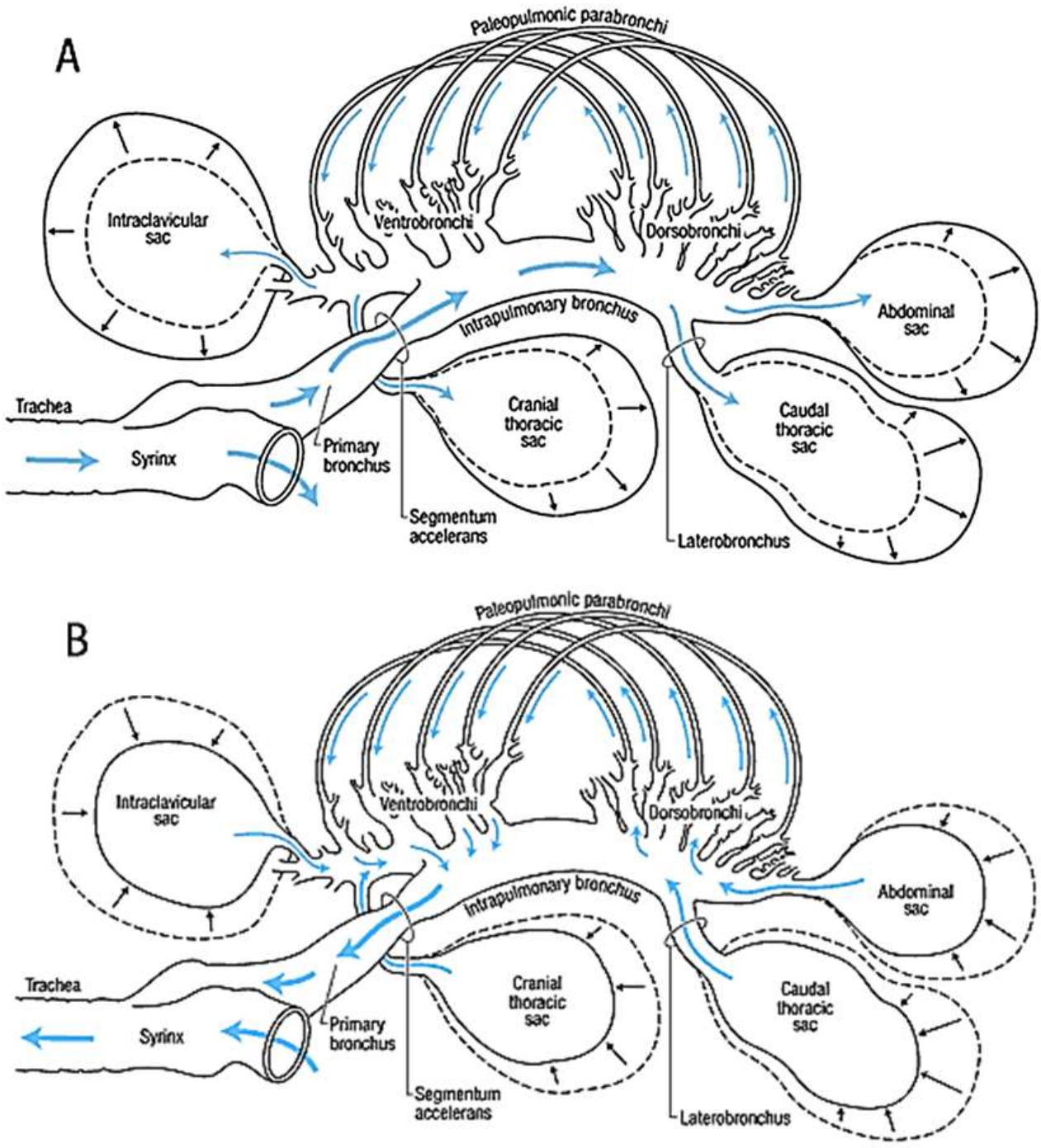

Figure 1.6 A schematic representation of the avian respiratory system and the air flow during inspiration (A) and expiration (B) (Reprinted with permission from Dr. William Ludders of Cornell University. Full attribution given to Michael Simmons and Cornell University as requested by Dr. Ludders.)

1.9 Aerosol delivery of therapeutics and vaccines

Humans have been using therapeutic aerosols since the ancient Egyptian era of ~ 1554 BC in recorded history. Thereafter, the use of therapeutic aerosol inhalation has been practiced by the Indian physicians Charaka and Sushruta to treat asthma as recorded around ~ 600 BC. Hippocrates the famous Greek physician also has used a device that allowed the vapour of burnt resins and herbs to escape through a small hole that can be inhaled. The development of aerosol based therapies improved with time (373) resulting in the modern day nebulizers that allowed us to use in our studies.

1.9.1 Nebulizer devices and aerosolization technology

Atomizer devices break down a liquid into fine particles that would result in a coarse spray that the particles generated will not be small enough to deposit all the way in the lungs. In nebulizers there is a special system (baffle) that helps to remove coarse droplets away from the air stream so that the aerosol particles created are small enough to deposit in lungs. The baffle system in the nebulizer blocks the large droplets so that they could drop back to the reservoir and recycle (300). Considering the mechanism of the modern day nebulizers that assist respiratory drug delivery, there are three methods to mechanically break up the drug solution (265). Air-blast atomization or "jet nebulization" is the oldest method used in the current nebulizer devices. In this method, a compressor brings air at a high speed to the liquid, breaking down the liquid into droplets. These initial droplets are too large to inhale into the lungs so the primary droplets are hit against a baffle to create smaller droplets (107,234). High frequency vibration is the next method in which there is no need of a high-pressure air supply. A bulk piezoelectric head submerged in the liquid can vibrate to create droplets and this type is known as "ultrasonic nebulizers" (435). A much modern technique uses a piezoelectric crystal that creates surface acoustic waves which produces aerosol droplets (Figure 1.7). This is much higher in frequency, lower power and has been found to deliver a higher proportion of solution to the lung. Rajapaksha *et al*. reported the successful pulmonary delivery of plasmid DNA into sheep lungs using a surface acoustic wave nebulizer while keeping the plasmid integrity (331). The other improved type is the vibrating mesh nebulizers. Vibration of a mesh containing thousands of tiny holes makes the solution exit as atomized droplets. Few advantages of that are quicker dosing, lesser residual volume and smaller in size compared to the jet nebulizers. However, the holes can

get clogged with micro particles which is a disadvantage (414). A much rather newer technology is used by "colliding jet nebulizers". Extrusion of ultra-small volumes of liquid solutions through tiny opposing nozzles causes a collision that leads to the formation of an aerosol in these nebulizers (265).

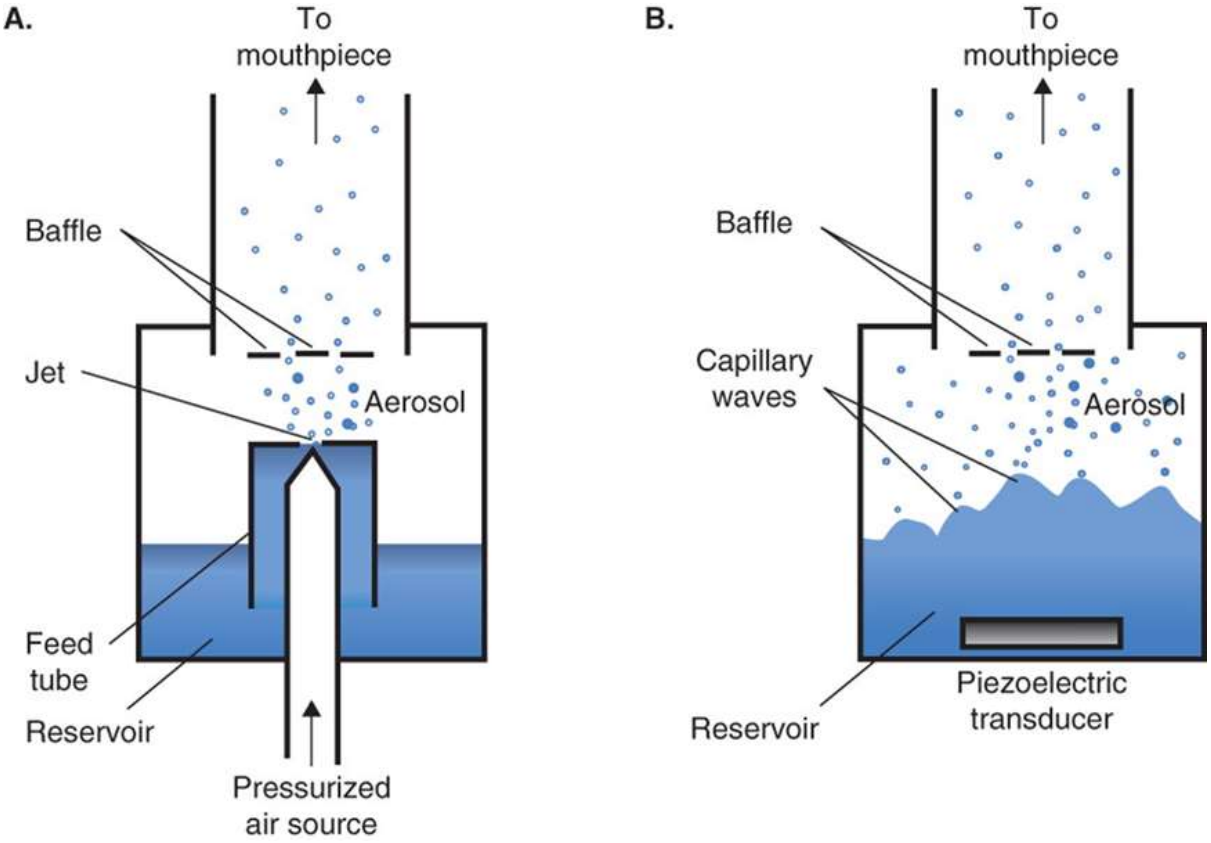

Figure 1.7 Schematic illustration of a typical jet nebulizer (A) and ultrasonic nebulizer (B) (Reprinted with permission from the publisher. Courtesy to Leslie Y. Yeo *et al*., 2010, Ultrasonic nebulization platforms for pulmonary drug delivery, Expert Opinion on Drug Delivery)

1.9.2 Factors influencing pulmonary aerosol delivery

Several factors such as particles size (aerodynamic diameter), size distribution, shape of the particle and its density affect the ability of a particle to deposit in the airway. Many researchers mentioned that particle size plays the biggest role and most of the medical aerosol in reality are heterodisperse, containing a range of particle sizes rather than being monodisperse (228). Calderon-Nieva *et al.*'s review on veterinary vaccine nanotechnology discussed that the three aerodynamic properties; impaction, sedimentation and diffusion that cause the settlement of aerosolized particles in the respiratory system depends on the particle size distribution that is

caused by the type of device that generated the particles as well as the type of breathing pattern (49). In fact, Lu and Hickey mentioned >5μm (3-5μm) particle size causes particles to deposit in the upper and middle airway by the mechanism of inertial impaction. If a deep forceful breath is taken, these large particles cannot change direction due to higher density so they end up depositing in the mouth, pharynx and tracheal mucus and eventually get removed by swallowing (49). <3μm particles that have not deposited by impaction, move to lower airways and bronchioles upon slower air velocity or breathing and deposit by sedimentation (gravity). Submicron size particles (<1μm) deposit in the lower airway by diffusion. However, when we consider vaccine or therapeutic molecules, such theories on particle size may not apply due to their aqueous nature (49,250). Martin and Finley discussed about the factors that affect the particle size in their review. Evaporation and condensation are two factors that can significantly change the diameter of an aqueous aerosol droplet which will affect its deposition in the intended region of the lung. If the aerosol is introduced to low humidity air just before they enter into the airway, the droplets can shrink and become smaller so that they could pass all the way to inner airway rather than depositing in extra thoracic airways (106,265).

1.9.3 Aerosol delivery of therapeutics and vaccines in poultry

In the poultry industry, a number of vaccines against infectious diseases currently utilize spray vaccination method in hatcheries as well as in bird houses. They include infectious bronchitis (343), Newcastle disease (87) and coccidiosis (12) vaccines administered in the hatchery and infectious bursal disease (30), infectious bronchitis (184), infectious laryngotracheitis (116) and Newcastle disease vaccines (409) administered on-farm (262). While live influenza vaccines are not allowed for aerosol administration due to its zoonotic potential, there are inactivated vaccines administered as intranasal sprays although they were identified poorly immunogenic (120). More recently, a spray freeze dried, powdered, inactivated and non-adjuvanted avian influenza vaccine was reported to be 100% protective against a high pathogenic avian influenza challenge when it was delivered to the syrinx (318). Other than these vaccines, spray vaccination against *E. coli* and *Salmonella* are currently in practice with a live bacterial vaccine (Poulvac® by Zoeitis) (105).

Currently used coarse spray and aerosol vaccinators produce a broad range of droplet size distribution. As a result, small vaccine droplets of a primary coarse spray vaccine could deposit

in the deeper air ways causing disease. Concurrently, scarcity of large droplets in a fine aerosol vaccine could result in poor immunogenicity. Based on previous studies, Landmark *et al.* mentioned that vaccine formulation as a dry powder would be better than liquid in order to maintain a narrow size distribution. Other researchers have also confirmed that experimentally (69). Although regarding immunogenicity and adverse effects, Landmark *et al.* did not observe a difference between powder or liquid forms in his Newcastle disease virus vaccine experiments (230).

In companion bird species, nebulization of aqueous therapeutics is in practice (274). Antibiotics (186), antifungals (346) and mucolytic agents are administered to small birds such as budgerigars using nebulization when it comes to emergency and critical care (101). Although aerosol delivery is used with the purpose of intrapulmonary deposition of therapeutics in birds, not many studies have been conducted to explore the degree of particle deposition upon nebulization. Lisa A. Tell *et al.* conducted studies to detect the distribution of 1μm aerodynamic equivalent diameter particles (fluorescent spheres) in the avian respiratory system when they were nebulized over different time durations. They found that particle deposition in the respiratory tract increased with exposure time (more after 2-4 hours) and more particles were deposited in the lung than the air sacs (391). Their previous study reported that in anaesthetized pigeons nebulized microspheres of 1-3μm diameter were distributed throughout the respiratory tract with a majority deposited in secondary bronchi and ostia. Larger particles of 6 and 10 μm were confined to the trachea and primary bronchi (390). With the available data from previous studies, nebulization seems to be an effective mode of therapeutic delivery to the avian lung if the optimum particle size is maintained.

1.10 Hypothesis

Mortality during the first week of life due to bacterial infections is causing major economic losses to poultry producers. Although the previous practice was to use prophylactic antibiotics, public health concerns such as the emergence of antibiotic resistant bacteria has raised questions regarding that. As a result, Canadian poultry industry has initiated to voluntarily withdraw from the prophylactic use of antibiotics. Although public health is addressed, the degree of health and welfare of the poultry birds affected without alternative strategies is concerning. As a result, the industry is actively seeking safe and effective alternatives to ensure

poultry health and welfare. CpG-ODNs have been identified immunostimulatory and protective against several bacterial pathogens in chickens when administered in parenteral routes and *in ovo*. However, in the fast-paced industry that handles a large number of birds on a daily basis, IM or SC injections are not practical. *In ovo* delivery is well established in poultry hatcheries but the immunoprotection induced by CpG-ODN delivered in this route is short lived post-hatch. Mucosal immunization methods are currently practiced in poultry hatcheries particularly via coarse spray, but these techniques do not facilitate deeper lung delivery of droplets. As a result, we developed a technique to deliver aerosolized micro particles of CpG-ODN directly to the lungs of neonatal broiler chicks with the hypothesis that it would induce generalized innate immunity. We hypothesized that this technique would provoke longer lasting protective immunity against lethal bacterial infections in day-old broiler chicks and it could be developed as an industry feasible technique for the commercial poultry industry in the long run.

1.11 Objectives

1. To develop a laboratory scale IPL CpG-ODN delivery system and study the immune stimulatory effects in neonatal broiler chickens;
    a. Protective efficacy against lethal *E. coli* septicemia
    b. Dose titration and exposure time for optimum immune protection
    c. Duration of protection upon administration
2. To develop a commercial scale poultry nebulizer (CSPN) to deliver CpG-ODN to a large number of neonatal broiler chickens at commercial broiler hatcheries and study the immune protective efficacy using large scale field trials.
3. To evaluate the immune mechanisms resulting from IPL CpG-ODN delivery in neonatal broiler chickens:
    a. Kinetics of cytokine gene expression involved in the immune protective effect of CpG-ODN (multiplex assay for mRNA gene expression)
    b. Cellular mechanisms involved in the immune protective effect of CpG-ODN (flow cytometry)
4. To explore the metabolomics landscape of antimicrobial immunity induced by CpG-ODN in neonatal broiler chicks

PREFACE TO CHAPTER 2

Protecting neonatal broiler chicks from lethal bacterial infections is vital for the commercial poultry industry. Since the withdrawal of antibiotics, a surge of need and demand for alternative strategies has begun (268). It is understood that the protective shield against these infections must be launched as soon as possible after hatching order to defend the neonatal chicks prior to reaching the barn environment (86,385). Immunostimulatory CpG-ODNs have been able to protect broiler chicks from bacterial infections when it's administered via parenteral routes such as IM, SQ or *in ovo* injections (125,126,382). IM and SQ routes confer excellent innate immune stimulation leading to remarkable protection that lasts for up to 6 days (126,381) however; their practicality in the fast paced commercial poultry production is questionable. In contrast, *in ovo* delivery of CpG-ODN is more feasible due to the use of an existing technology; the automated egg injector which is used in most hatcheries to deliver vaccines (137). Despite the ease of delivery, the length of immunoprotection induced by *in ovo* delivery in the day 18 embryos is rather shorter. By the time these chicks hatch at day 21, their post-hatch protection is quite short lived for about 2-3 days. It would be ideal to administer CpG-ODN to newly hatched chicks just before they leave the hatchery using a technique that could be compatible with the mass production of chickens on a daily basis. Vaccine administration to day-old chicks against diseases like infectious bronchitis and coccidiosis is currently in practice using methods like coarse spray or gel administration using a spray cabinet (105,184). Due to the use of modified live viruses in vaccines, that technique only targets to deliver droplets to the upper respiratory tract or for coccidial vaccine, oral ingestion (184). Compared to that, CpG-ODN is a synthetic TLR agonist that does not have virulent properties, so our objective was to deliver it to the lung and thereby induce an immune modulation. Droplet size is directly correlated to the deposition site in the respiratory tract. According to previous studies, <5μm particles could travel all the way to the lung (69). We identified IPL delivery as a suitable technique because; aerosolization of microdroplets could assist the delivery of CpG-ODN to many chicks at the same time. Further, as a needle free application, we hypothesized that it could be used with minimum to no handling of the birds. As a result, we performed experiments to identify the protective efficacy of IPL CpG-ODN delivery against *E. coli* septicemia. The following chapter 2 discusses the protective efficacy, dose titration and the duration of protection induced by the IPL delivered CpG-ODN microdroplets against lethal *E. coli* septicemia in neonatal broiler chicks.

CHAPTER 2 INTRAPULMONARY DELIVERY OF CPG-ODN MICRODROPLETS PROVIDES PROTECTION AGAINST *ESCHERICHIA COLI* SEPTICEMIA IN NEONATAL BROILER CHICKENS

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Published in *Avian Diseases,* 2017. 61 (4): 503-511

Contribution: The conceptual idea was generated and experiments were designed by my self, Ms. Popowich and Dr. Gomis. The members of the research group assisted to conduct the laboratory experiments. I wrote the manuscript under Dr. Gomis's guidance and all the authors mentioned above contributed with their feedback.

2.1 Abstract

CpG-ODN motifs are effective immunostimulatory agents against a variety of viral, bacterial, and protozoan diseases in different animal species including poultry. We recently reported that in ovo injection of CpG-ODN triggers protection in neonatal chickens against bacterial septicemia. This study was designed to explore the effectiveness of needle-free IPL delivery of CpG-ODN micro-droplets against *E. coli* infection in neonatal chicks. In the present study, we used 840 chicks in total keeping 40 chicks per group. Chicks were IPL delivered CpG-ODN or saline at 1 d post-hatch. Two-days later chicks were challenged with two doses ($1x10^4$ (n=20) or $1x10^5$ (n=20) colony forming units (CFU) of *E. coli*. We observed significantly lower clinical signs and bacterial load in the chicks treated with CpG-ODN by the IPL route in contrast to the group treated with saline ($P<0.05$) . CpG-ODN treated groups were significantly protected against *E. coli* septicemia. The immunoprotective effect induced by the IPL delivery of CpG-ODN was dose- and exposure time-dependent. Our observation proved that IPL delivery of CpG-ODN can induce protective immunity as early as 6 hr and remain effective at least until five days following treatment. Furthermore, there were no adverse effects of IPL delivery of CpG-ODN on growth or mortality up to 42 d of age. Our findings suggest that IPL delivery of CpG-ODN can be identified as an assuring alternative to antibiotics for stimulating protective immunity in chicks during the critical first week of neonatal life.

2.2 Introduction

The commercial poultry industry is constantly searching for novel measures to combat infections to ensure the welfare of birds and food safety (423). High mortality associated with bacterial infections during the first week of a bird's life has devastating impacts on production (433). Of these bacterial infections, *E. coli* septicemia is a major cause of FWM in the broiler chicken industry worldwide (301). In addition to high mortality during the flock cycle, these bacterial infections result in a lack of uniformity of a flock, chronic infections and condemnation of carcasses at processing (45,301). To prevent losses due to bacterial infections in the poultry industry, prophylactic use of antibiotics is in common practice in some areas of the poultry industry. These industry practices risk the emergence of resistant strains of bacteria and antibiotic residues in poultry products (52,144). As a result, the poultry industry urgently needs alternatives to antibiotics (15,275).

Earlier studies have reported that specific DNA sequences containing CpG motifs in bacterial DNA as well as their synthetic counterparts, CpG-ODN possess immune stimulatory properties (222,293,429,430). In human and other mammalian cells, these bacterial CpG motifs or synthetic CpG-ODNs are recognized by intracellular TLR9 present in the immune cells (10,157,178,369)**.** Upon stimulation of immune cells, CpG-ODNs induce a Th1 type immune response by stimulating lymphocytes (B cells, T cells, and NK cells) to secrete IL6, IL12 and IFNγ ensuring the induction of a robust innate immune response (211). This immune response induced by CpG-ODN has been demonstrated to be effective in protecting animals against bacterial (221,334) viral (235) and protozoan (444) infections.

In chicken, TLR21 is an intracellular receptor and a functional orthologous to mammalian TLR9, stimulating macrophages upon binding to bacterial and synthetic DNA containing CpG motifs (47,192). The immune responses induced by CpG-ODN in chicken are a predominantly Th1 type (192,312). We have previously shown that CpG-ODNs produce significant immunoprotection against bacterial septicemia such as *E. coli* and *S.* Typhimurium when administered by the parenteral route to broiler chickens or by the *in ovo* injection to incubating eggs (125,382). Recently, we reported that CpG-ODN formulated with liposomes or carbon nanotubes could further enhance the immunoprotective activity of CpG-ODN (137).

We assumed that needle-free delivery such as IPL aerosol route might be more suitable for poultry industry for concerning the ease of large-scale application. As a result, this study intended to reconnoitre the effectiveness of IPL delivery of CpG-ODN as micro-droplets at hatch as an immunoprotective measure against the E. coli septicemia in neonatal broiler chickens.

2.3 Materials and methods

2.3.1 Animal housing and maintenance

This work was performed with the approval of the Animal Research Ethics Board, University of Saskatchewan, obeying the guidelines of the Canadian Council on Animal Care. Day-old broiler chickens or broiler hatching eggs were obtained from commercial hatcheries in Saskatchewan or British Columbia, Canada. Eggs were incubated at the Animal Care Unit (ACU) at the Western College of Veterinary Medicine, University of Saskatchewan, Canada. Chicks were randomly allocated into groups and placed in animal isolation rooms at the ACU. Water and commercial broiler feed were provided ad libitum. Air from each bird room was

exhausted through a high-efficiency particulate air filter, and non-recirculated intake air was supplied at a rate of 15–20 air changes/hr. Maintenance of air pressure differentials and strict sanitation were ensured in this isolation facility. Broilers were raised at 32 C for the first week of life; thereafter, the temperature was decreased by 0.5 C per day until a room temperature of 27.5 C was reached. Light was provided for 24 hr from days 0 to 2 post-hatch. Darkness was introduced at three days post-hatch with 1 hr of dark added daily until 4 hr of darkness was achieved.

2.3.2 *E. coli* culture and animal model

As previosuly discussed, the challenge strain used was a field isolate of *E. coli* which was isolated from a septicemic turkey (128,141). Briefly, one colony of *E. coli* was added to 100 mL of Luria broth (Difco LB broth Miller; Becton, Dickinson, and Company, Sparks, MD) in a 250 mL capacity Erlenmeyer flask. The bacteria were grown at 37 C for 16–18 hr, shaking at 150 rpm. This stationary phase culture contained approximately $1x10^9$ CFU of bacteria per mL that was then further diluted into saline to the concentration of bacteria required to challenge birds. The *E. coli* challenge dose was confirmed by plating serial dilutions in duplicate on 5% Columbia sheep blood agar plates, incubating for 18 hr at 37 C, then counting the number of colonies. The *E. coli* animal challenge studies were conducted as described previously (125). Briefly, birds were challenged with either $1x10^4$ or $1x10^5$ CFU of *E. coli* by the SQ route in the neck. Two doses of *E. coli* were given to groups of birds to simulate field conditions because; in reality birds in a commercial poultry barn will not be exposed to a consistent dose of *E. coli*. Following challenge, the chicks were evaluated three times daily during the critical stage (until three days post challenge) and twice thereafter for seven days post challenge. Each bird was observed for clinical signs, and a daily clinical score was assigned: 0 = normal; 0.5 = appearing slightly abnormal, slow to move; 1 = depressed, unwilling to move; 1.5 = unwilling to move, may drink water and peck some; 2 = unable to stand or reach for food or water; and 3 = found dead. Birds displaying a clinical score of 2 were euthanatized by cervical dislocation. At the end of the trial, each bird was given a cumulative clinical score (CCS) as a sum of daily clinical scores as previously described (125). Immediate necropsy was performed on the chicks that were found dead or euthanatized. On day seven post-challenge, the remainder of surviving birds were euthanatized by cervical dislocation. Bacterial swabs were collected from the air sacs of dead

and euthanatized and cultured on 5% Columbia sheep blood agar according to the quadrant streaking technique. A semi-quantitative evaluation of *E. coli* isolation was performed according to the growth on blood agar. Bacterial growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; few = less than 5 colonies; 1+ = bacterial growth on area 1; 2+ = bacterial growth on areas 1 and 2; 3+ = bacterial growth on areas 1, 2, and 3; and 4+ = bacterial growth on all areas 1, 2, 3, and 4 (315).

2.3.3 CpG-ODN and intrapulmony delivery

The CpG-ODN (TCGTCGTTGTCGTTTTGTCGTT, 2007) was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies Inc., Huntsville, AL). Synthetic CpG-ODN was diluted in sterile, nonpyrogenic saline. CpG-ODN was delivered by IPL route and was aerosolized as microdroplets (particle size of 0.5–5 μm) by using a Compressor Nebulizer (705-470) (AMG Medical Inc., Montreal, QC, Canada). Three doses (4 mg/chamber, 2 mg/chamber, or 0.4 mg/chamber) of CpG-ODN were aerosolized in a closed 0.036 m^3 acrylic chamber for 15 or 30 min (Figure 2.1). The control group of birds was aerosolized with saline for 30 min in the acrylic chamber by using the Compressor Nebulizer (AMG Medical Inc. Montreal, QC, Canada). The temperature was maintained at 28 – 30 C in the acrylic chamber during the administration of CpG-ODN or saline. Using fluorescent labeled CpG-ODN and confocal microscopy of the entire respiratory tract including trachea and lung tissues, we have confirmed the intrapulmonary delivery of CpG-ODN (unpublished data).

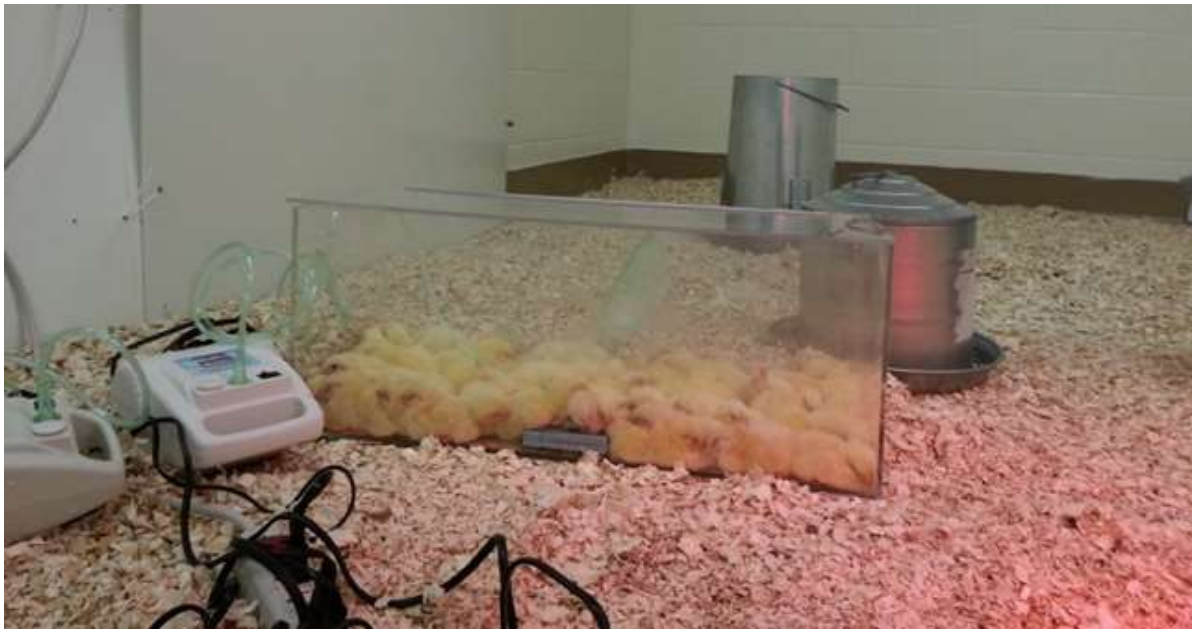

Figure 2.1 Intrapulmonary CpG-ODN administration to a group of 40 neonatal broiler chicks. The acrylic chamber ($0.036m^3$) containing chicks (n = 40) with tubing and mask is attached to a compressor nebulizer set up in the animal room.

2.3.4 Experimental design

2.3.4.1 Immunoprotective effects of CpG-ODN as intrapulmonary microdroplet against *E. coli* septicemia

The experiment consisted of two experimental groups: (a) IPL CpG-ODN (4 mg/chamber) microdroplets for 30 min on day 1 of hatch (n = 40) and (b) IPL saline for 30 min on day 1 of hatch (n = 40). Both groups were challenged with either $1x10^4$ (n = 20) or $1x10^5$ (n = 20) CFU of *E. coli* at 3 days post-hatch (3 days post–IPL delivery). Birds were examined for clinical signs for ten days post–*E. coli* challenge. The clinical signs and bacterial isolations were recorded as described above. Gross pathologic examination was conducted on dead birds.

2.3.4.2 Exposure time of CpG-ODN in neonatal broiler chickens for intrapulmonary microdroplet delivery

We next designed experiments to identify the exposure time of IPL CpG-ODN as microdroplets required to obtain significant immunoprotection against *E. coli* septicemia. Three groups of birds at day 1 of hatch were used: (a) IPL CpG-ODN (4 mg/chamber) as microdroplets for 15 min (n = 40), (b) IPL CpG-ODN (4 mg/ chamber) as microdroplets for 30 min (n = 40), and (c) IPL saline microdroplets for 30 min (n = 40). All groups were challenged with *E. coli* at

3 days post-administration of CpG-ODN with either $1x10^4$ (n = 20) or $1x10^5$ (n = 20) CFU of *E. coli*. The clinical signs and bacterial counts from air sacs were recorded as described above. Gross pathologic examination was conducted on dead birds.

2.3.4.3 Dose titration of CpG-ODN in neonatal broiler chickens for intrapulmonary microdroplet delivery

In another experiment, CpG-ODN was aerosolized using various doses including 4 mg/chamber, 2 mg/chamber, or 0.4 mg/chamber, in closed 0.036 m^3 acrylic chambers. The intention of this experiment was to identify the minimum effective dose of CpG-ODN that could protect against *E. coli*. Four experimental groups of birds at day 1 of hatch were included in the experiment: (a) IPL CpG-ODN as microdroplets for 30 min by using CpG-ODN 4 mg/chamber, (b) IPL CpG-ODN as microdroplets for 30 min at a concentration of 2 mg/chamber, (c) IPL CpG-ODN as microdroplets for 30 min by using CpG-ODN 0.4 mg/chamber, and (d) IPL saline microdroplets for 30 min. All groups were challenged with *E. coli* at 3 days post-administration of CpG-ODN with either $1x10^4$ (n = 20) or $1x10^5$ (n = 20) CFU of *E. coli*. The clinical signs and bacterial counts from air sacs were recorded as described above. Gross pathologic examination was conducted on dead birds.

2.3.4.4 Duration of immunoprotective effects of CpG-ODN as intrapulmonary microdroplets against *E. coli* septicemia

Here, the objective was to study the duration of immunoprotective effects of CpG-ODN following IPL microdroplet delivery. Broiler chickens at day 1 of hatch were randomly allocated into ten groups (n = 40). Of these ten groups, five received IPL CpG-ODN (4 mg/chamber) as microdroplets for 30 min while the other five groups received IPL saline as microdroplets for 30 min. Within each group, birds were challenged with *E. coli* at $1x10^4$ (n = 20) or $1x10^5$ (n = 20) CFU SQ in the neck at the following time points: (a) 6 hr, (b) 1 day, (c) 3 days, (d) 5 days, and (e) 7 days post-administration of either IPL CpG-ODN or IPL saline as microdroplets. The clinical signs and bacterial counts were recorded as described above. Gross pathologic examination was conducted on dead birds.

2.3.4.5 Cellular infiltration in the lungs and growth rate of chickens following intrapulmonary delivery of CpG-ODN

Two groups of broiler chickens at day 1 of hatch were exposed to (a) IPL CpG-ODN (4 mg/chamber) as microdroplets for 30 min (n = 60) or (b) IPL saline as microdroplets for 30 min (n = 60). All birds used for histopathology of lungs were raised in the same manner. In order to evaluate the pulmonary parenchyma at the microscopic level, sections of lungs were collected from five birds per group at 0, 3, 6, 12, 24, 48, and 72 hr post-administration of IPL CpG-ODN. These samples were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned in 5 μm, and stained with hematoxylin and eosin by using standard methods. Remaining birds (25 birds/ group) were monitored for health and clinical signs and at 42 days, were euthanatized. At the time of euthanasia, tissue samples (lung, liver, spleen, heart, bursa of Fabricius, thymus, and muscle) were collected for histopathologic examination. Body weight and bursal weight to body weight ratio (BBW) were calculated (214).

2.3.5 Statistical analysis

Clinical scores of each bird for the 10-day period were summed to generate a CCS. Kruskal Wallis nonparametric analysis of variance was used to calculate the significance of differences among groups. The significance of difference in survival analysis, bacteriologic scoring, and CCS were analyzed using Prism (Prism 5.0; GraphPad Software Inc., San Diego, CA). The relative risks of mortality compared to control subjects were calculated using Fisher exact test in Prism. The significance of differences among groups in survival patterns and median survival times were analyzed using the log-rank test and chi-square statistics.

2.4 Results

2.4.1 Immunoprotective effects of CpG-ODN as microdroplets against *E. coli* septicemia

A significantly higher survival proportion in the IPL CpG-ODN as microdroplets was noted when compared to the IPL saline as microdroplets group ($P<0.005$) (Figure 2.2). This group of birds experienced about half of the relative risk of mortality as did the birds that received saline 52%, $P=0.0072$). The dead or euthanatized birds were opened at the necropsy facility to identify pathologic lesions. On gross examination, the dead or euthanatized birds at 24

hr post-infection did not show pathologic changes other than mild to moderate splenomegaly. Fibrinous pericarditis appeared in a majority of birds together with splenomegaly after 48 hr of infection. By 72 hr post-infection, common findings of bacterial infections such as fibrinous pericarditis, air sacculitis, and mild to moderate perihepatitis started to show. Beyond 72 hr, dead and euthanatized birds had moderate to severe fibrinous polyserositis with caseous material deposited all over the celomic cavity. Some birds had ascites containing clear to cloudy, yellowish fluid. Dead birds in both groups showed similar gross lesions. The groups that received IPL CpG-ODN as microdroplets had significantly lower CCS ($P<0.05$) compared to IPL saline as microdroplets (Figure 2.3). Low counts of bacteria were isolated from the groups that received IPL CpG-ODN as microdroplets compared to IPL saline (Figure 2.4).

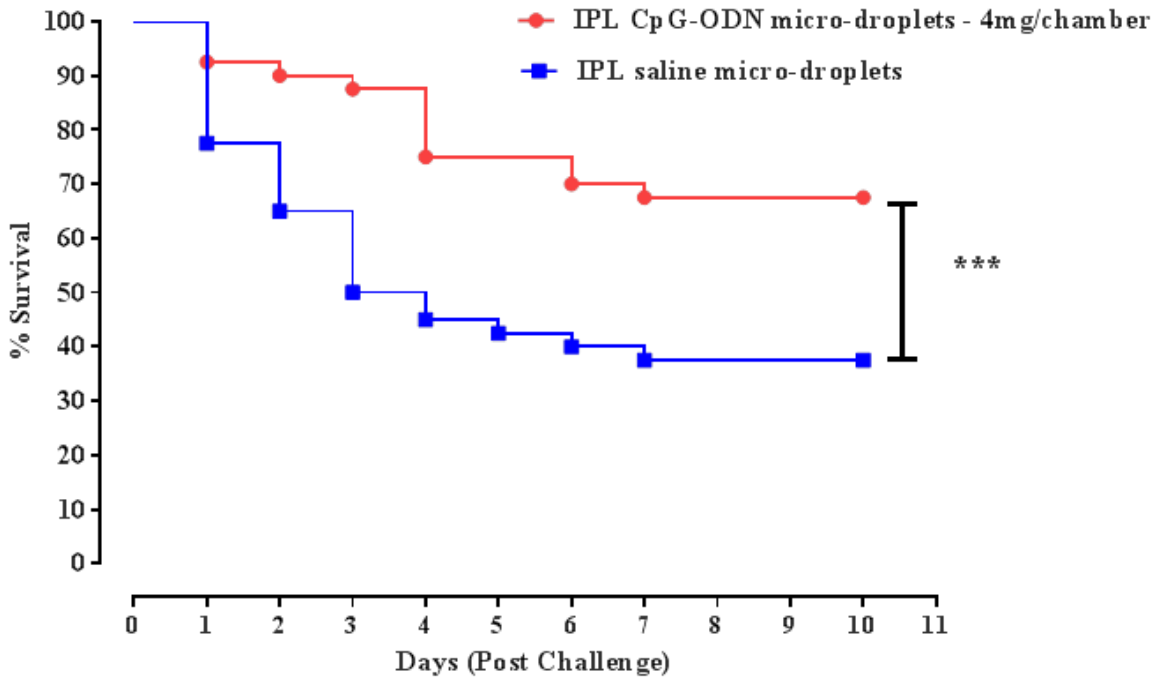

Figure 2.2 Survival percentages following *E. coli* challenge Survival of neonatal broiler chickens delivered with IPL CpG-ODN prior to a lethal *E. coli* challenge [50% of birds of each group received $1x10^4$ CFU (n=20) and the remaining 50% of birds received $1x10^5$ CFU (n=20) *E. coli*]. Birds that received IPL CpG-ODN as micro-droplets had a significantly higher ($P<0.05$) survival compared to the IPL saline control.

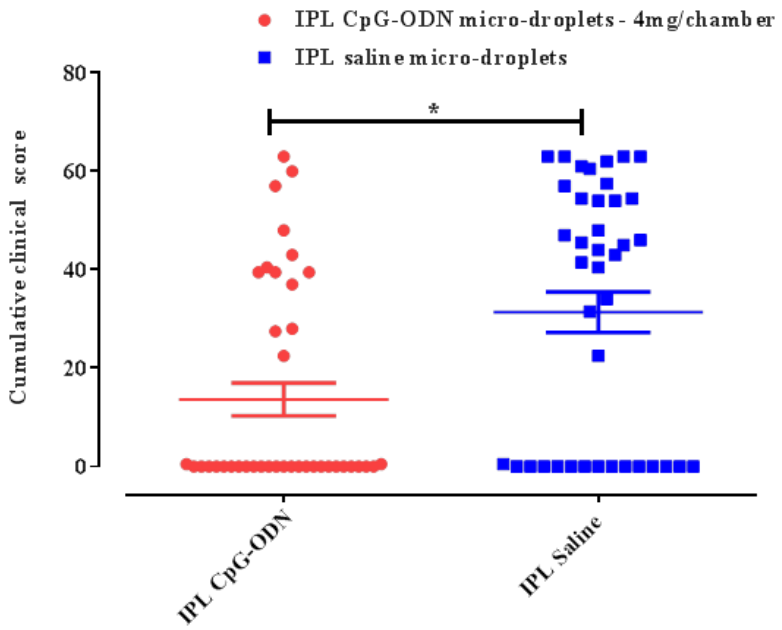

Figure 2.3 Cumulative clinical score following *E. coli* challenge CCS of neonatal broiler chickens following CpG-ODN treatment and *E. coli* challenge. CCS in the birds treated with IPL CpG-ODN were significantly lower than the IPL saline control group ($P<0.05$).

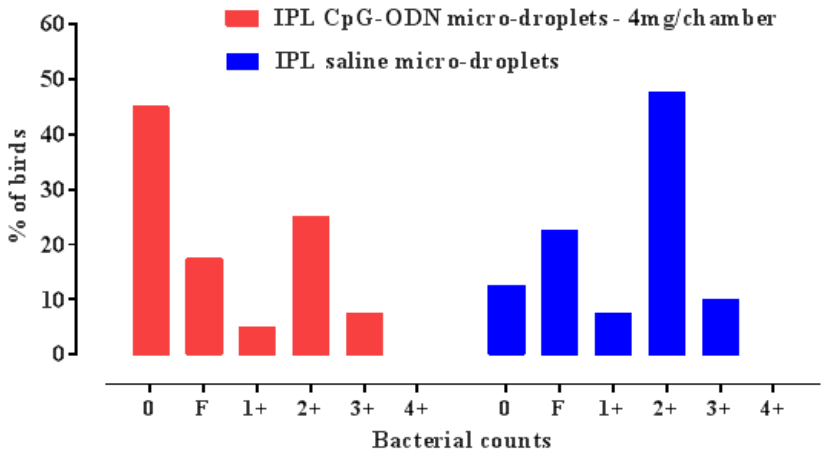

Figure 2.4 Bacterial loads from the air sacs Bacterial isolations from air sacs of neonatal broiler chickens following IPL CpG-ODN as micro-droplets and *E. coli* challenge. The IPL CpG-ODN micro-droplet group had the lowest bacterial isolations compared to other groups. (n = 40).

2.4.2 Exposure time and dose titration of intrapulmonary CpG-ODN in neonatal broiler chicks

Exposure of birds to IPL CpG-ODN as microdroplets for 15 or 30 min showed significantly higher survivability compared to control group IPL saline ($P<0.05$) (Figure 2.5). The birds that were exposed to 15 min of CpG-ODN by the IPL route experienced about half the relative risk of mortality (47%, $P = 0.029$) compared to the IPL saline group. In this experiment, when the birds were exposed to CpG-ODN for 30 min by the IPL route, they experienced approximately a quarter of the relative risk of mortality (24%, $P = 0.001$) as did the IPL saline control birds. Although birds that were given 30 min exposure to IPL CpG-ODN as microdroplets had numerically better survival compared to those with 15 min of IPL CpG-ODN as microdroplets, the difference was not statistically significant. The CCS of birds exposed to IPL CpG-ODN as microdroplets at either 15 or 30 min was significantly lower compared to the IPL saline control group ($P<0.05$) (Figure 2.6). More birds had lower bacterial counts in the group treated with IPL CpG-ODN as microdroplets (Figure 2.7) than in the other groups.

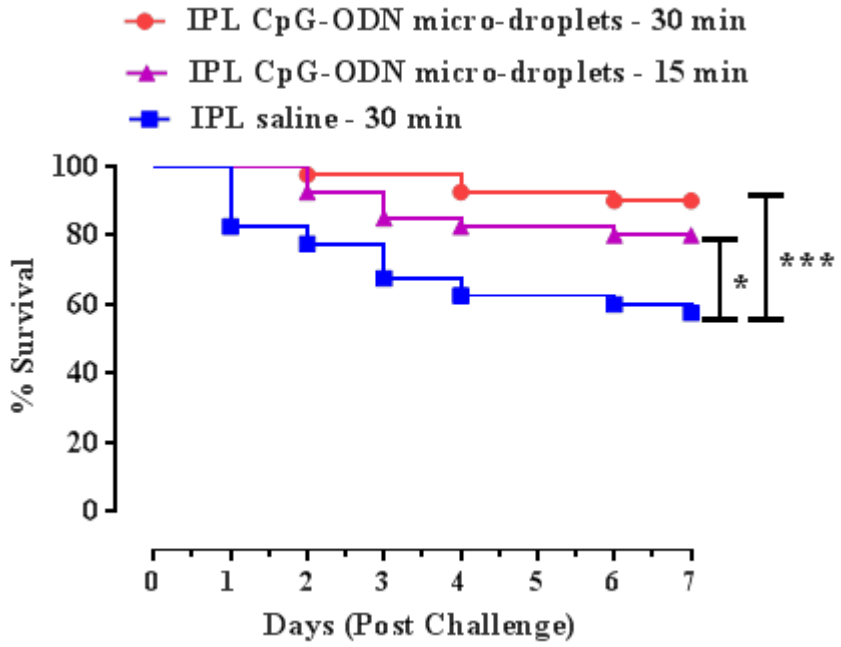

Figure 2.5 CpG-ODN dose titration by controlling the duration of exposure. Birds exposed to CpG-ODN by the IPL route at a concentration of 4 mg/chamber for either 15 or 30 min prior to *E. coli* challenge [50% of birds of each group received $1x10^4$ CFU (n=20) and the remaining 50% of birds received $1x10^5$ CFU *E. coli* (n=20)] had significantly higher survivability compared to the IPL saline control group ($P<0.05$).

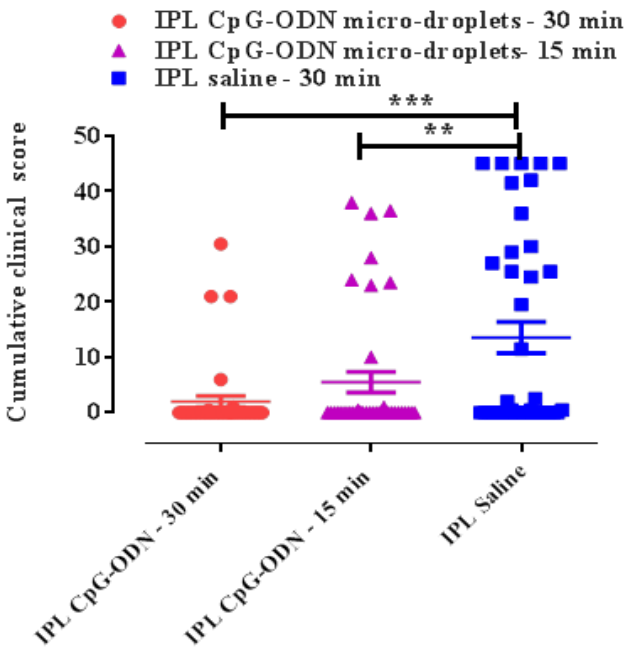

Figure 2.6 Cumulative clinical score corresponding to the dose titration by changing the duration of exposure. CCS of neonatal broiler chickens following intrapulmonary (IPL) CpG-ODN and *E. coli* challenge as described in Figure 12. CCS of the birds treated with IPL CpG-ODN as micro-droplets at either 15 or 30 min had a significantly lower CCS compared to the IPL saline control group ($P<0.05$). (Bar = Median).

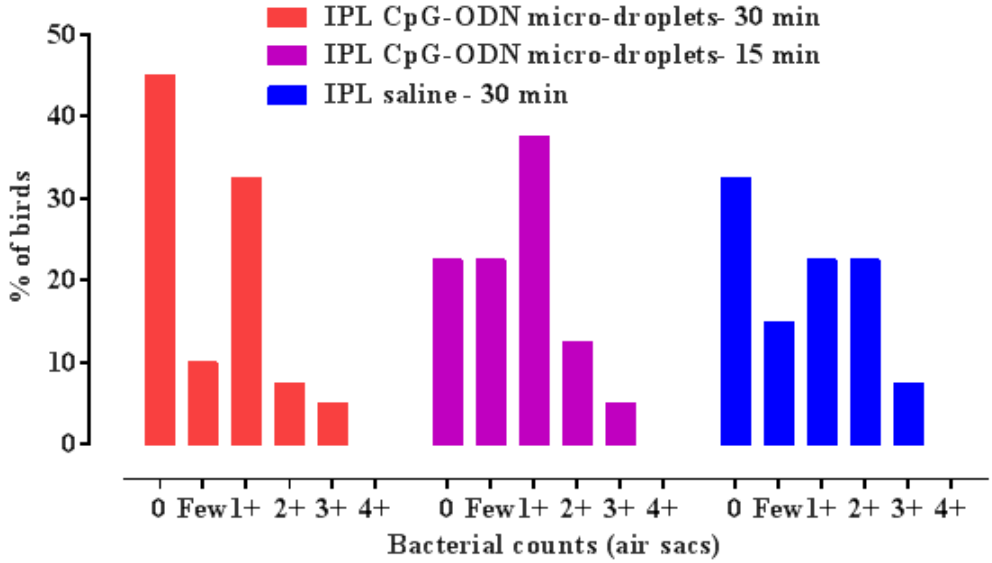

Figure 2.7 Bacterial loads corresponding to dose titration by changing the duration of exposure. Bacterial isolations from air sacs of neonatal broiler chickens following IPL CpG-ODN as micro-droplets and *E. coli* challenge. The IPL CpG-ODN as micro-droplets had lower counts of bacteria compared to IPL saline as micro-droplets (n=40)

Birds exposed to IPL CpG-ODN as microdroplets at the concentration of 4 mg/chamber or 2 mg/chamber had significantly higher survival compared to the IPL saline as the microdroplet group ($P<0.05$) (Figure 2.8). The clinical signs and bacterial counts in the two groups that received IPL CpG-ODN as microdroplets were similar, which were significantly lower when compared to the IPL saline control group ($P<0.05$) (data not shown). Birds exposed to the concentration of 0.4 mg/chamber of IPL CpG-ODN as microdroplets for 30 min were not protected from the *E. coli* challenge ($P>0.05$) (data not shown). However, dead birds in all groups showed similar gross lesions.

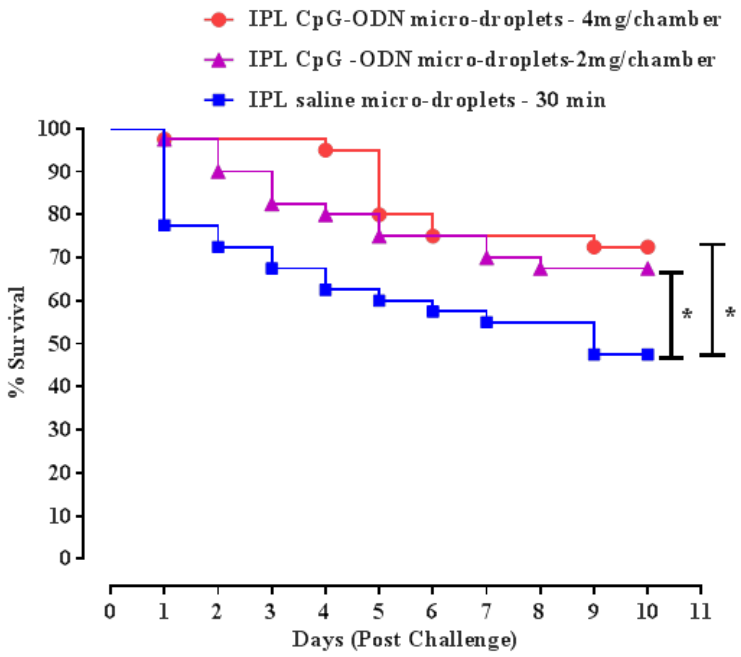

Figure 2.8 Dose titration by administering different CpG-ODN concentrations. Survival of chickens following *E. coli* challenge in groups treated with IPL CpG-ODN as micro-droplets at concentrations of 4 mg/chamber and 2 mg/chamber. Groups exposed to 4 mg/chamber or 2 mg/chamber for 30 min were significantly protected against *E. coli* challenge ($P<0.05$).

2.4.3 Duration of protective immunity against *E. coli* in chickens following IPL CpG-ODN administration

Groups that received IPL CpG-ODN as microdroplets for 30 min showed significantly higher survival against *E. coli* challenge as early as 6 hr post-administration of CpG-ODN (Figure 2.9A), and continued to have statistically significant protection until 5 days (Figure 2.9B–D) but not at 7 days (Figure 2.9E) post–CpG-ODN treatment, compared to the IPL saline

control (P < 0.05) (Figure 2.9). The dead birds showed similar lesions irrespective of the treatments (data not shown).

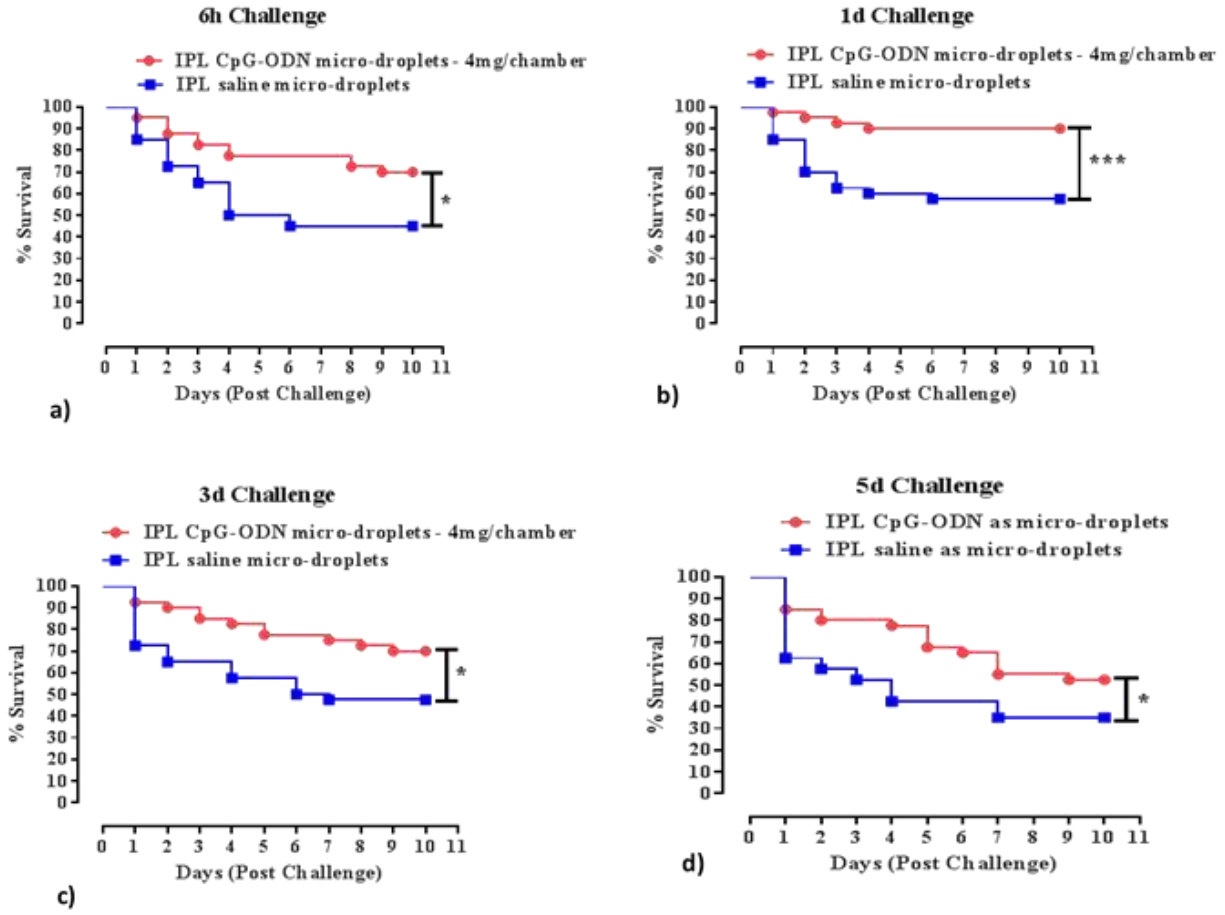

Figure 2.9 Duration of intrapulmonary CpG-ODN mediated immunoprotection. Duration of immunoprotection following *E. coli* challenge in groups (n=40) treated with IPL CpG-ODN as micro-droplets for 30 min at the concentration of 4 mg/chamber. Groups of chickens challenged with *E. coli* following IPL CpG-ODN as micro-droplets 6 h, 1 d, 3 d and 5 d had significantly higher survival compared to the IPL saline control group (P<0.05).

2.4.4 Cellular infiltrations in the lungs and growth of chickens following CpG-ODN delivery

Histopathologic examination of the lungs revealed infiltration of inflammatory cells that were predominantly mononuclear cells with occasional heterophils in the pulmonary parenchyma in groups treated with IPL CpG-ODN as microdroplets at 24 hr post-administration of CpG-ODN (Figure 2.10). No microscopic changes were detected by histopathology in any of the

organs (i.e. lungs, liver, spleen, heart, bursa, thymus, and muscle) when they were examined 42 days following IPL CpG-ODN as microdroplets. The BBW did not have a significant difference (P >0.05) between the IPL CpG-ODN as microdroplet and IPL saline control groups. The average body weight of IPL CpG-ODN as microdroplets group was 2.39 kg (SD 353.7), whereas the IPL saline group was 2.37 kg (SD 284.2) by the end of 42 days post-hatch. Total mortality was zero in both the IPL CpG-ODN and IPL saline control groups.

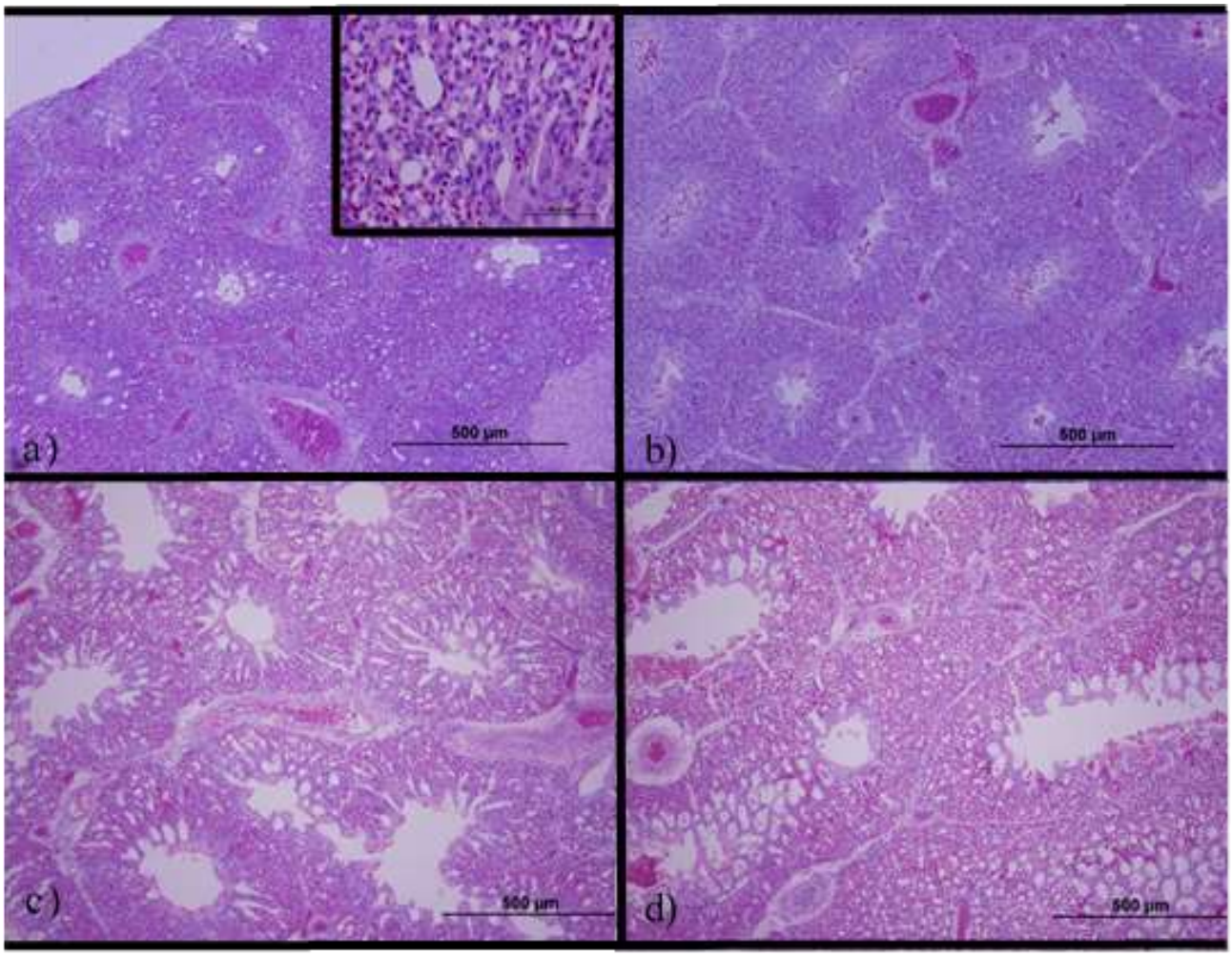

Figure 2.10 Cellular infiltrations into the lung following intrapulmonary CpG-ODN microdroplet delivery. IPL CpG-ODN as micro-droplets (a=24 hr post-administration; b=72 hr post-administration) compared to IPL saline as micro-droplets (c=24 hr post-administration; d=72 hr post-administration). Lungs show high cellular infiltrations at 24 and 72 hr post-administration of IPL CpG-ODN compared to IPL saline as micro-droplets treated lungs (10x). Higher magnification (100x) of the lung at 24 hr post-administration of CpG-ODN by the IPL route (a; insert) demonstrating influx of mononuclear cells.

2.5 Discussion

Amidst growing public concern about antibiotic residues and antibiotic-resistant pathogens in poultry products, the chicken industry worldwide is trying to come up with some suitable strategies to limit or abandon the use of antibiotics in poultry production (85,119,225,231). Recent attempts of antibiotic-free farming in Canadian commercial chicken farms resulted in poor growth performance and greater incidence of *C. perfringens* and *C. jejuni* infections (119). This finding suggests that the antibiotic-free farming without an alternative strategy for controlling bacterial infections might increase the risk of contamination of poultry products with foodborne illness–causing bacteria (96,119,177). To date, several strategies have been investigated as alternatives to antibiotics in poultry (70) such as vaccination (83), prebiotics (36,314), probiotics (332,351), cationic peptides (214), cytokines (24), bacteriocins (380), and bacteriophages (167,440).

Most bacterial infections in poultry occur during the first week of neonatal life that adversely influence the remaining production cycle and growth of broiler chickens (137). It has been documented that mortality over 2% at the end of the first seven days of age is associated with poor growth, loss of uniformity, and chronic infections within the flock and higher condemnations at processing (301). We have previously demonstrated that CpG-ODN alone can be used as an immunoprotective agent to control bacterial septicemia in neonatal chickens (125,126,382). To our best knowledge, the present investigation is the first study that systematically investigated the effectiveness of needle-free delivery of CpG-ODN microdroplets by IPL aerosol route at hatch as an immunoprotective measure against the *E. coli* septicemia in neonatal chickens.

In this study, we first designed a closed acrylic chamber (0.036 m^3) fitted with a Compressor Nebulizer (AMG Medical Inc.) that can aerosolize particles of 0.5–5 μm size for delivering CpG-ODN microdroplets by IPL route to neonatal chicks at hatch. We next examined if CpG-ODN (4 mg/chamber) or saline microdroplets delivered for 30 min at day 1 of hatch by IPL route can provide protection against *E. coli* septicemia. Three days after the IPL delivery of CpG-ODN or saline treatment, chicks were challenged with two doses ($1x10^4$, n=20 or $1x10^5$, n=20 CFU) of *E. coli*. Mortality and clinical signs were recorded over 10 days post *E. coli* challenge. We found that CpG-ODN treatment significantly increased the survival of chicks compared to saline control group. The clinical scores and bacterial load were significantly low in

CpG-ODN group. These data clearly showed that CpG-ODN microdroplets delivery by IPL route significantly protected neonatal chicks against *E. coli* septicemia.

We next planned experiments to identify an optimum exposure time of IPL CpG-ODN microdroplets that can provide immuno-protection against *E. coli* septicemia. Three groups (n=40/group) of day-old chicks were used: (a) IPL CpG-ODN (4 mg/chamber) for 15 min, (b) IPL CpG-ODN (4 mg/chamber) for 30 min, and (c) IPL saline for 30 min. Chicks were challenged with *E. coli* as described in the previous experiment. Results showed significantly higher survival rate in CpG-ODN treatment groups (15 min and 30 min) compared to the saline group. The chicks that were exposed to 15 min or 30 min of CpG-ODN experienced about half or quarter of the relative risk of mortality (47%, P=0.029 or 24%, P=0.001) compared to the saline group, respectively. Our results suggest that CpG-ODN exposure time, a correlate of dose, does influence the disease outcome. Overall, this experiment suggests that even 15 min exposure of chicks to CpG-ODN by IPL route can significantly provide protection against *E. coli* septicemia.

We next tested the immunoprotective effects of different doses of CpG-ODN against *E. coli* septicemia. Here, we used three different doses of CpG-ODN for IPL delivery including 4 mg/chamber, 2 mg/chamber, and 0.4 mg/chamber. Our results showed that IPL delivery of CpG-ODN by 4 mg/chamber and 2 mg/chamber doses significantly protected chicks against *E. coli* septicemia compared to the saline group. In contrast, 0.4 mg/chamber failed to provide protection after the *E. coli* challenge (data not shown). These data suggest that CpG-ODN IPL delivery provides a dose dependent protection in chicks.

We further sought to examine the duration of protective immunity that CpG-ODN induces in neonatal chicks following the IPL delivery at hatch. To investigate this issue, we performed IPL delivery of CpG-ODN (4 mg/chamber) at hatch, and then chicks were challenged with *E. coli* at various time points (6 hr, 1 day, 3 days, 5 days, and 7 days) after IPL delivery of CpG-ODN or saline. The clinical signs and bacterial counts were recorded as described above. We discovered that IPL delivery of CpG-ODN is able to induce protective immunity as early as 6 hr and that it remains effective at least up to day 5 post-treatment. Furthermore, we found that the protective effect of CpG-ODN decreases by day 7, as evidenced by the poor survivability of the chicks challenged 7 days post–CpG-ODN administration. Based on these findings, we suggest that CpG-ODN delivery by IPL route will induce protective immunity in neonatal

chickens during the critical first week of neonatal life starting from the time of their placement in a poultry barn.

Histopathologic examination of lungs collected at various time points after IPL delivery of CpG-ODN revealed cellular infiltration in lungs. The cellular infiltration consisted predominantly of mononuclear cells with few heterophils at 24 hr. Previous studies have shown increased influx of macrophages following the intratracheal administration of *E. coli* (398) and *P. multocida* (397), and increased influx correlated with enhanced protection. Further, it was shown that CpG can have immunostimulatory effects on monocyte-derived APCs (114,241). CpG-ODN treatment can also enhance immune responses through cytokines and chemokines activation in chickens (77,113,392). Therefore, we hypothesize that IPL CpG-ODN treatment possibly increases cellular infiltration, immune activation, and cytokine and chemokines secretion, enabling activated immune cells and secreted cytokines to launch prompt and strong immune responses against the systemic bacterial infection. We hypothesize that CpG-ODN treatment possibly increases cellular infiltration enabling prompt and strong immune response to occur against the bacterial infection. However, further studies are necessary to explicate the mechanisms that provide protection against *E. coli* septicemia in neonatal broiler chicken following IPL delivery of CpG-ODN. Moreover, there were no macroscopic or microscopic lesions, abnormalities, or toxic effects in any of the internal organs examined at 42 days of age that could be ascribed to IPL CpG-ODN delivery.

Overall, we found that IPL delivery of CpG-ODN alone as microdroplets can provide significant protection in neonatal chicks against *E. coli* septicemia. The CpG-ODN delivered by the IPL route significantly reduces mortality, clinical signs, and bacterial load in birds, which is in agreement with previous studies that delivered CpG-ODN by intramuscular or *in ovo* route (125,137,381). A recent study on aerosol vaccination of CpG-ODN adjuvanted avian influenza vaccine also supports an immunostimulatory role for intrapulmonary delivered CpG-ODN (365).

Several studies used probiotics as alternatives to antibiotics in poultry (70,332,351). The use of probiotics resulted in the reduction of colonization and shedding of pathogenic bacteria (284,309,404). *C. jejuni* shedding (284) and *Salmonella enteritidis* colonization (404) were significantly reduced following the administration of probiotics in chicks. A limited number of studies have investigated the effects of probiotics on the clinical aspect and mortality following *E. coli* challenge. However, in a set of field trials and a controlled trial, Timmerman *et al.* (393)

reported that probiotics as growth promoters significantly improved feed conversions, but the reduction in the mortality following *E. coli* challenge was not statistically significant (393). Besides, studies conducted on prebiotics showed that the use of prebiotics helped birds in overcoming the stress elicited by the *E. coli* challenge and transportation compared to the non-treated control group. However, the isolation of the challenged strain and generic strains of E. *coli* from the air sacs was not statistically different between groups (166). In our studies, we found that CpG-ODN was able to reduce the *E. coli* isolation from air sacs while significantly improving the survivability and clinical conditions of birds.

Taken together, our findings demonstrate that IPL delivery of CpG-ODN microdroplets can protect neonatal broiler chickens against bacterial infections during the critical first week of age when birds are more vulnerable to bacterial infections. Further studies involving field efficacy trials would be very valuable. The use of CpG-ODN as an alternative to antibiotics is promising and has great potential in the poultry industry.

PREFACE TO CHAPTER 3

Infections caused by bacterial pathogens such as *E. coli*, *Salmonella* and *Enterococcus* during the first week of life in neonatal broiler chicks cause severe mortalities and poor weight gains in chicks (191,433) as well as lesions leading to condemnations at processing (18). With the current resistance to the antibiotic use in production animals and CFC's decision to voluntarily withdraw prophylactic antibiotics, an apparent need for alternative strategies have become a priority. In chapter two we demonstrated that the IPL delivery of TLR agonist, CpG-ODN was able to induce significant immunoprotection in neonatal broiler chicks against *E. coli* septicemia in laboratory scale experiments (128). We identified this technique as an alternative to antibiotics and the most appropriate stage to apply it in the commercial poultry industry is the commercial broiler hatcheries. A large number of broiler chicks are produced in broiler hatcheries across Canada on a daily basis. The immune system of a newly hatched broiler chick is naïve and immature until exposed to pathogens, making them susceptible for a large number of bacterial and viral infections (360). As a result, it is critical that these neonatal birds leave the hatchery with an activated immune system in order to fight bacterial infections encountered as soon as they are placed in the barns (3). Hence, the administration of CpG-ODN at the hatchery seemed like the most applicable choice. It was imperative that the IPL CpG-ODN delivery technique didn't cause disruption or delay in the normal work flow of the hatchery. Thus, we collaborated with engineers and developed a CSPN with the holding capacity of 8000 birds. Large scale field experiments were conducted to evaluate its efficacy in the delivery of CpG-ODN in the IPL route at commercial settings under different weather conditions. In chapter 3, we have discussed the findings of the field experiments.

CHAPTER 3 PROTECTION OF NEONATAL BROILER CHICKENS AGAINST LETHAL ESCHERICHIA COLI SEPTICEMIA BY INTRAPULMONARY DELIVERY OF CPG-ODN USING A COMMERCIAL-SCALE PROTOTYPE NEBULIZER UNDER FIELD CONDITIONS

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The work described in this manuscript has been submitted to *Avian Diseases* Journal.

Contribution: The conceptual ideas were generated my self, Ms. Popowich and Dr. Gomis from laboratory scale to commercial scale. The commercial scale neubulizer was designed and developed by RMD engineering Inc. and Dr. Simonson, Mr. Kamali and Mr. Boire provided engineering expertise to the design team. Mr. Gebhardt and Mr. Roberts from RMD Engineering Inc.conducted the field experiments with me, Ms. Popowich and Dr. Gomis and all the research group members assisted at different points of the field and laboratory experiments. Dr. Ambrose coordinated the field experiments in BC. Mr. Gebhardt provided the computer assited design illustrations and Dr. Simonson explained the engineering concepts. I performed data analysis and wrote the manuscript. Ms. Popowich provided the climatic data graphs. The manuscript was written under Dr. Gomis's guidance. All the authors mentioned above contributed with their feedback.

3.1 Abstract

CpG-ODNs are potent innate immune stimulators in neonatal and adult broiler chickens against bacterial septicemia. We have recently demonstrated that IPL delivery of CpG-ODN as micro droplets can protect neonatal broiler chickens against lethal *E. coli* septicemia under laboratory conditions. The objectives of this study were to develop a commercial-scale prototype nebulizer to deliver CpG-ODN as micro-droplets in neonatal broiler chickens at hatch and to study the efficacy of IPL delivery under different environmental conditions in two geographical locations in Canada. Field experiments were conducted in commercial poultry hatcheries during different seasons of the year in Saskatchewan and British Columbia, Canada. Neonatal broiler chicks (n= 8,000) received CpG-ODN by the IPL route in the multi-level commercial-scale poultry nebulizer (CSPN) for 30 min and control broiler chicks received distilled water (DW) for 30 min. Broiler chicks were sampled from different locations of the CSPN following nebulization and challenged with a lethal dose of *E. coli* to study efficacy of CpG-ODN by the IPL delivery. Broiler chicks were protected at a significant level against *E. coli* challenge following IPL delivery of CpG-ODN in the CSPN ($P<0.05$). It was critical to control the temperature, humidity and humidex of the CSPN to ensure efficacy of IPL delivery of CpG-ODN. We were able to deliver CpG-ODN by the IPL route to protect neonatal broiler chicks against lethal *E. coli* septicemia at a significant level when the humidex was at or below 28 C and relative humidity (RH) was 40-60% ($P<0.05$) in the CSPN. Immunoprotection of IPL delivery of CpG-ODN against *E. coli* septicemia declined ($P>0.05$) when the humidex was 29 C or RH was 70% in the CSPN. Results of this study confirmed that IPL delivery of CpG-ODN against septicemia in neonatal broiler chickens was industrially feasible and effective under different weather conditions in different geographical locations provided the temperature and humidity in the CSPN can be controlled.

3.2 Introduction

E. coli causes a variety of disease syndromes in poultry including YSI, omphalitis, respiratory tract infection and septicemia (301). *E. coli* infections in neonatal poultry are characterized, in acute form, by septicemia resulting in death and in subacute form, by pericarditis, airsacculitis, and perihepatitis (252,301). Many *E. coli* isolates commonly associated with commercial broiler chickens belong to serogroups O1, O2, and O78 (98,127).

Similarly paratyphoid *Salmonella* species cause yolk sac infections and septicemia in newly hatched broiler chicks triggering increased FWM resulting in significant economic losses to the poultry industry (194,433). Although prophylactic antibiotics were used to control mortality associated with *E. coli* and *Salmonella* infections of neonatal poultry in the past, the commercial poultry industry is searching for alternatives to antibiotics because of consumer demand and to reduce emergence of antibiotic resistant bacteria. Recently we have demonstrated the utility of immune stimulatory oligodeoxynucleotides containing CpG-ODN as an alternative to antibiotics in broiler chickens under laboratory conditions by delivering CpG-ODN by IM and *in ovo* routes against *E. coli* and *S.* Typhimurium septicemia (125,382). Furthermore, we have reported that IPL delivery of CpG-ODN as aerosolized micro droplets can protect neonatal broiler chicks against lethal *E. coli* septicemia under laboratory conditions (128). We hypothesized that IPL delivery of CpG-ODN is an industrially feasible method in poultry hatcheries, and can protect neonatal broiler chickens against lethal bacterial septicemia.

Although, *in ovo* and aerosol delivery of a number of vaccines against common infectious diseases of broiler chickens is practiced in many countries, no alternatives to antibiotics are available commercially to prevent bacterial infections of neonatal broiler chickens. It has also been demonstrated that the utility of probiotics could improve the health of neonatal poultry. However, probiotics were not efficacious to minimize death and clinical signs associated with bacterial infections of neonatal broiler chickens (393,417). In contrast, we demonstrated CpG-ODN as a potent innate immune stimulator that induces the innate immune system of broiler chickens against common bacterial infections through induction of Th1 and Th2 type immune responses mediated by secreting IFN-γ, IL1β, IL6, IL8 and IL18 and lipopolysaccharide-induced TNF (136,312). Although the innate immune system of neonatal broiler chickens is developed at hatch, they are susceptible to bacterial infections (80). Synthetic molecules such as CpG-ODN have a great potential for inducing danger signals and activating the innate immune system in neonatal broiler chickens as a preventative measure to minimize common bacterial infections (126,384). We have recently demonstrated that IPL delivery of CpG-ODN can protect neonatal broiler chickens against lethal bacterial septicemia as early as 6 hours following CpG-ODN administration (128). Although, IPL delivery of CpG-ODN protects neonatal broiler chickens under laboratory conditions, it is essential to demonstrate the utility of IPL delivery of CpG-ODN under different climatic and weather conditions during the year in different geographical

regions. The objective of this study was to develop a CSPN to deliver CpG-ODN by the IPL route and to demonstrate protection of neonatal broiler chickens against lethal septicemia under different environment conditions during different seasons of the year in Canada.

3.3 Materials and method

3.3.1 Capacity and features of the commercial-scale poultry nebulizer

A CSPN was designed and manufactured to deliver CpG-ODN by the IPL route to a batch of 8,000 newly hatched broiler chicks (Figure 3.1). The CSPN including a nebulizer unit, an air conditioning unit, fans and a chick enclosure was designed to deliver a calculated amount of CpG-ODN, while maintaining temperature, humidex and RH and monitoring carbon dioxide (CO_2) concentration in the chick enclosure. The chick enclosure had a volume of 7.55 m^3 and received conditioned air from the nebulizer unit of the CSPN (where CpG-ODN was aerosolized). Before the air entered the nebulizer unit it was conditions by the air conditioning and fan units (where the temperature, airflow rate, RH and humidex were controlled). Temperature and RH sensors were installed at the air inlet of the chick enclosure. A CO_2 sensor was installed at the air outlet of the chamber. The environmental parameters (temperature and RH) were monitored every second within the enclosure.

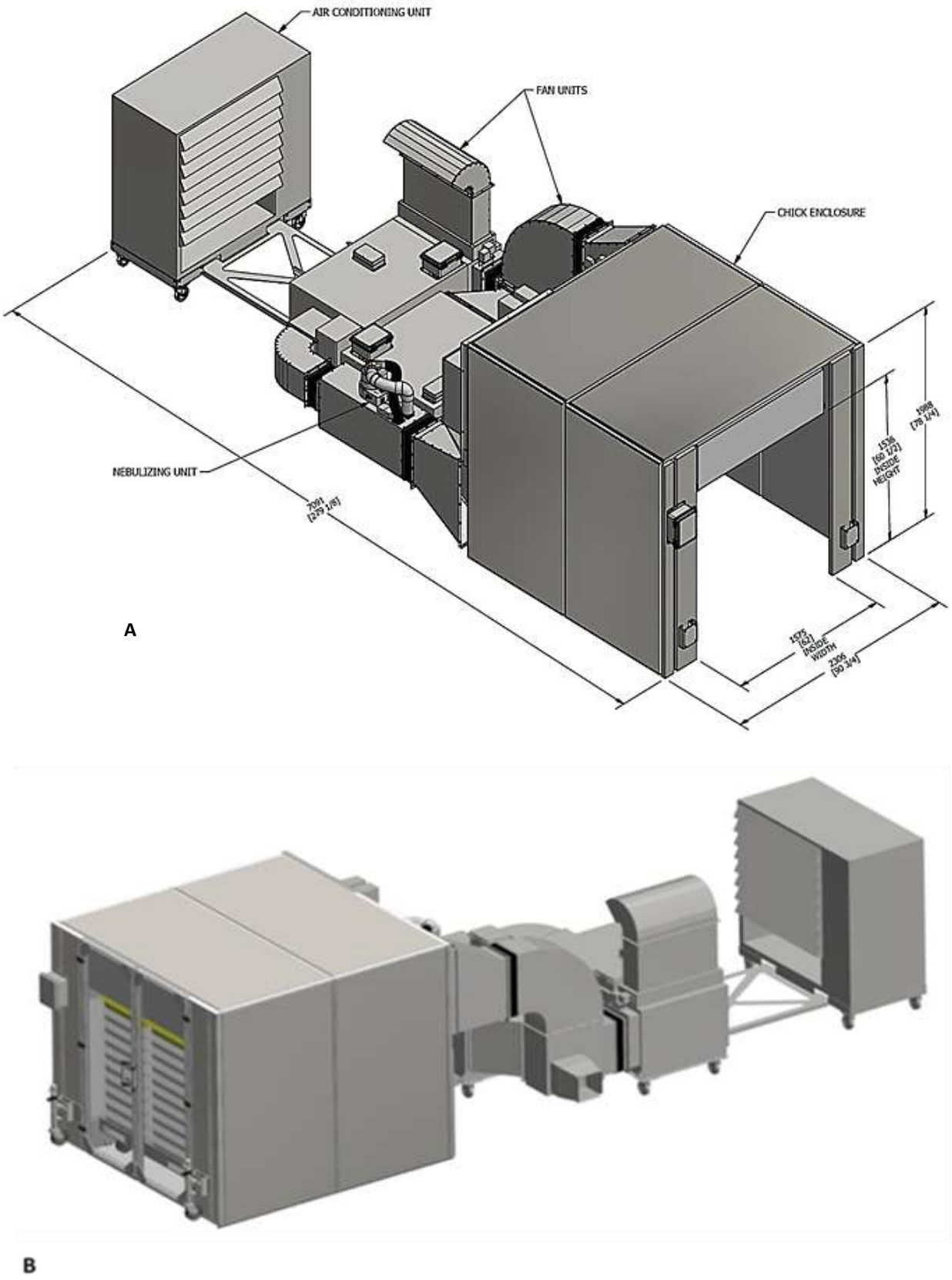

Figure 3.1 Conceptual design of the commercial scale poultry nebulizer. Left view (A) and right view (B).

The nebulizer consisted of an ultrasonic array system, capable of delivering CpG-ODN at a particle size of 0.5-5µm. An electric field applied across piezoelectric ceramic plate generated high frequency ultrasonic waves to convert the CpG-ODN solution into aerosol droplets (Figure 3.2). The nebulizer system was located close to the air inlet of the chamber. Between each batch of broiler chicks, the CSPN was purged with fresh outside air to remove CO_2. The CSPN was designed and manufactured at RMD Engineering Inc. Saskatoon SK, Canada.

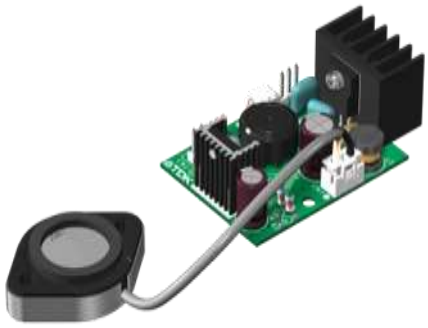

Figure 3.2 An individual ultrasonic type nebulizer Each consisted of a piezoelectric ceramic plate with the ability to generate high frequency ultrasonic waves to convert the CpG-ODN solution into aerosol droplets. An array of these was used.

Broiler chicks (commercial broilers, Ross) were placed in the chick enclosure of the CSPN in chick baskets containing 102-104 chicks and stacked as columns; each containing 10 baskets (9 baskets with chicks and one empty basket on top). Eight stacks of chick baskets were loaded in the chick enclosure of CSPN for each nebulization test. An average of 70 mL CpG-ODN at a concentration of 12 mg/mL was nebulized for 30 min during each run.

3.3.2 Sampling of chicks for *E. coli* challenge

Following nebulization, broiler chicks from the top, middle and bottom baskets from each of the eight stacks of chick baskets were collected to study the uniformity of dispersion of CpG-ODN (Figure 3.3). Broiler chicks were individually tagged to record the basket of origin. A total of 10 broiler chicks per basket were collected from the 24 baskets labelled 1 to 24 in Figure 3.3. Chicks from the 24 baskets were combined into 6 separate groups according the color coding in Figure 3.3. The chicks from 4 baskets were combined into each group, where baskets 17-20 formed group 1 (bottom left of the chick enclosure), baskets 21-24 formed group 2 (bottom

right), baskets 9-12 formed group 3 (middle left), baskets 13-16 formed group 4 (middle right), baskets 1-4 formed group 5 (top left) and baskets 5-8 formed group 6 (top right). Additionally, a group of birds (n=40) that was exposed to nebulized (aerosolized) DW was included as group 7. The selected and tagged broiler chicks were transported to ACU, Western College of Veterinary Medicine, University of Saskatchewan, Canada, to test the efficacy of the preventive treatment by challenging them with *E. coli* as described below.

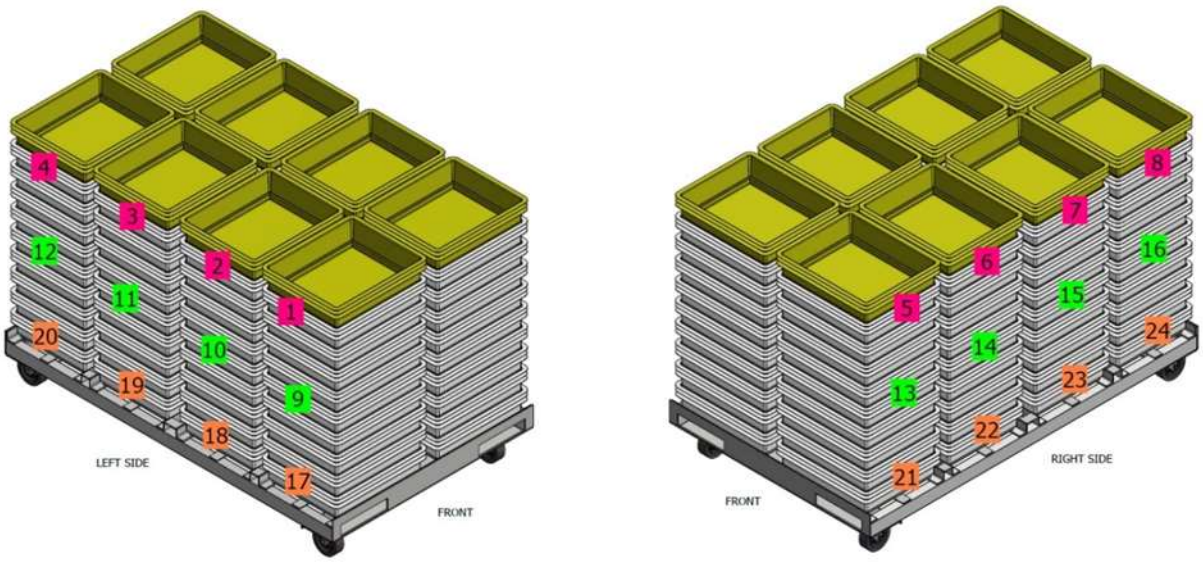

Figure 3.3 Stacks of broiler chick baskets and sampling scheme following nebulization.. Day old broiler chicks were sampled from individual chick baskets (n=10) numbered 1 to 24 from the top, middle and bottom and left and right side stacks for lethal *E. coli* challenge following CpG-ODN delivery by the IPL route in the chick enclosure of CSPN

3.3.3 Ethics statement

The animal experiment was approved by the University Committee on Animal Care and Supply Animal Research Ethics Board at the University of Saskatchewan and conducted following the guidelines of Canadian Council on Animal Care.

3.3.4 Animal housing

Water and commercial broiler feed were provided *ad libitum* during the experimental observation period of seven days. Broiler chicks were raised at 32 C for the first week of life and thereafter the temperature was decreased 0.5 C per day until a room temperature of 27.5 C was

reached. Light was provided for 24 h/day for 0 to 2 days post-hatch. Darkness was introduced at 3 days post-hatch with 1 hr of dark added daily until 4 hr of darkness was achieved. All animal experiments were approved by the Animal Research Ethics Board, University of Saskatchewan.

3.3.5 *E. coli* challenge

The challenge strain used was a field isolate of *E. coli* from a turkey with septicemia. Preparation of the *E. coli* challenge was done as described previously (125,128). Briefly, one colony of *E. coli* was taken and mixed in 100 mL of Luria broth (Difco LB broth, Miller, Becton Dickinson and Company; Sparks, MD, USA). It was incubated at 37 C for 16-18 hr, shaking at 150 rpm. A serial dilution of the diluted culture was plated in duplicates on 5% Columbia sheep blood agar plates, incubated for 18 hr at 37 C and the number of colonies were counted in order to confirm the *E. coli* challenge dose.

The *E. coli* challenge procedure was conducted as previously described (128). Briefly, at day-two post CpG-ODN treatment; birds were challenged with either $1x10^5$ or $1x10^6$ CFU of *E. coli* by the SQ route in the neck. Two doses of *E. coli* were given to birds to simulate field conditions since all birds in a commercial poultry barn are not exposed to a consistent dose of *E. coli*. Birds were evaluated three times daily at the critical stage (first 3 days post-challenge) and twice thereafter for 8-10 days post-challenge. Each bird was observed for clinical signs and a daily clinical score was assigned: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1= depressed, unwilling to move; 1.5 = unwilling to move, may take a drink of water and peck some; 2 = unable to stand or reach for food or water; and 3 = found dead. Birds that received a clinical score of 2 were euthanized by cervical dislocation. At the end of the trial, each bird was given a CCS; sum of daily clinical scores as previously described (125,128). Chicks that were found dead or euthanized were necropsied immediately. On 8-10 days post-challenge, the remaining birds were euthanized by cervical dislocation. Bacterial swabs were taken from the air sacs of dead and euthanized birds and cultured on 5% Columbia sheep blood agar according to the quadrant streaking technique. A semi-quantitative estimate of *E. coli* isolation was conducted according to the growth on blood agar. Growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; few = less than 5 colonies; 1+ = bacterial growth on area 1; 2+ = bacterial growth on areas 1 and 2; 3+ = bacterial growth on areas 1, 2, and 3; and 4+ = bacterial growth on areas 1, 2, 3, and 4 (160). Data from three individual experiments are described in

this manuscript in order to demonstrate the importance of RH and humidex in the chick enclosure when broiler chicks were nebulized with CpG-ODN using the CSPN. Experiment 1 was conducted in Saskatchewan while experiments 2 and 3 were conducted in British Columbia, Canada.

3.3.6 CpG-ODN

The CpG-ODN (2007) sequence was 5' – TCGTCGTTGTCGTTTTGTCGTT – 3' and was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies Inc., Huntsville, AL). The CpG-ODN was dissolved in sterile DW at a concentration of 12mg/mL.

3.3.7 Statistical analysis

Clinical scores of broiler chicks following *E. coli* challenge were assigned as previously described (128). The significance of differences among groups in survival, bacteriological scoring, CCS, were analyzed and graphically presented using Prism (Prism 6.0, GraphPad Software Inc; San Diego, CA, USA) with a significance level of $P<0.05$. The significance among groups in survival patterns and median survival times were analyzed using the log-rank test. Significance of differences in CCS among groups was tested using Kruskal Wallis nonparametric analysis of variance when 3 or more groups were compared (i.e., to compare the effect of the location of the chick in the enclosure) or Mann Whitney nonparametric test when 2 groups were compared (i.e., to compare the exposure to aerosolized CpG-ODN with exposure to aerosolized DW).

3.4 Results

3.4.1 Efficacy of the commercial scale poultry nebulizer

Experiment 1 was conducted in Saskatchewan, Canada during the winter of 2017. The outdoor conditions during the experiment were -3 C and 76% RH, giving a humidity ratio of 2.2 g/kg and a dew point temperature of -6.2 C. The command module of the CSPN was set to maintain humidex of 28 and temperature of 24 C at the beginning of the experiment 1. The air conditions in the chick enclosure of CSPN were maintained at 25.5 C and 42% RH giving a humidex of 27.6 and humidity ratio of 8.6 g/kg (Figure 3.4A). The CO_2 concentration at the start

of the test was 1,000 ppm and increased to 4,085 ppm at the end of 30 min nebulization period. The CO_2 concentration increases because air in the chamber is recirculated; there is no fresh outdoor air supplied during the tests. Experiment 2 was conducted in British Columbia, Canada during the summer of 2017. The outdoor conditions during the experiment were 14 C and 81% RH, giving a humidity ratio of 8.1 g/kg and a dew point temperature of 10.8 C. The command module of the CSPN was set to maintain humidex of 28 and temperature of 22 C at the beginning of the experiment 2. Temperature inside the chick enclosure of CSPN was maintained at 23.7 C, RH was maintained at 61% and humidex was maintained at 28.0 with humidity ratio of 10.7 g/kg (Figure 3.4B). CO_2 level reached to 5,794 ppm at the end of 30 min nebulization. Experiment 3 was conducted in British Colombia, Canada during the winter of 2018 where the outdoor conditions were 5.5 C and 99% RH, giving a humidity ratio of 5.6 g/kg and a dew point temperature of -5.4 C. The command module of the CSPN was set to maintain humidex of 29 and temperature of 23 C at the beginning of the experiment 3. Temperature of the CSPN was maintained at 22.9 C, RH was maintained at 71% and humidex was 29.3, humidity ratio = 12.5 g/kg (Figure 3.4C). CO_2 level reached to 8,000 ppm at the end of 30 min period of nebulization. Air flow was maintained at 1,100 cubic feet per minute (CFM) in all three experiments. The intra–enclosure climatic conditions (average ± SD) maintained during each experiment are summarized in Table 3.1.

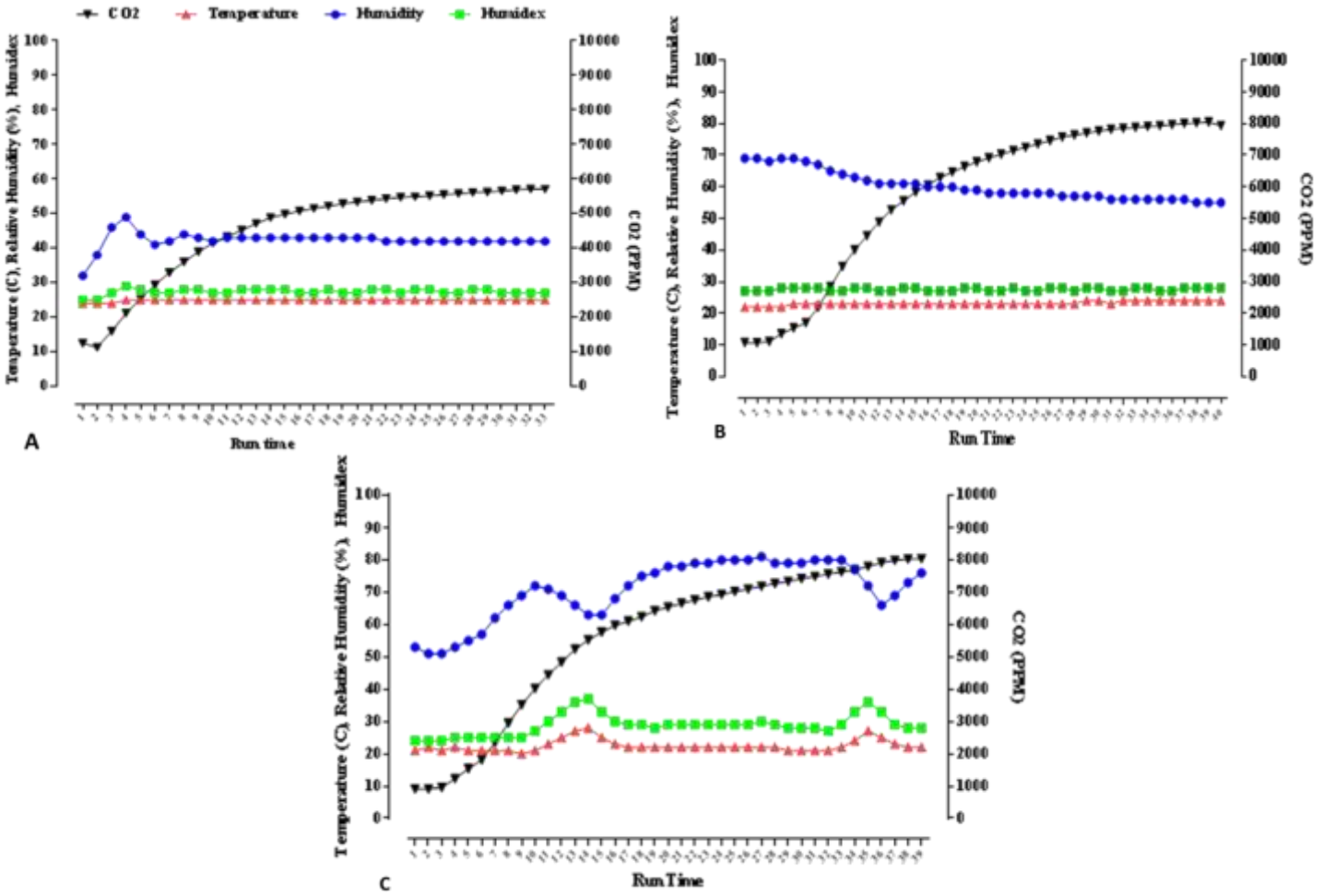

Figure 3.4 Temperature, humidity, humidex and CO_2 levels in the commercial scale poultry nebulizer from experiment 1 (A), experiment 2 (B), and experiment 3 (C). Experiment 1 was conducted in Saskatchewan while experiments 2 and 3 were conducted in British Columbia. Humidex was set to 28, while RH was set between 40-60% in experiments 1 and 2. RH was set to >60% in experiment 3.

Table 3.1 Temperature, relative humidity, humidex, humidity ratio and CO_2 in the chick enclosure of the commercial scale poultry nebulizer for individual experiment. (T=temperature; RH=relative humidity; W=humidity ratio; CO_2 in the chick enclosure of the CSPN)

Experiment	T ± SD	RH ± SD	Humidex ± SD	W	CO_2
1	25.5 ± 0.29	42 ± 2.48	27.6 ± 0.82	8.6	5,000
2	23.7± 0.59	61 ± 4.54	28.0 ± 0.5	10.7	8,000
3	22.9 ± 1.83	71 ± 9.33	29.3 ± 3.29	12.5	8,000

3.4.2 Efficacy of intrapulmonary delivery of CpG-ODN by the commercial scale poultry nebulizer

The group of broiler chicks that was administered CpG-ODN by the IPL route in the chamber of the CSPN during experiment 1, had significantly higher survival ($P<0.05$) compared to the group of broiler chicks that received DW (Figure 3.5A). Chicks administered CpG-ODN showed an average survival of 69% whereas the group given DW had a survival of 43% following *E. coli* challenge. IPL CpG-ODN-administered chicks had a significantly lower CCS score compared to the group that received DW (Figure 3.6A). The same group of chicks demonstrated lower bacterial load in the thoracic cavity compared to the group that received DW (Figure 3.7A).

In experiment 2, the chicks that received CpG-ODN by the IPL route in the chamber of CSPN had significantly higher survival ($P<0.05$) compared to the group of birds that were administered DW (Figure 3.5B). Chicks administered CpG-ODN by the IPL route had 51.7% survival following *E. coli* challenge. In contrast, the group that received DW by the IPL route had 25% survival. IPL CpG-ODN-administered birds had a significantly lower CCS score compared to the DW control (Figure 3.6B). The birds that were administered CpG-ODN by the IPL route had a lower bacterial load compared the DW control group (Figure 3.7B).

No significant protection ($P>0.05$) was seen in experiment 3 between groups that received CpG-ODN or DW by the IPL route (Figure 3.5C). No significant difference was noted in CCS between groups of broiler chicks that received CpG-ODN or DW by the IPL route (Figure 3.6C). There was no difference between bacterial loads in groups of birds that received CpG-ODN or DW by the IPL route (Figure 3.7C).

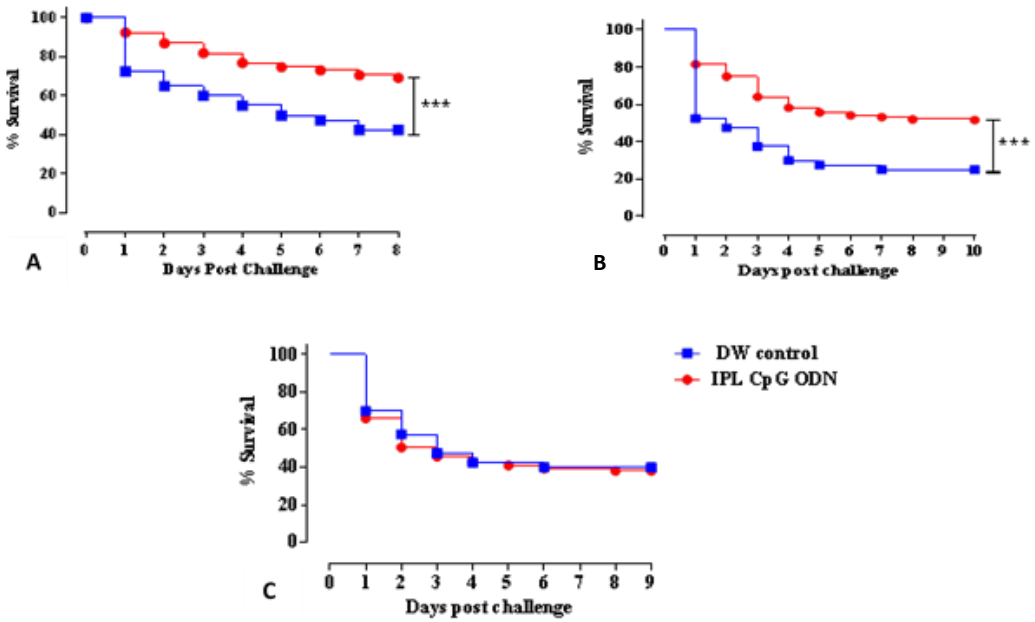

Figure 3.5 Survival of birds following lethal *E. coli* challenge from experiment 1 (A), experiment 2 (B) and experiment 3 (C). Broiler chicks administered with CpG-ODN via the intrapulmonary (IPL) route showed significantly better survival (P<0.05) compared to the distilled water (DW) control in experiments 1 and 2. No significant protection was seen between the DW control and IPL CpG-ODN groups (P>0.05) in experiment 3.

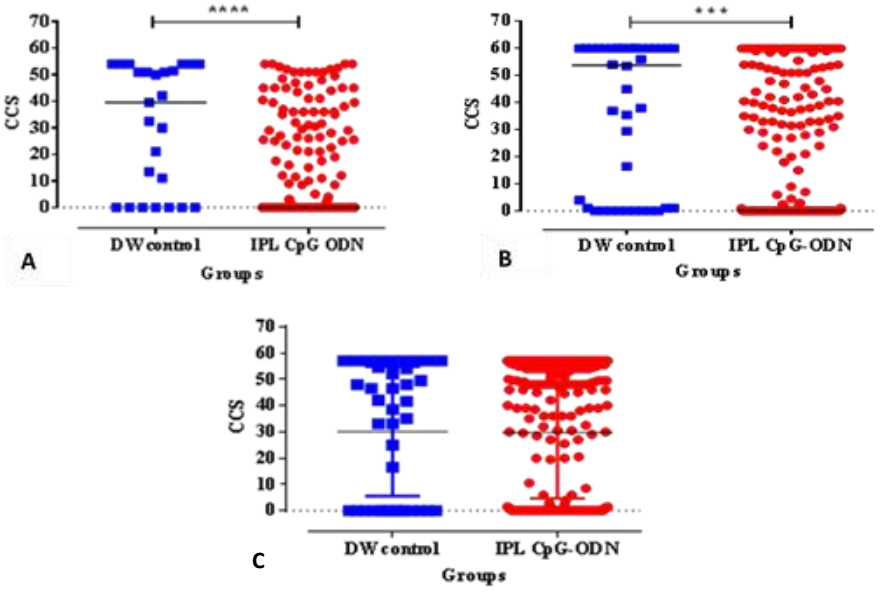

Figure 3.6 Cumulative clinical score of broiler chicks following lethal *E. coli* challenge. Birds that received CpG-ODN by the IPL route had a significantly low CCS (P<0.05) compared to birds that received DW in 1 (A) and 2 (B). (C) Birds that received CpG-ODN or DW by the IPL route did not have a significant difference in CCS (P>0.05) in the experiment 3.

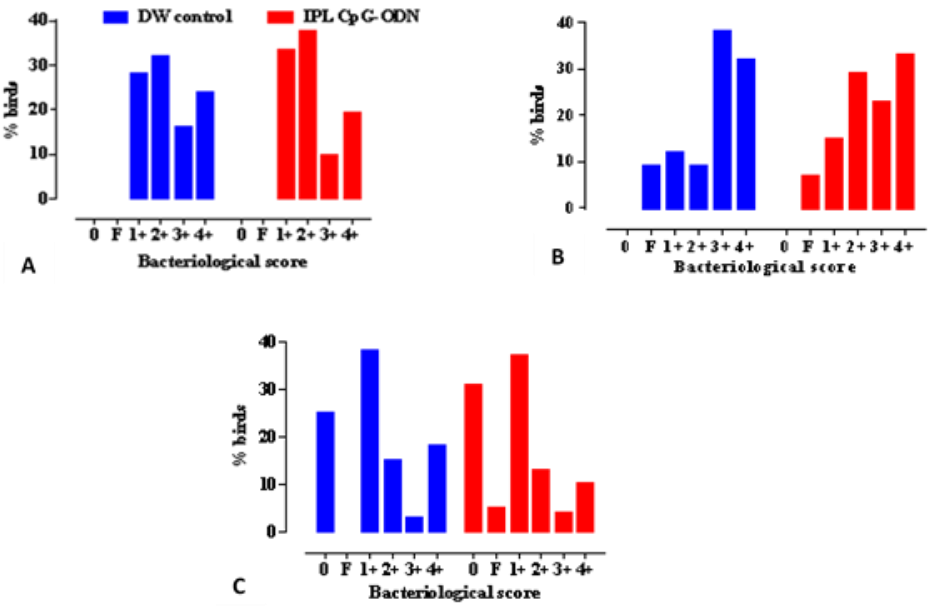

Figure 3.7 Bacterial score of broiler chicks following a lethal *E. coli* challenge. Birds that received CpG-ODN by the IPL route in CSPN in experiment 1 (A) and experiment 2 (B) tended to have lower bacterial loads compared to the birds of the DW control group. This tendency was not as apparent in experiment 3 (C) where the bacterial loads appear similarly in the birds administered CpG-ODN in the CSPN and the DW control

3.5 Discussion

Importance of alternatives to antibiotics has been a high priority for the poultry industry in the recent past due to increased consumer demand for antibiotic-free poultry products, potential association of emergence of antibiotic-resistant bacteria due to imprudent use of antibiotics in animal agriculture and increased incidence of antibiotic-resistant bacterial infections in human are some of the driving forces (268). Food safety and human health are intricately associated with food animal production especially with broiler chicken production. We have previously demonstrated the efficacy of immune stimulatory effects of CpG-ODN against *E. coli* and *S.* Typhimurium septicemia in neonatal broiler chickens via subcutaneous, IM and *in ovo* routes (125,126,382). Although *in ovo* delivery is an industrially feasible technique, stimulation of the innate immune system with CpG-ODN lasts only for six days. Hence, the efficacy of *in ovo* delivery of CpG-ODN in day-18 of incubating eggs lasts only three days following hatch (137,382). As a result, we have recently demonstrated IPL delivery of CpG-ODN at hatch as an effective industrially feasible technique to protect neonatal broiler chicks against *E. coli* septicemia (128). Moreover, IPL delivery of CpG-ODN induces a significant immunoprotection against *E. coli* septicemia as early as 6 hrs following IPL delivery (128); in

contrast it takes 24 hrs to stimulate the innate immune system of broiler chickens if CpG-ODN is delivered by the SQ or IM routes (126,382). We have also demonstrated that immuno-protection against *E. coli* septicemia by IPL delivery of CpG-ODN lasts up to 5 days under laboratory conditions (128). We have previously demonstrated a reduction of clinical signs and bacterial loads in organs following *E. coli* challenge in broiler chicks treated with CpG-ODN by different routes (125,126,128,382). Similarly, we were able to demonstrate a reduction of clinical signs and bacterial loads in broiler chicks delivered with CpG-ODN by the IPL route using CSPN under field conditions.

In order to align with demands and activities of commercial poultry hatcheries, it was imperative that an IPL delivery technique of CpG-ODN would not interfere with pace and efficiency of routine operations of a commercial hatchery. As a result, we designed a large-scale chamber with a holding capacity of 8,000 chicks and a nebulizer array to aerosolize CpG-ODN to that maximum capacity of birds during each run. A climate control system was designed and fabricated in order to assist with the regulation of the climate inside the chamber to ensure optimal delivery of CpG-ODN, bird comfort and welfare. We performed several field experiments to understand the efficacy of this technique, compliance and demands of a commercial poultry hatchery while maintaining bird's welfare and comfort inside the chamber of the CSPN. The field experiments were carried out in two commercial broiler hatcheries in Saskatchewan and British Columbia, Canada in order to test the efficacy of the technique under different environmental conditions. We have used our well-established *E. coli* animal model to test the immune protective effect of CpG-ODN following IPL delivery using CSPN (128).

Here, we have demonstrated a significant level of immunoprotection of neonatal broiler chickens against *E. coli* by delivering CpG-ODN under different field conditions in different seasons of Saskatchewan and British Colombia, Canada where climatic conditions are significantly different. We have conducted field experiments in winter months in Saskatchewan and winter and summer months in British Colombia, Canada where we had different temperature and humidity conditions but we have demonstrated that our CSPN can deliver CpG-ODN very efficiently under different outdoor conditions.

The ambient environmental conditions in the CSPN chick enclosure had an impact on aerosolization dynamics as well as the inhalation efficacy of chicks. Our experiments confirmed that humidex of 28 and RH below 60% is critical for nebulization of broiler chicks in order to

protect them against bacterial infections and lethal septicemia. In order to prove the importance of humidex in the chick enclosure, we increased RH above 60% and humidex above 28 inside the chick enclosure of the CSPN. As a result, protection against *E. coli* septicemia did not occur following CpG-ODN nebulization.

CpG-ODN mimics activities of bacterial DNA by initiating danger signals to immune cells that have the ability to stimulate the innate immune system through TLR mediated pathways (215). In chickens, CpG-ODNs are likely recognized via intracellular TLR21 receptors in a number of cell types (47). We have recently demonstrated that CpG-ODN mediates Th1 and Th2 type immune response in chickens following CpG-ODN administration by upregulation of IFNγ and proinflammatory cytokines such as IL1β, IL6, IL8 and IL18 (312). We also demonstrated that *in ovo* delivery of CpG-ODN to day 18 embryos elevated the proinflammatory cytokines; particularly lipopolysaccharide-induced tumor necrosis factor in the lungs and spleens of the embryos and day old chicks (136). On further analysis we witnessed the upregulation of antigen presenting cells such as macrophages and dendritic cells as well as CD4+ and CD8+ T lymphocyte populations indicating their role in mediating the antibacterial immunity (136).

As a majority of poultry pathogens, including *E. coli*, enter the systemic circulation through the respiratory system of chickens (121), the respiratory tract-associated mucosal immune system is important for bacterial clearance (101). Avian lung is more efficient in air exchange and diffusion of aerosolized compounds compared to the mammalian lung, thus making avian lung an excellent route for delivery of therapeutic agents (336). Furthermore, poultry vaccines are successfully administered through the respiratory route as aerosols and coarse sprays (12,105,318). However, droplet or particle size plays a critical role in determining their dispersion and deposition in airways (68,390). A study conducted in pigeons using fluorescent microspheres demonstrated that <6μm particles were distributed throughout the respiratory tract including deeper lung tissues while larger particles were deposited in the upper airway (390). Vaccines containing live microorganisms need a coarse spray since microorganisms should not go below the level of the trachea (30,49) while immunotherapeutic agents such as CpG-ODN need to enter the blood stream through air capillaries of the tertiary bronchioles of the avian lung; hence the particle size of CpG-ODN droplets should be 0.5-5μm in the CSPN.

It has been demonstrated that one hr exposure to 50,000 ppm of CO_2 started causing difficulty in breathing in 6-8 week old chickens, indicating CO_2 levels below 50,000 would be relatively tolerable to chickens (19). Hatching-eggs are exposed to controlled concentrations of CO_2 during the incubation period in order to facilitate embryonic growth (102,254). This embryonic exposure to moderate concentrations of CO_2 must be making neonatal broiler chicks tolerant to higher concentrations while they are young (97). According to one study, exposing hatching eggs to 4,000-10,000 ppm CO_2 levels did not have any adverse effects on the growth of the chicks (102). Furthermore, researchers who exposed neonatal chicks to 600,000-900,000 ppm CO_2 in residual air found that they needed higher concentrations of CO_2 and longer exposure times for successful euthanasia of the neonatal chicks (329,330). Loss of consciousness in newly hatched chicks was achieved by exposing to 200,000-400,000 ppm CO_2 with argon and 20,000 ppm residual oxygen or 900,000 CO_2 in the air (329). A recent study discussed that the exposure of chicks to 750,000-900,000 of CO_2 induced loss of posture and motion (142). In contrast, during our experiments, CO_2 levels were maintained between 4,000-6,000 ppm inside the chamber of CSPN and the chicks did not show any signs of discomfort during the nebulization process, indicating that the CSPN was able to maintain CO_2 levels inside the chamber at a safe level.

Although we nebulized birds with CpG-ODN for 30 min using CSPN, we could potentially reduce this duration by 50% as we have demonstrated under laboratory conditions using our laboratory scale nebulizer (128). Decreasing the time required for nebulization is likely an important aspect in the commercial hatchery process, as hatchery staff does not need to hold birds for a long time before birds are transported. Although we have demonstrated that 15 min is sufficient time to nebulize broiler chicks under laboratory conditions, we need to prove this under field conditions using CSPN in future experiments.

In summary, the Canadian broiler chicken industry has taken steps to reduce antimicrobial use and is searching for alternatives to use of antibiotics to improve flock health. It is promising that CpG-ODN is an excellent alternative to antibiotics and can be used under field conditions by delivering via the IPL route. Since the CSPN can be used in different climatic conditions in different seasons of the year in Canada, it is very likely that CSPN can be used in any country in the world with different climatic conditions. We believe that the utilization of this

technique with the reduction of antibiotic use will improve poultry health and welfare while protecting public health by minimizing the emergence of antibiotic resistant bacteria.

PREFACE TO CHAPTER 4

CpG-ODN are synthetic counterparts of bacterial DNA that have immunostimulatory properties in mammalian (150,243,356,402,407), avian (125) and certain lower animal species (377). In chapters 2 and 3 we have determined that the IPL delivery of CpG-ODN was able to induce protective immunity in neonatal broiler chicks against lethal *E. coli* septicemia. The immunomodulatory mechanisms leading to this systemic outcome, following mucosal administration of CpG-ODN is yet to be fathomed. Previous studies have revealed that the IM delivery of CpG-ODN induced predominantly Th1 type cytokine genes to upregulate in the spleens (312). Meanwhile, *in ovo* delivery of CpG-ODN has resulted in the expression of both Th1 and Th2 cytokine genes in multiple organs such as the lungs, spleen and bursa of fabricius (77,136,349). We were curious to discover the cytokines involved in the protective immune response and their expression profiles locally in the lungs as well as systemically in the spleen resulting from IPL CpG-ODN administration. Hence, the work in the first part of chapter 4 was conducted to understand that mechanistic insight, prioritizing cytokine gene expression levels. Further on, studies indicated that CpG-ODN delivery significantly increased the number of T and B lymphocytes in systemic lymphoid organs such as the spleen and bursa of fabricius (62). Dar *et al.* demonstrated downstream upregulation of cytokines and chemokines that could possibly mature and attract more APC and lymphocytes to systemic lymphoid organs upon CpG-ODN stimulation in broiler chicks (75). The most recent publication by our group showed that CpG-ODN delivered by the *in ovo* route was able to increase the number of APC such as macrophages and CD4+ and CD8+ T lymphocytes in lungs and spleens of neonatal broiler chicks (136). With the novel IPL CpG-ODN administration route, we were curious to explore the downstream cellular activation, maturation and infiltration profiles both in the local site of the lung and in the systemic immune organs. As a result, the second part of the chapter 4 discusses the cellular infiltration profiles studied using flow cytometric analysis and histopathology tools.

CHAPTER 4 MUCOSAL DELIVERY OF CPG-ODN MIMICKING BACTERIAL DNA VIA THE INTRAPULMONARY ROUTE INDUCES SYSTEMIC ANTIMICROBIAL IMMUNE RESPONSES IN NEONATAL CHICKS

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The work described in this manuscript has been submitted to Scientific Reports Journal.

Contribution: The experiments in this chapter were conceptualized collectively by my self, Dr. Gunawardana, Dr. Ahmed and Dr. Gomis. I conducted the experiments together with Dr. Gunawardana and all the members of the research group assisted at various steps. Ms. Lockerbie assisted to conduct Bioplex analysis and Dr. Ahmed assisted to conduct flow cytometry and its data analysis. I performed data analysis and wrote the manuscript with help from Dr. Ahmed. All the authors mentioned above contributed by providing their feedback.

4.1 Abstract

The transition to antibiotic-free poultry production in the face of pathogenic threats is a very challenging task. We recently demonstrated that mucosal delivery of CpG-ODN alone by the IPL has potential as an effective alternative to antibiotics in neonatal chicks against Escherichia coli septicemia. How exactly mucosal delivery of CpG-ODN elicits protective antibacterial immunity is poorly understood. In this study, CpG-ODN or distilled water was delivered via the intrapulmonary route to day-old chicks (n=80/group) using a compressor nebulizer in an acrylic chamber (1mg/mL CpG-ODN for 15 min). In the initial part of the study, two days after mucosal CpG-ODN delivery, 40 chicks from each group were challenged SQ with $1x10^5$ CFU (n=20) or $1x10^6$ CFU (n=20) of *E. coli* and the mortality pattern was monitored for 7 days. We found significantly higher survival, better clinical conditions and lower bacterial loads in chicks that received mucosal CpG-ODN. In order to explore the mechanisms behind this protective immunity, we first looked at the kinetics of the cytokine gene expression (3 birds/ group/ time for 10 time-points) in the lungs and spleens. Multiplex gene analysis demonstrated a significant elevation of pro-inflammatory cytokine genes [IL18, IL1β, IL6 and LITAF (TNFα factor)] mRNA in the CpG-ODN group. IL1β was robustly upregulated many fold in the lung after CpG-ODN delivery. LITAF and IL18 showed expression for a longer period of time in the lungs. Anti-inflammatory cytokine IL10 was upregulated in both lungs and spleen, whereas IL4 showed upregulation in the lungs. Flow cytometry and histology were also performed at 24, 48 and 72 hrs post-delivery of CpG-ODN to investigate the kinetics of immune enrichment in the lungs and spleens. We detected a significant enrichment of CD4+ and CD8+ T-cell subsets, along with monocytes/macrophages with mature phenotypes (high expression of CD40 and MHCII) in the target organ lungs. Importantly, mucosal delivery of CpG-ODN via the IPL route also significantly modified immune development in the spleen as well, suggesting its ability to produce a systemic effect in neonatal chicks. Altogether, mucosal delivery of CpG-ODN enhances not only mucosal immunity but also the systemic immune responses to bolster protective immunity against *E. coli* septicemia.

4.2 Introduction

The appearance of antimicrobial resistant bacterial strains due to antimicrobial use in animal production is a global problem. Antimicrobial resistance is quintessentially a one-health

issue. The Canadian chicken industry is implementing antimicrobial use reduction strategies to control antimicrobial resistance development. However, studies suggest that withdrawal of prophylactic antibiotic use led to a substantial increase in therapeutic antibiotic use in the poultry industry (52). Broiler chickens are most susceptible to bacterial infections during the first-week of their life and these infections adversely influence the remaining production cycle and growth of chickens (306,433). As a result, the poultry industry is urgently looking for suitable alternatives to antibiotics for disease prevention. Our research group has been studying immunomodulation using CpG-ODN in chickens over a decade. We provided the first *in vivo* evidence for standalone antimicrobial function of CpG-ODN in chicks against *E. coli* and *Salmonella* infections using *in ovo* (embryo injection) (137,382), IM and SQ (125,126) routes of delivery.

CpG-ODNs are short, single stranded DNA that have the ability to stimulate the immune system by acting as PAMPs (66,140,141). CpG-ODN initiates immune modulation by binding to specific PRRs called TLR9 (mammalian species) or TLR21 (avian species) in APCs such as DCs, macrophages and B lymphocytes (35,47,192,388). Functionally, both TLR9 and TLR21 are similar in CpG-ODN pattern recognition although there is species specific variability in recognizing different contexts of nucleotide sequences in the CpG motifs (434). Murine TLR9 recognizes the GACGTT motifs whereas human TLR9 and chicken TLR21 recognizes the GTCGTT motifs comprising CpG-ODN (215). Both human and avian CpG-ODN elicits similar cytokine induction and activation of immune cells leading to innate and adaptive immune responses (61,62,148). Furthermore, immune stimulatory ability of CpG-ODN can be potentiated by formulating it with nanoparticles (48,137,140,365). A study conducted using a chicken macrophage cell line (HD11 cells) reported that CpG-ODN induced strong IL6 and NO secretion while killing *S. enteritidis* in the activated cells (428). There is evidence that CpG-ODNs innate immune stimulation results in high nitric oxide production in monocytes, which may be directly associated with its ability to control microbial infections (154). Other studies have reported that CpG-ODN stimulates the expression of IFNγ, IL1β, IL6, and IL8 in spleens (14, 44). A study by our group showed that *in ovo* CpG-ODN injection in 18-day old embryonated eggs elicited a significantly higher expression of pro-inflammatory cytokines (136).

We recently reported that mucosal delivery of aerosolized CpG-ODN micro-droplets via the IPL route can protect neonatal broiler chicks against lethal *E. coli* septicemia (128).

However, the mechanisms by which mucosal delivery of aerosolized CpG-ODN alone provides protection in chickens against *E. coli* septicemia are not completely understood. The present study was designed to gain greater insights into the antimicrobial protective mechanisms resulting from mucosal delivery of aerosolized CpG-ODN in neonatal chicks.

4.3 Materials and methods

4.3.1 CpG-ODN

The sequence of CpG-ODN used was 5'-TCGTCGTTGTCGTTTTGTCGTT-3'. It was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies, Inc; Huntsville, AL, USA).

4.3.2 Bacterial strain and culture for challenge

The challenge bacterial strain used here was a field isolate of *E. coli* from a septicemic turkey. This *E. coli* belonged to serogroup O2 and it was non-hemolytic, serum-resistant, aerobactin-producing, with a K1 capsule and Type 1 pili. Aliquots of bacteria were stored at -80 C in 50% brain–heart infusion broth (Difco, Detroit, MI) containing supplement of 25% (w/v) glycerol (VWR Scientific, Inc., Montreal, Quebec). Bacterial culture was prepared as previously described (128). Briefly, bacteria used for challenge were cultured on 5% Columbia sheep blood agar plates for 18-24 hrs at 37 C. One medium sized colony was mixed in 100 mL of Luria broth in a 250 mL capacity Erlenmeyer flask. The bacteria were grown at 37 C for 13 hr with shaking at 150 rpm. Stationary phase culture contained approximately 1×10^9 CFU of bacteria per mL. The cultures were diluted in sterile saline to obtain the concentrations of bacteria required for challenge (1×10^5 or 1×10^6 CFU/bird). Determination of bacterial viability and count was done by plating serial dilutions of the diluted culture in duplicate on 5% Columbia sheep blood agar plates, incubating for 18-24 hr at 37 C; then counting the number of colonies.

4.3.3 Chickens, animal housing and maintenance

This work was conducted with the approval of the Animal Research Ethics Board, University of Saskatchewan, in accordance with the Canadian Council on Animal Care guidelines. Hatching eggs were obtained from a commercial hatchery in Saskatchewan, Canada and incubated at the ACU, Western College of Veterinary Medicine, University of

Saskatchewan. Hatched chicks were allocated randomly into an animal isolation room at the ACU with wood shaving on the floor as litter. A tag with an identification number was placed on the neck to identify the groups. Water and commercial broiler feed were provided *ad libitum*. Ventilation of the animal isolation rooms was maintained with filtered, non-recirculated air at a rate of 10–12 changes/hr. Moreover, air pressure differentials and strict sanitation were maintained in this isolation facility. Broilers were raised at 32 C for the first week of life; thereafter the temperature was decreased 0.5 C per day until a room temperature of 27.5 C was reached. Light was provided for 24 hr/day during days 0 to 2 post-hatch. Darkness was introduced at 3 days post-hatch with 1 hr of dark added daily until 4 hr of darkness was achieved.

4.3.4 *E. coli* challenge of neonatal chicks

The *E. coli* animal challenge studies were carried out following the procedure as described previously (128). Briefly, 2 days post-treatment, birds were challenged SQ in the neck with either $1x10^5$ or $1x10^6$ CFU of *E. coli*. Two doses of *E. coli* were used in our study to simulate field conditions wherein all birds are not usually exposed to a consistent dose of *E. coli*. Birds were evaluated three times daily at the critical stage (until 3 days post-challenge) and twice thereafter up to 7 days post-challenge. Each bird was observed for their clinical presentation and a daily clinical score was assigned: 0 = normal; 0.5 = appearing slightly abnormal, slow to move; 1= depressed, unwilling to move; 1.5 = unwilling to move, may take a drink and peck some; 2 = unable to stand or reach for food or water; and 3 = dead. Birds that were assigned a clinical score of 2 were euthanized by cervical dislocation. At the end of the trial, each bird was given a CCS as a sum of daily clinical scores as previously described (126,128). Chicks that were found dead or euthanized were subjected to immediate necropsy. On day 7 post-challenge, the remaining birds were humanely euthanized by cervical dislocation. Bacterial swabs were taken from the air sacs of dead and euthanized birds, and cultured on 5% Columbia sheep blood agar according to the quadrant streaking technique. In quadrant streaking method, bacterial reduction occurs as streaking moves clockwise from quadrant 1 to quadrant 4. A semi quantitative estimate of *E. coli* isolation was conducted according to the growth on blood agar. Growth on these plates was recorded on a scale from 0 to 4+, where 0 = no growth; few = less than 5 colonies; 1+ = bacterial growth on quadrant 1 only; 2+ = bacterial growth on quadrants 1-2; 3+ = bacterial growth on quadrants 1-3; and 4+ = bacterial growth on quadrants 1- 4 (160).

4.3.5 Preparation of cells for flow cytometry

Preparation of cells and antibody staining for flow cytometry was done as previously described with slight modifications (11,138,224). Briefly, lung and spleen were harvested from chicks at 24, 48 and 72 hr post CpG-ODN treatment and processed for single cell preparation. Each spleen was gently pressed against a metal strainer by applying gentle pressure using a syringe plunger in ~3 mL of phosphate buffered saline (PBS) and cells were collected in a 15 mL centrifuge tube. The lung was manually chopped into small pieces using a surgical blade and incubated at 37 C for 30 min with ~1 mL of collagenase (1 mg/mL) dissolved in Dulbecco's Modified Eagle Medium. After incubation, these tissues were similarly pushed through a metal strainer to obtain a single cell suspension and washed twice with PBS. Then, lung and spleen cells were incubated for 15-20 min with red blood cells lysis buffer. These cells were washed three times with wash buffer (PBS containing 2% fetal bovine serum and 0.1% sodium azide). Then the cells were used for antibody staining and analyzed by flow cytometry.

4.3.6 Antibodies for flow cytometry

Monoclonal antibodies for chicken monocyte/ macrophages [mouse anti-chicken monocyte/ macrophages-phycoerythrin (PE)], CD4 (mouse anti-chicken CD4-PE) and CD8 [mouse anti-chicken CD8α-fluoroscein isothiocyante (FITC)] were obtained from Southern Biotechnology (Birmingham, Ala, USA). The primary antibodies used were mouse anti-chicken CD40 and mouse anti-chicken CD86 monoclonal antibodies (Bio-Rad, Raleigh, NC, USA). The secondary antibody used was Anti-mouse-FITC IgG antibody. Goat anti-mouse IgG together with Streptavidin-PerCP/Cy5.5 and Mouse IgG1 isotype control were obtained from Bio Legend (San Diego, CA, USA).

4.3.7 Experimental design

4.3.7.1 Mucosal delivery of CpG-ODN via the intrapulmonary route

Synthetic CpG-ODN was diluted in sterile, DW and using a Compressor Nebulizer (705-470*)* unit (AMG Medical Inc; Montreal, QC, Canada), mucosal delivery of CpG-ODN was performed as aerosolized micro-droplets (particle size of 0.5–5 µm) in a closed 0.036 m^3 acrylic chamber containing 40 birds/chamber for 15 min (4 mg CpG-ODN/ chamber). The control group

of birds was aerosolized with sterile DW for 15 min in the acrylic chamber using a similar compressor nebulizer. Each group had a total of 80 birds (n=80). The temperature was maintained at 28-30 C in the acrylic chamber during administration of CpG-ODN or DW.

4.3.7.2 *E. coli* challenge

Two days post IPL delivery of CpG-ODN (day-2 post-hatch), birds were challenged with either $1x10^5$ (n=20/group) or $1x10^6$ CFU (n=20/group) of a virulent strain of *E. coli* by SQ injection in the neck, following the previosuly published challenge model (128). Data were collected on mortality, clinical signs, pathology and bacterial isolations from the air sacs for 7 days following challenge with *E. coli*.

4.3.7.3 Sample collection

QuantiGene Plex assay: Spleen and lung samples from three chicks per group were collected at 10 time points post treatment (0, 3, 6, 12, 24, 32, 48, 72 hr, day 5 and day 7) in 1.5 mL tubes, flash frozen in dry ice and ethanol slurry and stored in -80 C.

4.3.7.4 Flow cytometry

Three chicks from each group were humanely euthanized at 24, 48 and 72 hr post treatment by cervical dislocation. Spleen and lung tissues were collected into 1.5 mL tubes, rinsed with PBS and kept on ice. They were processed on the same day.

4.3.7.5 Analysis of cytokine gene expression

Expression of mRNA of IFNα, IL18, IFNγ, IL1β, LITAF, IL4, IL6, and IL10 cytokine genes in the lung and spleen were measured by commercially available probes for avian cytokines QuantiGene Plex 2.0® (Panomics/Affymetrix Inc., Fremont, CA, USA). Table 4.1 lists the genes of interest and their accession numbers.

Table 4.1 Genes of interest (*housekeeping genes- HKGs)

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

Frozen spleen and lung tissues were processed to prepare the tissue homogenates according to the manufacturer's instructions with some modifications. Briefly, 5 mg of tissue was chopped in 300 µL of homogenization solution containing 3 µL of Proteinase K. At 65 C the tissue lysate was digested to release the mRNA and then it was centrifuged to remove the debri by precipitation. The tissue lysates supernatants were collected and saved at -80 C for future use. The oligonucleotide capture probes used for mRNA and the label probes were designed by the manufacturer. The tissue homogenates (40 µL/well) were added to a 96-well plate that was pre-loaded with 210 µL of the capture reagent per well and the respective probe set. After overnight hybridization at 54±1 C, further processing was carried out according to the manufacturer's instructions by hybridizing with bDNA pre-amplifier 2.0, bDNA amplifier 2.0, biotinylated probe and substrate. Luminescence was measured using a Luminex instrument (Bio-Rad, USA). Signals recorded as the mean fluorescence intensity (MFI) generated from each bead is proportional to the amount of mRNA captured on the surface of each generated specific probe set (455). The gene expressions were normalized with that of the housekeeping genes (HKGs), tubulin beta 1 (Tubb1) and Hypoxanthine-guanine phosphoribosyl transferase 1 (Hprt 1). For analysis, MFI data of each gene was divided with the average HKGs (Hprt 1 and Tubb 1) to normalize the data. Then, using normalized MFI data fold expression of CpG-ODN group in comparison to saline control group at each time point was calculated.

4.3.7.6 Flow cytometry

Cell populations isolated from lung and spleen were stained with cell marker specific antibodies in order to identify the appearance of monocyte/macrophages, CD4+ and CD8+ T cells. The monocyte/macrophages were further examined for the expression of maturation markers (CD40 and CD86). Briefly, ~5x10^5 cells were incubated with mouse anti-chicken monocyte/macrophage (PE) antibody at 4 C for 30 min for detecting APCs. Cells from the previous step were washed three times and incubated separately with either mouse anti-chicken CD40 or CD86 primary antibodies at 4 C for 30 min to identify maturation markers on the APCs. Following three washing steps with PBS, the cells were stained with PerCP/Cy5.5 goat anti-mouse IgG secondary antibody and kept at 4 C for 30 min. In order to detect the ratio of CD4+ and CD8+ T cells, another set of ~5x105 cells were incubated with mouse anti-chicken CD8 (FITC) and CD4 (PE) together at 4 C for 30 min. Ultimately, the washed cells were suspended in 300 µL of PBS in glass flow cytometry tubes and flow cytometry analysis was performed using the Epics XL (Beckman Coulter) and FACS Caliber (BD Bioscience). Acquired data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

4.3.8 Statistical analysis

The significance of difference in survival analysis, bacteriological scoring, CCS, difference of gene expression and cell populations between groups were analyzed using Prism (Prism 6.0, GraphPad Software Inc; San Diego, CA, USA) with a significance level of $P<0.05$. The survival data of both 1x10^5 CFU/bird and 1x10^6 CFU/bird of *E. coli* were combined for clarity of analysis and presentation. The significance of differences among groups in survival patterns, median survival times and relative risk of mortality were analyzed using the log-rank test and chi-square statistics. Clinical scores of each bird for the 10 day period were summed to generate a CCS and the significance of differences among groups was tested using Kruskal Wallis nonparametric analysis of variance. Sidak multiple comparisons test following Ordinary One Way ANOVA was used to test the significance in the differences of gene expression between CpG-ODN group and the saline control group at each time point. The percentages of CD4+ and CD8+ T lymphocyte subsets were combined to compare the total T cell infiltration percentage in the lung and spleen between groups. In order to test the difference of CD4+ and

CD8+ T lymphocyte expression and APC expression between groups, Student-T test with Welch's correction for unequal variance was used, with a significant difference of $P<0.05$.

4.4 Results

4.4.1 Immunoprotective effect of mucosal delivery of CpG-ODN against *E. coli* septicemia

During the 7 days post *E. coli* challenge, the group that received mucosal CpG-ODN as micro droplets through the intrapulmonary route showed a significantly higher survival ($P=0.0249$) compared to the distilled water control group (Figure 4.1A). The CCS of the birds following *E. coli* challenge with $1x10^5$ or $1x10^6$ CFU/bird exhibited that the group that received CpG-ODN through mucosal route had a significantly lower CCS ($P=0.0384$) compared to the distilled water control group (Figure 4.1B). Isolation of bacteria from the thoracic air sacs of the birds tended to show a higher bacterial load in the control chicks compared to the CpG-ODN group (Figure 4.1C). The relative risk of mortality following the *E. coli* challenge was reduced by 53.92% following CpG-ODN treatment.

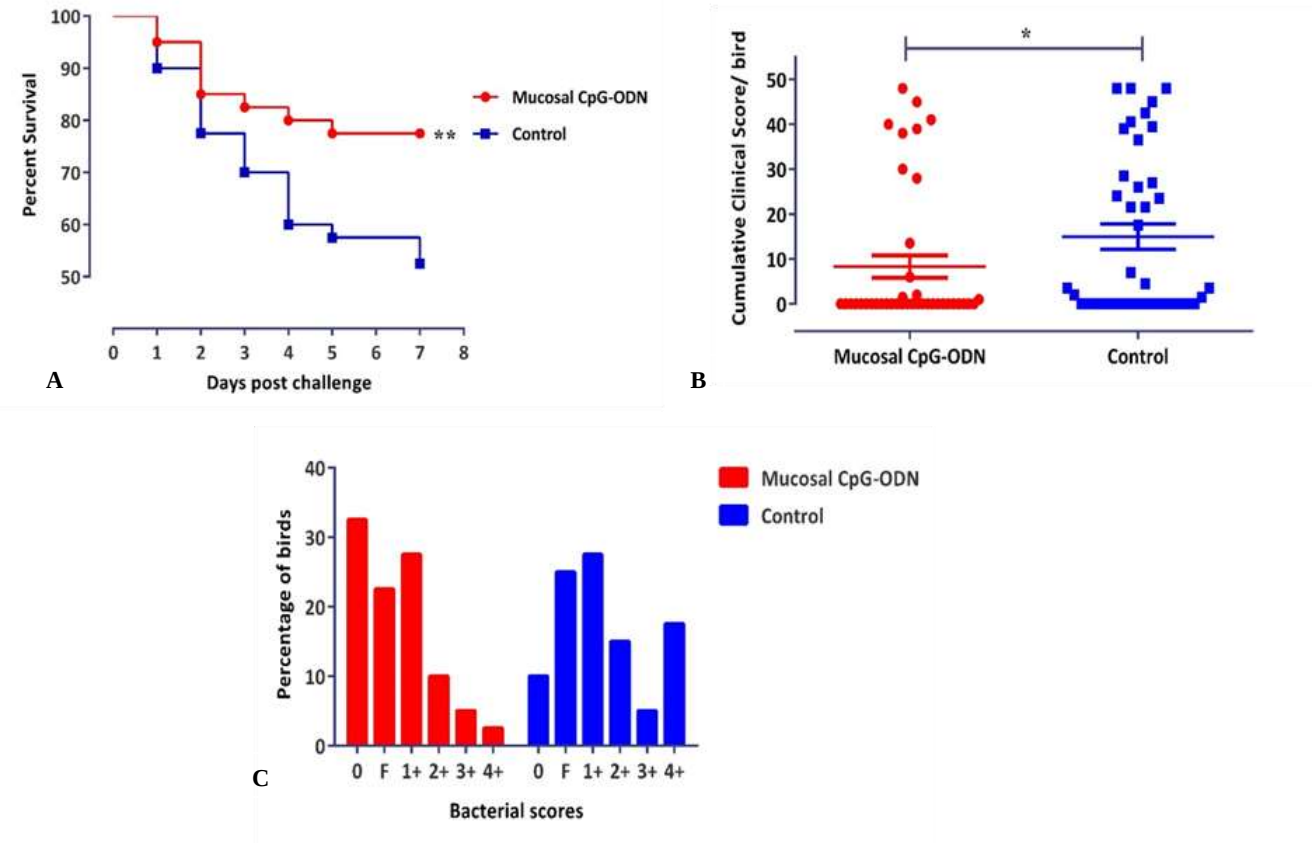

Figure 4.1 Survival percentages, cumulative clinical score and bacterial scores of birds that received mucosal CpG-ODN and/or distilled water followed by a lethal *E. coli* challenge. A. Birds that received CpG-ODN (n=40) showed significantly higher survival compared to the distilled water (n=40) control group (P = 0.0249). B. CCS values following the *E. coli* challenge depicted significantly lower CCS in the CpG-ODN received group compared to the distilled water control (P = 0.0384). C. Birds following $1X10^5$ CFU or $1X10^6$ CFU/bird *E. coli* challenge indicated a heavier bacterial load in the distilled water control birds compared to the IPL CpG-ODN treated birds.

4.4.2 Analysis of cytokine gene expression

CpG-ODN mediated immune modulation was elucidated by measuring the expression array of pro-inflammatory (IL1β, IL6, LITAF, IL18), Th-1 type (IFNγ, IFNα) and Th2 type (IL4, IL10) cytokines in spleen and lung at various time points post mucosal CpG-ODN delivery. It was identified that the fold changes of mRNA levels were 1 or >1 almost all times, in the chicks that received IPL CpG-ODN compared to the control group. There was no specific consistent pattern of gene expression; however, pro-inflammatory cytokine genes (IL1β, IL6, LITAF, and IL18) exhibited relatively higher levels in the mucosal CpG-ODN group compared to the control. Level of expression of IL1β gene was significantly many folds higher in the lung compared to that of the spleen starting from 6 through 72 hr peaking at 12 and 24 hr. LITAF

expression increased 24 to 48 hr post treatment in both lung and spleen whereas IL18 elevated from 12 to 48 hr (Figure 4.2). Both Th1 type cytokine genes (IFNγ, IFNα), and Th2 type cytokines (IL4, IL10) were generally highly expressed in the lung compared to spleen in the mucosal CpG-ODN treated birds, peaking at 24 hr post treatment (Figure 4.3).

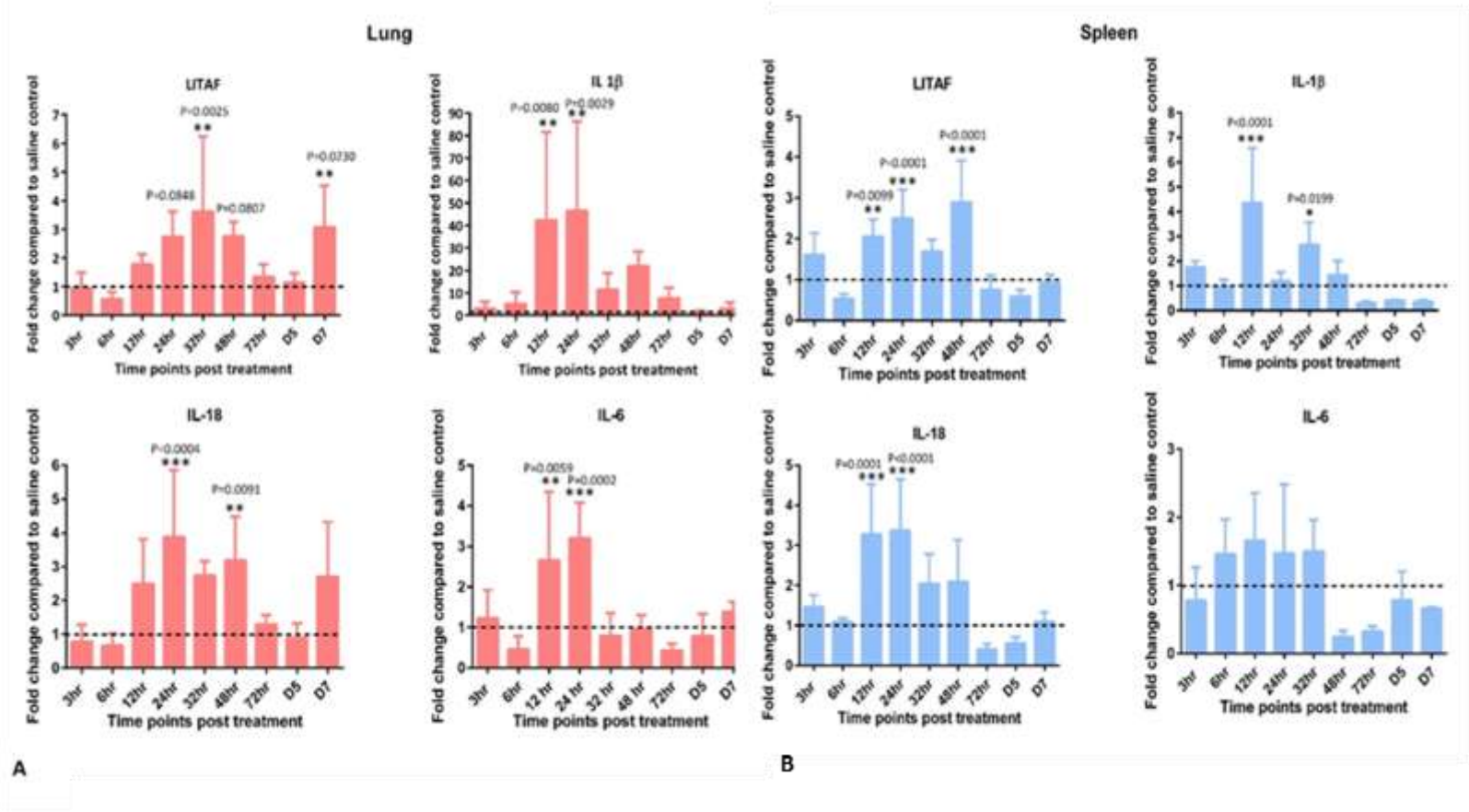

Figure 4.2 Pro-inflammatory cytokine profiles in lungs (A) and spleen (B). MFI of each cytokine gene was normalized to the averages of HKGs (Hprt 1 and Tubb 1). Fold changes were calculated compared to the saline control at each time point. (Broken lines indicate the fold change of one, which shows no change compared to the control group). Sidak multiple comparisons test following ANOVA was used to analyse the significance of gene expression between CpG-ODN and the distilled water control group at each time point. Asterisks indicate the fold changes that were significantly different ($P<0.05$).

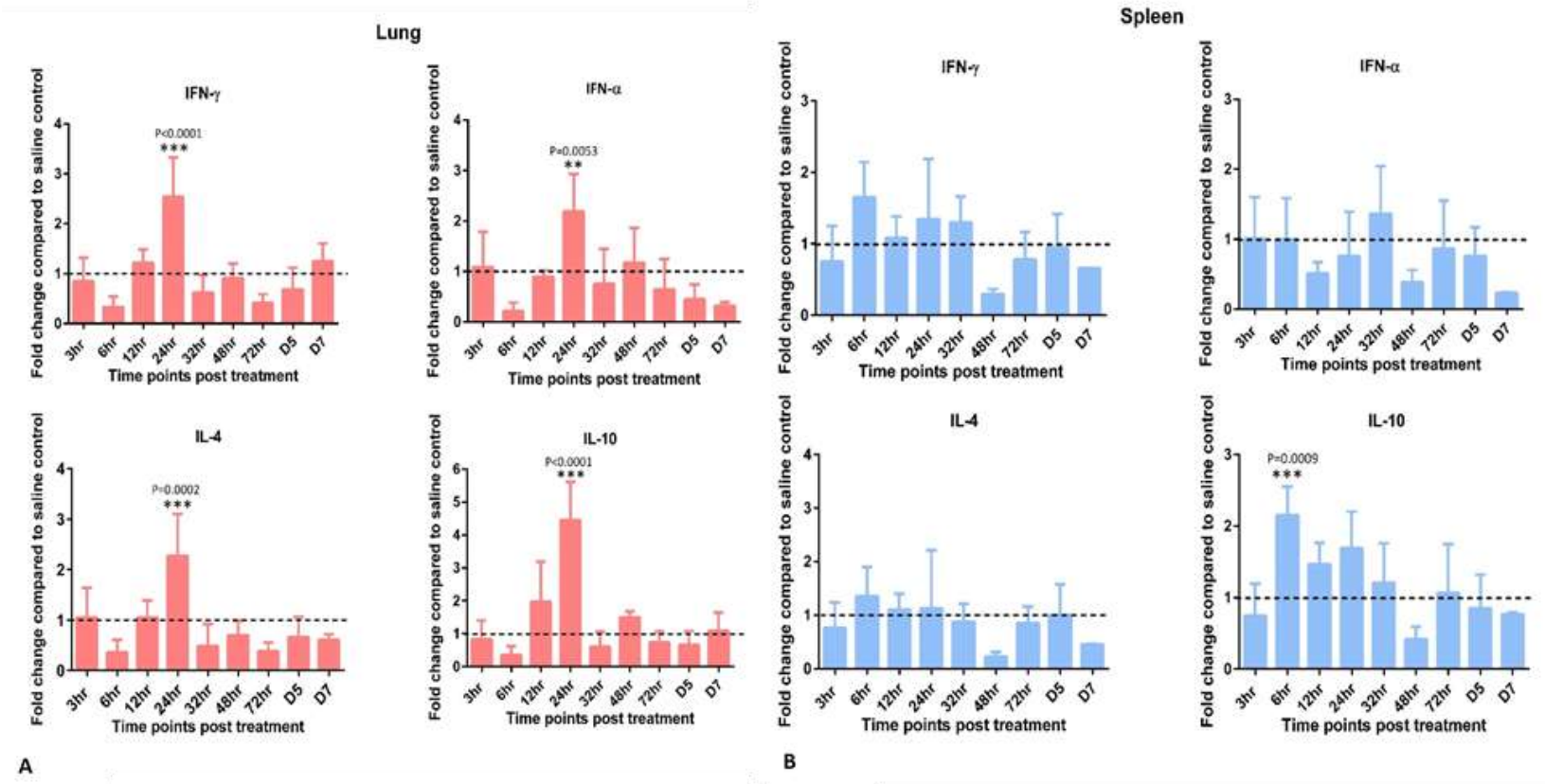

Figure 4.3 Interferon and regulatory cytokine profiles in lung (A) and spleen (B). MFI of each cytokine gene was normalized to the averages of HKGs (Hprt 1 and Tubb 1). Fold changes were calculated compared to the saline control at each time point. (Broken lines indicate the fold change of one, which shows no change compared to the control group). Sidak multiple comparisons test following ANOVA was used to analyse the significance of gene expression between CpG-ODN and the distilled water control group at each time point. Asterisks indicate the fold changes that were significantly different ($P<0.05$).

4.4.3 Flow cytometry analysis and histology

Flow cytometry analysis revealed that mucosal delivery of CpG-ODN via the intrapulmonary route significantly influenced the infiltration of APCs such as monocytes/macrophages with mature phenotypes as well as the T cell populations in the lungs and spleen.

4.4.4 Immune cell infiltration in the spleen and lung tissues following CpG-ODN mucosal delivery

Flow cytometry data at 24 hr post CpG-ODN treatment revealed a significant infiltration of monocytes/macrophages in the spleen (Figure 4.4A). However, we did not observe an upregulation of costimulatory molecule CD40 in these spleen-infiltrating monocytes/macrophages (Figure 4.4B). The combined percentage of CD4+ and CD8+ T cell subsets were markedly increased in spleen of CpG-ODN treated chicks compared to the control (Figure 4.4C). Flow cytometry data of the lung showed significantly greater infiltration of monocytes/macrophages (Figure 4.5A). Moreover, CD40 was highly upregulated in the lung-infiltrating monocytes/macrophages indicating maturation of these APCs (Figure 4.5B). Also in the lung, the combined percentage of $CD4^+$ and $CD8^+$ T cell subsets were significantly higher in the CpG-ODN group (Figure 4.5C). The histological examination of the lung showed very high infiltrations of monocytes/macrophages and lymphocytes in CpG-ODN group (Figure 4.6B and D) compared to control (Figure 4.6A and C).

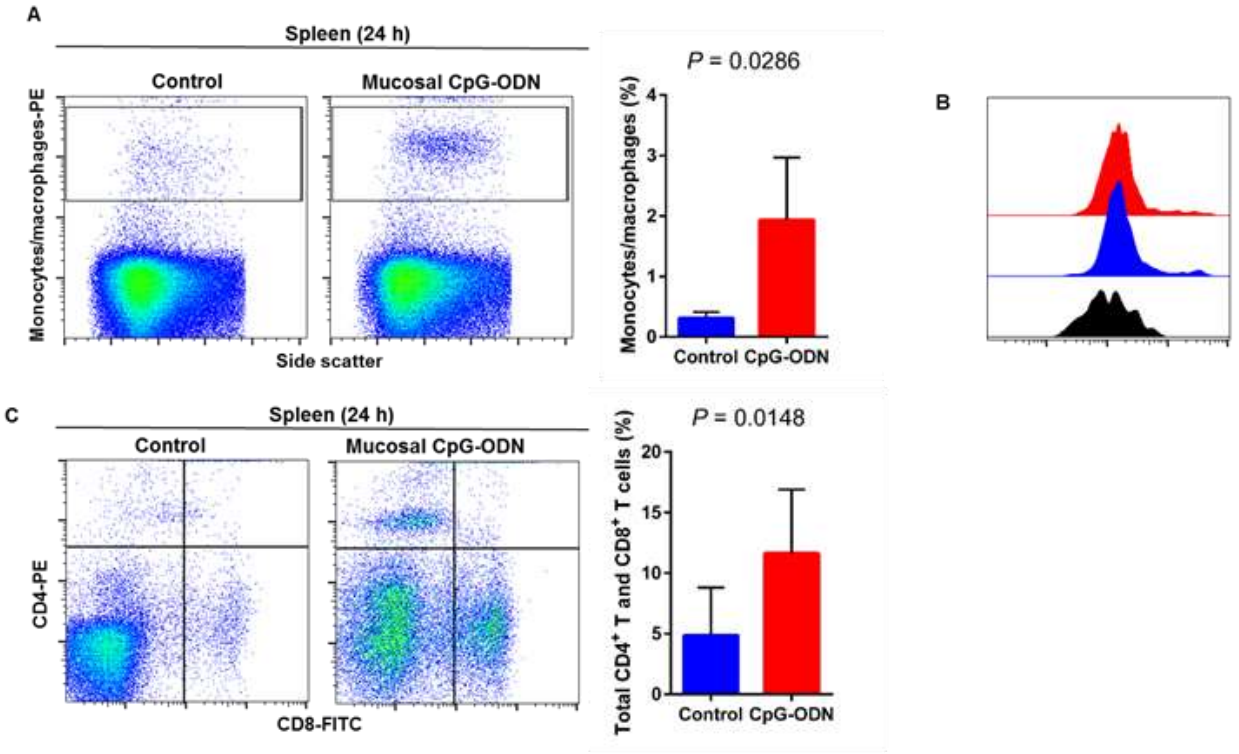

Figure 4.4 Flowcytometric analysis of spleen cells at 24 hours post intrapulmonary CpG-ODN or distilled water treatment. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the spleen following IPL CpG-ODN treatment. Standard Error of Mean (SEM) is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant. B. Histogram panel displays the MFI of CD40 (costimulatory molecule found on APC) expression on the APCs. C. T lymphocyte population in the spleen analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8 monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the spleen following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant.

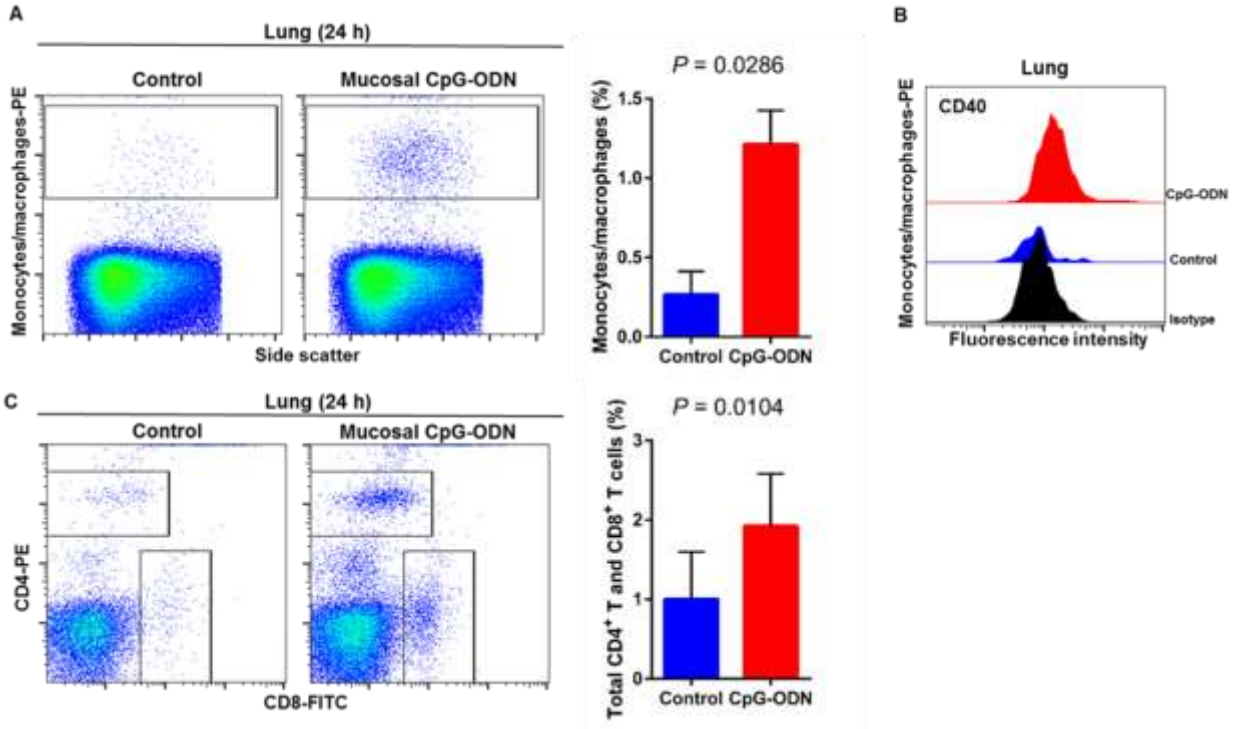

Figure 4.5 Flow cytometric analysis of lung cells at 24 hours post intrapulmonary CpG-ODN or distilled water treatment. A. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the lungs following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant. B. Histogram panel displays the MFI of CD40 expression on the APCs. C. T lymphocyte population in the lung analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8 monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the lungs following IPL CpG-ODN or DW treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant.

Figure 4.6 Histology of lungs 24 hours post treatment with intrapulmonary distilled water (A and C) or intrapulmonary CpG-ODN (B and D). Low power images (10X) indicated a clearly visible high degree of immune cell infiltration in the CpG-ODN treated lung (B) compared to the less cellularity and clearly visible air spaces in the DW control lungs (A). At higher power (20X and 100X), the IPL CpG-ODN received lung showed a distinctly higher number of lymphocytes (black arrow heads in the insert) and large mononuclear cells resembling monocytes/ macrophages (yellow arrows in the insert) infiltrated into the parenchyma (D) compared to the less number of those cells visible in the DW control lung (C).

At 48 hr post CpG-ODN delivery, flow cytometry showed increased infiltration of monocytes/macrophages in the spleen (Figure 4.7A). However, like the 24 hr time-point, we did not find a significant increase in CD40 expression by infiltrating splenic monocytes/macrophages at the 48 hr time-point (Figure 4.7B). At the 48 hr time point, the combined percentage of $CD4^+$ and $CD8^+$ T cell subsets distinctly increased in spleen of CpG-ODN treated chicks (Figure 4.7C). In the lung, monocytes/macrophages at the 48 hr time-point was significantly high (Figure 4.8A) as well as the level of CD40 expression was also significant (Figure 4.8B). The lung showed significantly high infiltration of T cell subsets ($CD4^+$ and $CD8^+$ T cell combined) 48 hr after CpG-ODN treatment (Figure 4.8C). The histological examination also supports higher infiltration of monocytes/macrophages and lymphocytes in the lungs of CpG-ODN group (Figure 4.9B and D) compared to control (Figure 4.9 A and C).

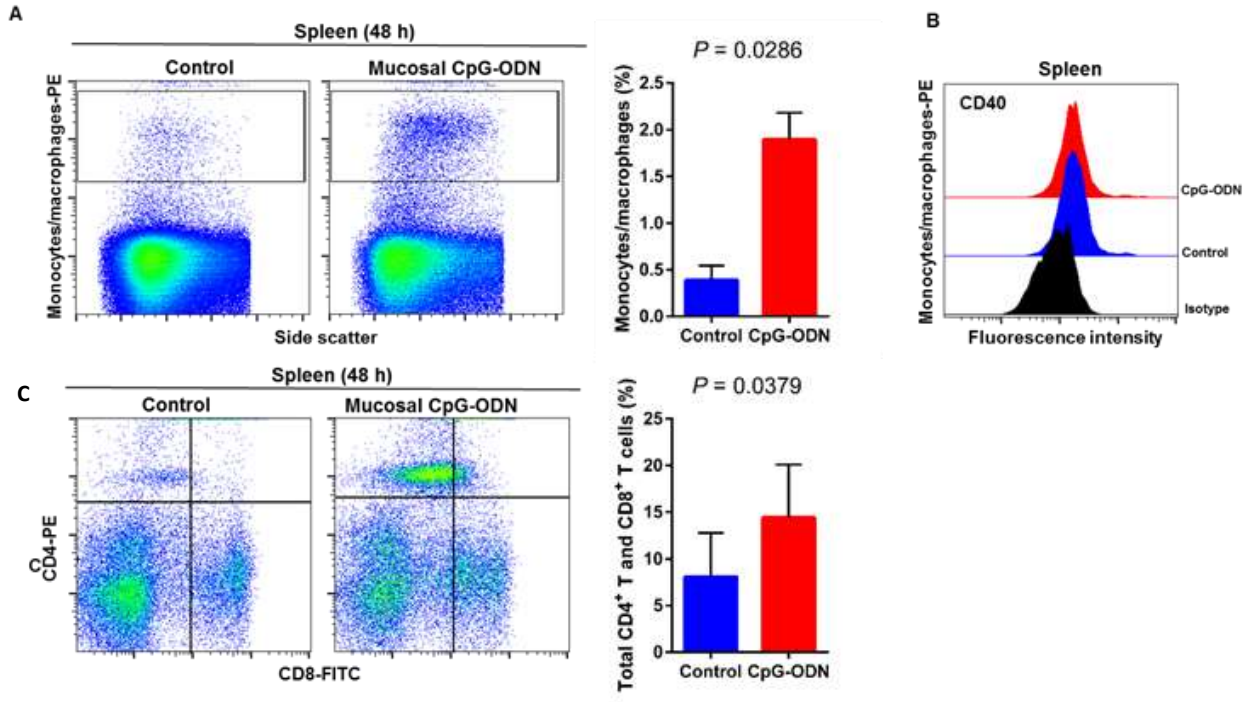

Figure 4.7 Flow cytometric analysis of spleen cells at 48 hours post intrapulmonary CpG-ODN or distilled water treatment. A. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the spleen following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with $P < 0.05$ being statistically significant. B. Histogram panel displays the MFI of CD40 expression on the APCs. C. T lymphocyte population in the spleen analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8

monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the spleen following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant.

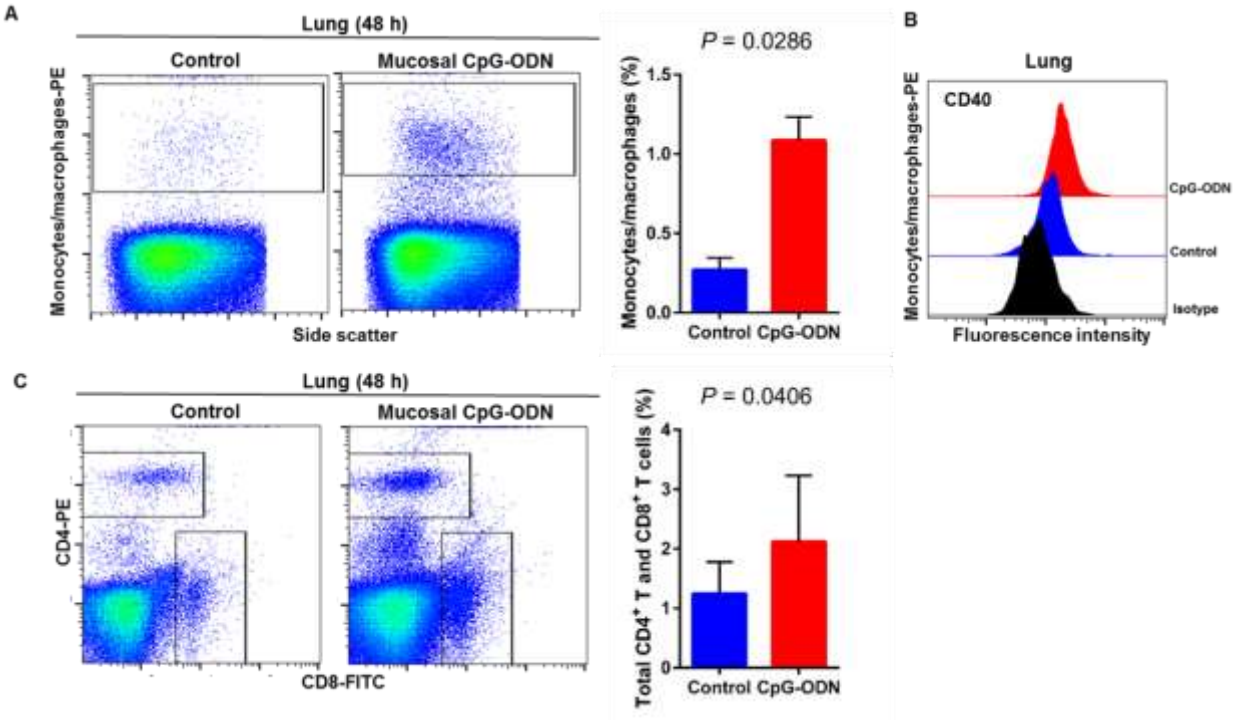

Figure 4.8 Flow cytometric analysis of lung cells at 48 hours post intrapulmonary CpG-ODN or distilled water treatment. A. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the lungs following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant. B. Histogram panel displays the MFI of CD40 expression on the APCs. C. T lymphocyte population in the lung analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8 monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the lungs following IPL CpG-ODN or DW treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant.

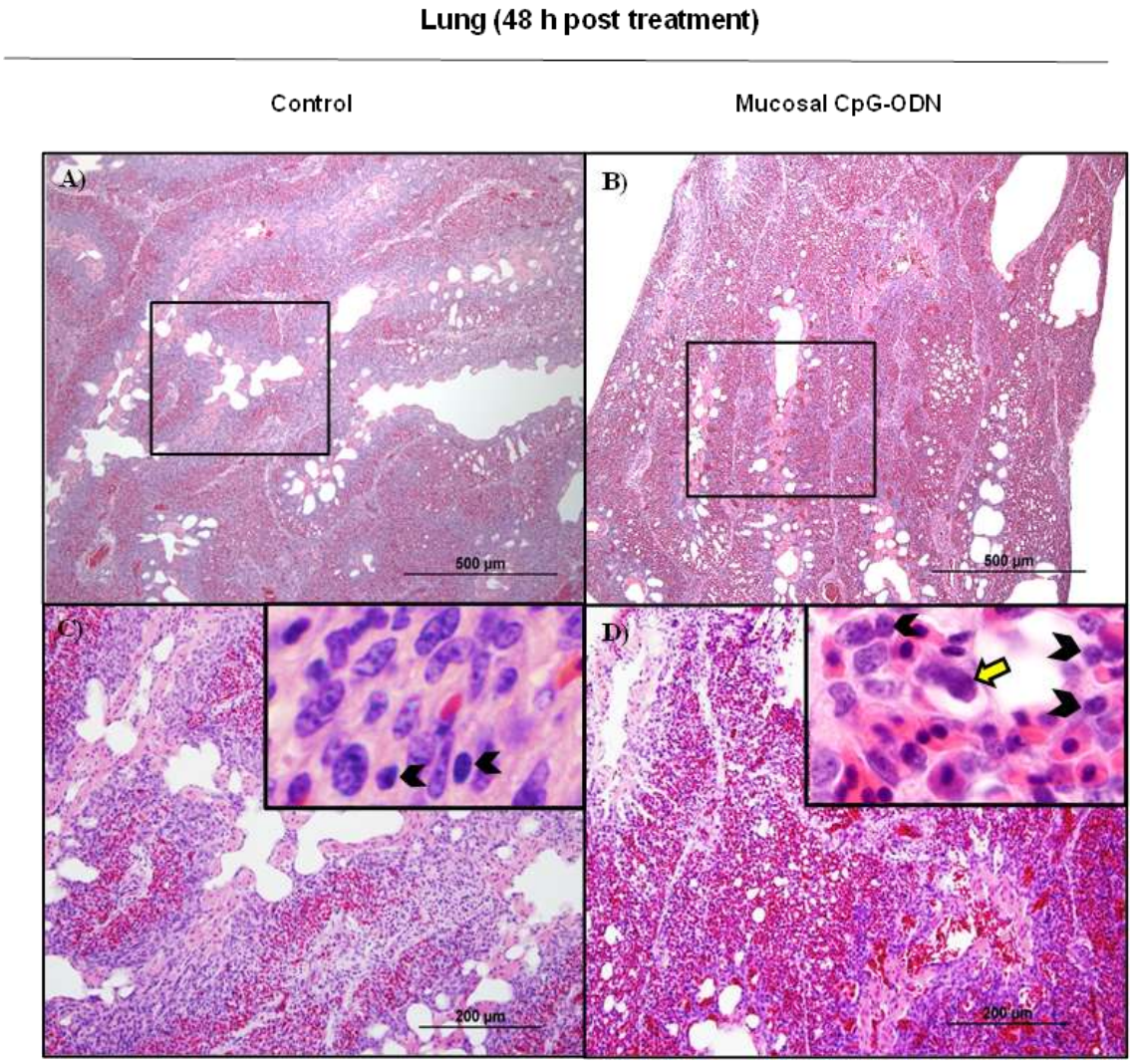

Figure 4.9 Histology of the lungs 48 hours following intrapulmonary distilled water (A and C) and intrapulmonary CpG-ODN delivery (B and D). At lower magnification (10X), DW the CpG-ODN treated lungs appeared more red and infiltrated with large numbers of lymphocytes (B) however visually, the difference between saline control (A) was less apparent compared to the 24 hour time point. At higher magnification (20X and 100X), more lymphocytes (black arrow heads in the insert) and mononuclear cells resembling monocytes and macrophages (yellow arrows in the insert) were seen in the parenchyma of IPL CpG-ODN lungs (D) compared to the DW control lungs (C).

At the 72 hr time point, flow cytometry revealed not only greater infiltration of monocytes/macrophages (Figure 4.10A) but also significantly upregulated CD40 expression in the splenic monocytes/macrophages (Figure 4.10B). This data indicated that mucosal delivery of CpG-ODN via the intrapulmonary route is able to induce maturation of monocytes/macrophages in the spleen, though with some delay. At 72 hr post CpG-ODN treatment infiltration of T cell subsets ($CD4^+$ and $CD8^+$ T cell combined) in the spleen appeared higher in the CpG-ODN group but it was not statistically different than control (Figure 4.10 C). Flow cytometry data from lung

tissues at 72 hr post CpG-ODN delivery demonstrated significantly greater infiltrations of monocytes/macrophages (Figure 4.11A, top). Interestingly, the expression of antigen presenting molecule MHCII by these infiltrating monocytes/macrophages was significantly higher in CpG-ODN group (Figure 4.11A, bottom). Moreover, our finding of significantly higher level of CD40 and MHCII expression in monocytes/macrophages of the lung at 72 hr provide evidence of strong maturation of these sentinel immune cells (Figure 4.11B). Flow cytometry of lung tissues at the 72 hr time-point demonstrated markedly higher infiltration of T cell subsets ($CD4^+$ and $CD8^+$ T cell combined) in the CpG-ODN group (Figure 4.11C). The histological examination of lung showed higher infiltrations of immune cells in the CpG-ODN group (Figure 4.12B and D) compared to control (Figure 4.12A and C). Altogether, CpG-ODN mucosal delivery was able to accelerate immunological development in neonatal broilers by orchestrating the enrichment of immunological niches in the spleen and the lung of neonatal chicks.

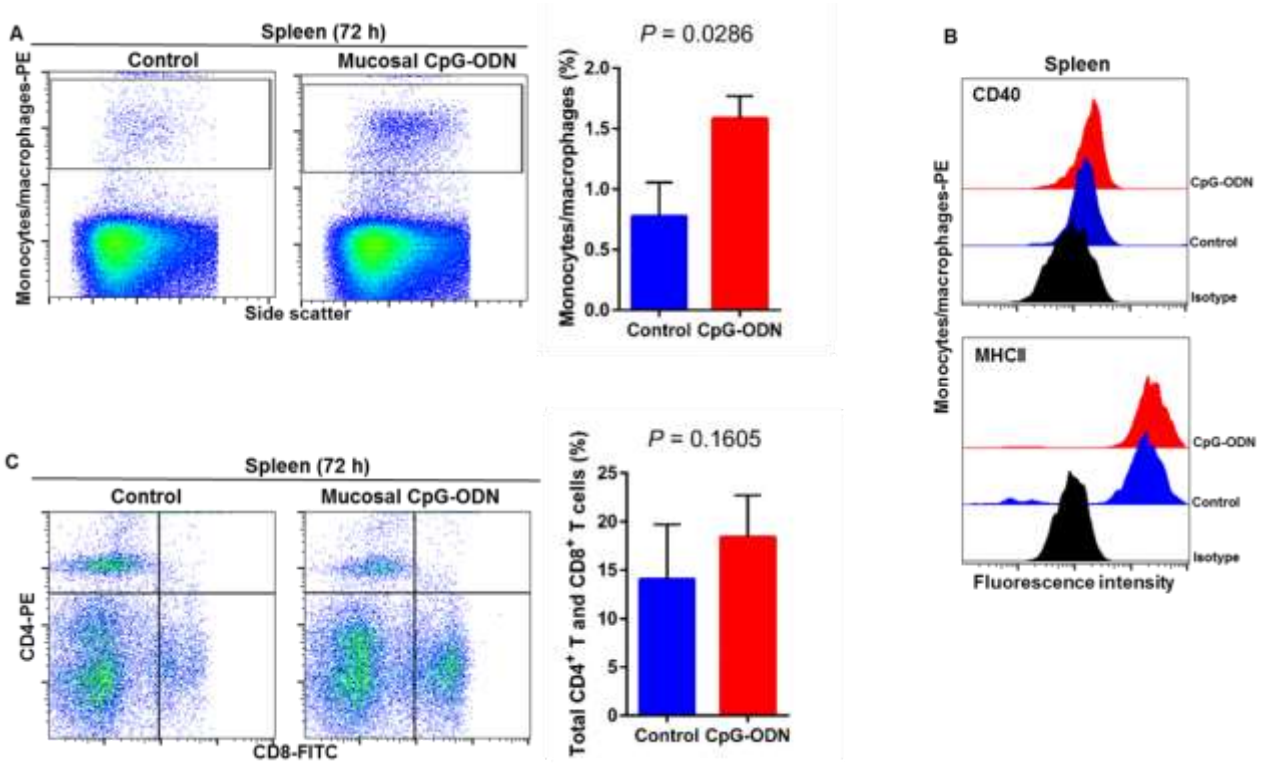

Figure 4.10 Flow cytometric analysis of spleen cells at 72 hours post intrapulmonary CpG-ODN or distilled water treatment. A. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the spleen following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with $P < 0.05$ being statistically significant. B. Histogram panel displays the MFI of CD40 and MHCII expression on the APCs. C. T lymphocyte population in the spleen analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8 monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the spleen following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with $P < 0.05$ being statistically significant.

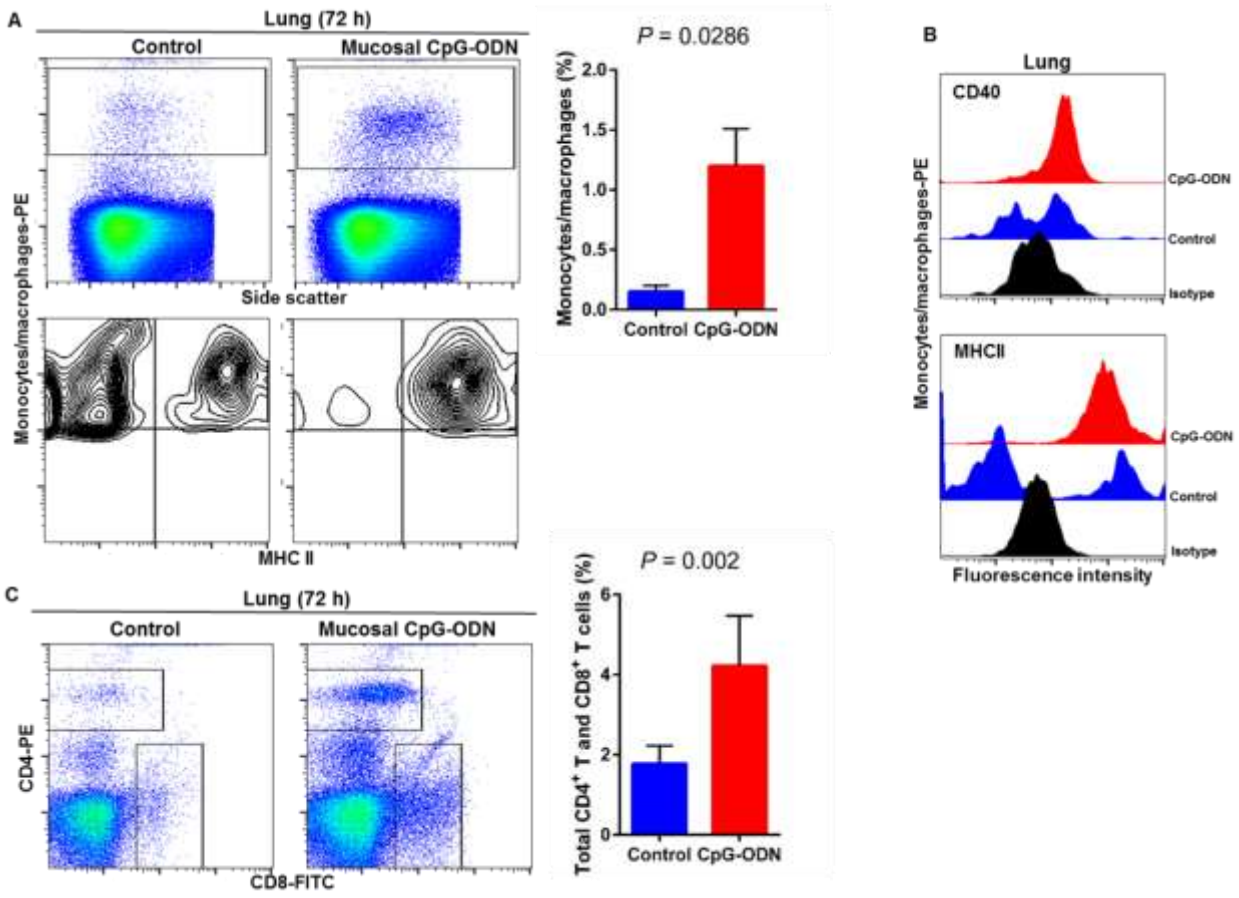

Figure 4.11 Flow cytometric analysis of lung cells at 72 hours post intrapulmonary CpG-ODN or distilled water treatment. A. Macrophage analysis was performed by gating on monocyte/ macrophage population based on forward and side scatter. Bar diagram indicates the mean monocyte/ macrophage APC percentages in the lungs following IPL CpG-ODN treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant. B. Histogram panel displays the MFI of CD40 and MHCII expression on the APCs. C. T lymphocyte population in the lung analysed by gating on the lymphocyte population based on forward and side scatter plot. Quantification of CD4+ and CD8+ T lymphocytes was done by using PE-labeled mouse anti-chicken CD4 and FITC-labeled mouse anti-chicken CD8 monoclonal antibodies. Bar diagram indicates the mean T lymphocyte percentages in the lungs following IPL CpG-ODN or DW treatment. SEM is indicated by the vertical line and horizontal bar, (n=3). Two-way ANOVA with Bonferroni post-hoc analysis was conducted to compare means of APC percentages in the spleens of CpG-ODN administered group vs DW control group, with P <0.05 being statistically significant

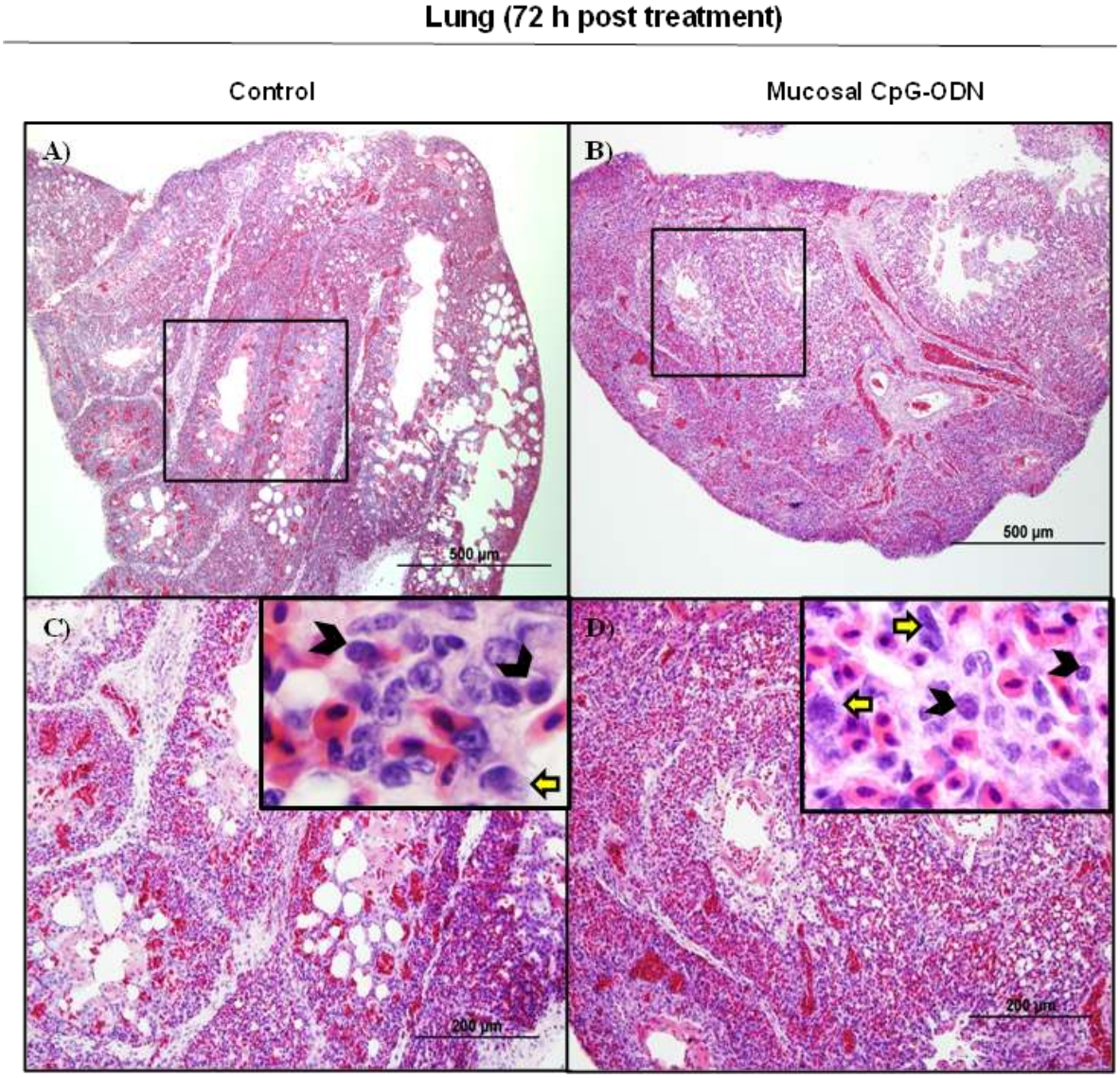

Figure 4.12 Histology of lungs 72 hours following intrapulmonary distilled water (A and C) and intrapulmonary CpG-ODN (B and D) delivery. At lower magnification (10X), the DW control lung showed slightly less cellularity and more air spaces (A) compared to the CpG-ODN treated lung appearing compact and more cellular (B), although the contrast was less striking 72 hours post treatment. At higher magnification (20X and 100X), the IPL CpG-ODN treated lung (D) contained visibly more lymphocytes (black arrow heads in the insert) and large mononuclear cells resembling monocytes/ macrophages (yellow arrows in the insert) compared to the DW control lungs (C).

4.5 Discussion

The chicken industry worldwide, including Canada, is trying to come up with some suitable strategies as alternatives to antibiotics to mitigate antimicrobial resistance development (86,120,231,238). In poultry, most bacterial infections occur during the first-week of life with devastating impacts on production (142). In quest of an alternative to antibiotics, our laboratory

pioneered the use of CpG-ODN alone as an immune protective agent against bacterial infections in chickens (127,128,395). We have demonstrated that CpG-ODN protects chickens against E. coli (126,128) and S. Typhimurium infection (395). In mice, CpG-ODN has been able to control E. coli (349) and Helicobacter pylori infections (336), successfully indicating its antibacterial efficacy across species. In chicken, CpG-ODN has been applied using several routes of delivery such as in ovo (142,263), IM (127) and SQ (128) routes. The lung is a major site of entry for bacterial and viral pathogens in the chicken. We recently used mucosal delivery of aerosolized CpG-ODN alone through IPL route, found that the antibacterial protection kicks off as soon as 6 hrs post mucosal delivery of CpG-ODN and remains effective at least for 5 days in neonatal chicks (128). Also in this study, we found significant protection in chicks following mucosal delivery of CpG-ODN indicating the effectiveness of mucosal route in inducing protective immunity against bacterial infections. Our mucosal delivery of CpG-ODN directly targeted the lung. Therefore, the current study was designed to understand the mechanisms by which the mucosal delivery of aerosolized CpG-ODN alone provides protection against E. coli septicemia. We were interested to investigate cytokine responses and cellular infiltration levels at the site of CpG-ODN treatment that is the lung, to understand the local immune modulatory effects and also the spleen as a secondary immune organ to examine the systemic immune response.

When a bacterial pathogen enters the body, pathogens are sensed by specific pattern recognition receptors, predominantly TLRs, which recognize PAMPs such as LPS, flagellin and unmethylated CpG motifs. This recognition leads to the secretion of pro inflammatory cytokines like IL1β and IL6 as well as chemokines that attract phagocytic heterophils and macrophages to the site of infections as a response (438). Since synthetic CpG-ODNs mimic bacterial unmethylated CpG motifs, similar innate and adaptive downstream mechanisms are expected. Using in vitro experiments, He et al. showed that CpG-ODN stimulates significant amounts of NO by chicken monocytes (159) as well as pro-inflammatory cytokine mRNA IL1β and IL6. At the same time, significant induction of chemokine mRNA such as IL8 and MIP1α within 2 hrs post treatment was observed in chicken monocytes indicating their potential role in trafficking leukocytes to infected sites (161). Previously, Patel et al. demonstrated that in ovo delivered CpG-ODN induced predominantly a Th1 type of immune response in the spleens of chicks (321). However, a previous report (397) and our recent study (139) demonstrated expression of both Th1 and Th2 types of cytokines in chickens in response to CpG-ODN treatment.

In this study, we investigated the expression levels of 8 cytokine genes; pro-inflammatory cytokines (IL1β, IL6, LITAF, IL18), Th1 type (IFNγ, IFNα) and Th2 type (IL4, IL10) in order to understand the pathways of anti-bacterial effects following mucosal delivery of CpG-ODN via the IPL route. For exploring the cytokine mRNA expression, we used QuantiGene Plex 2.0® assay technique; a multiplex assay that measures the expression levels of multiple cytokine genes in a single well using probe hybridization technique and without polymerase chain reaction amplification. Our results indicated that all cytokines noticeably increased in both lungs and spleens following CpG-ODN administration. In our study, we observed upregulation of both Th1 and Th2 cytokines in the lungs and spleens following mucosal delivery of CpG-ODN. This observation agrees with our recent data that demonstrated both Th1 and Th2 cytokine expression following in ovo delivery of CpG-ODN in eighteen days old embryonated eggs (139). Another study also supports the finding that CpG-ODN induces both Th1 and Th2 types of cytokines in chickens (397). Both IL4 and IL10 are anti-inflammatory cytokines (13). In our study, IL10 was upregulated at significant levels, both in lungs and spleens whereas IL4 was significantly elevated in the lungs. This finding could suggest that both IL4 and IL10 are possibly involved in controlling potential inflammatory damage during mucosal delivery of CpG-ODN. Type II IFNs (IFNγ) is an important cytokine in the Th1 type immune response and it plays a vital role in controlling viral and intracellular bacterial infections. IFNγ activates macrophages and thereby increases phagocytosis and the production of potent antimicrobial products such as N), oxygen free radicals and hydrogen peroxide. Furthermore, IFNγ helps naïve CD4+ and CD8+ T lymphocytes to become mature Th1 effector cells to combat pathogens in cell mediated immunity (370). It was reported that recombinant chicken IFNγ treatment during an E. coli infection increased phagocytes in lungs and blood of chickens with more MHCII bearing cells in the air sacs and elevated IL6 levels (185). High levels of IFNγ is associated with a superior antiviral response through CD8+ T cells and NK cell activation. This antiviral property has been highlighted in many studies that used CpG-ODN against viral pathogens (34,35). A study that used chicken Harderian gland cells demonstrated that CpG-ODN could induce IFNγ secretion indicating its potential of using in mucosal vaccines (62).

Other studies using in ovo delivery of CpG-ODN in chickens indicated elevated levels of both IFNγ and Type I IFN; IFNα in the spleens representing a Th1 type immune response (321,359). In our study, we identified a statistically significant upregulation of IFNγ levels

particularly in the lungs within 24 hrs of treatment as well as noticeably higher levels in the spleens following mucosal delivery of CpG-ODN. The statistically higher levels of cytokine gene expressions in the lung could be due to the direct delivery of CpG-ODN in lungs. IFNα has been greatly identified as a potent antiviral agent against a multitude of viral pathogens in chickens (363). An in vitro study demonstrated that IFNγ with IFNα synergistically enhanced antiviral effects and NO production in a chicken macrophage cell line (372). We observed statistically significant upregulation of IFNα mRNA in the lungs 24 hrs post CpG-ODN treatment. In agreement with previous studies (321,359), both IFNα and IFNγ were upregulated following mucosal delivery of CpG-ODN in our study.

During an infection, pro-inflammatory cytokines secreted from phagocytic and non-immune cells of the tissue elicit local and systemic immune responses. Cytokines IL1β, IL6 and IL12 together with TNFα act synergistically to eliminate the infection (213). Our study indicated that mucosal delivery of CpG-ODN promoted the upregulation of pro-inflammatory cytokine genes; IL1β, IL6, LITAF and IL18 significantly in the lung and spleen. Overall the cytokine gene expression was significantly higher in the lungs compared to the spleens which may suggest that CpG-ODN is a potent mucosal immune stimulator when delivered through the IPL route. Our results support our recent study (136) and other reports of CpG-ODN-mediated induction of pro-inflammatory cytokine genes in various organs (32,349,368) as well as in avian thrombocytes (372). We observed a remarkable upregulation of IL1β mRNA expression which was many fold higher than the expression of other cytokine genes. IL1β is an important pro-inflammatory cytokine secreted largely by macrophages as well as NK cells, B lymphocytes, dendritic cells, fibroblasts and epithelial cells. Its major function is recruitment of effector immune cells to the site of infection. It initiates macrophage activation and NO production thereby promoting microbial killing (21). In a study that used tracheal organ cultures, it was shown that CpG-ODN treatment stimulated tracheas to produce elevated levels of IL1β and the supernatants were able to stimulate macrophages to produce NO (32). A study on human Leishmaniasis indicated that IL1β signalling is crucial for the activation of macrophages and NO mediated intracellular parasitic killing (240). IL1β contributes to inflammation by increasing vascular permeability and expressing endothelial adhesion molecules (292). Furthermore, IL1β is known to stimulate the release of other pro-inflammatory cytokines such as IL6 and TNFα (292).

IL6 is well known for being synthesized during an acute infection and providing immune protection via synthesis of acute phase proteins, haematopoiesis and differentiation of naïve T lymphocytes (389). Elevation of IL6 mRNA expression within the first 24 hrs post CpG-ODN treatment in our study, particularly in the lungs, suggests its contribution to the immunological defense against *E. coli* infection. We observed statistically significant elevation of IL18 mRNA gene expression in both lungs and the spleen of the CpG-ODN treated birds. As one of the important pro-inflammatory cytokines, IL18 is identified as the driving force of Th1 type immune response (88). IL18 mainly induces IFNγ production from T cells and NK cells which is important in antimicrobial defense. Structurally and chemically similar to IL1, IL18 promotes the gene expression and synthesis of TNF, IL1, Fas ligands and certain chemokines (88). The orthologue of mammalian TNFα, chicken LITAF, is primarily secreted by macrophages (344). It is noteworthy that LITAF and IL18 remained upregulated in both lungs and spleen for 48 hr following CpG-ODN treatment. Importantly, out of all the cytokines tested here, LITAF and IL18 expression again upregulated at day 7 post-treatment in lungs. It was reported earlier that IL18 promotes monocytes TNFα production (71). TNFα is a multifunctional cytokine that induces upregulation of CD40 and other costimulatory molecules in antigen presenting cells such as macrophages and dendritic cells (364). Mammalian TNF is important in the control of intracellular bacterial infections as well as initiation of inflammation. In agreement with our recent study that used *in ovo* (embryo injection) delivery of CpG-ODN, the present data using mucosal delivery of CpG-ODN also highlights the importance of LITAF in CpG-ODN-induced immunoprotective mechanisms.

We next explored the recruitment kinetics of immune cells using flow cytometry in the non-lymphoid target organ, that is lung, as well as in the lymphoid organ, spleen, following mucosal delivery of CpG-ODN via the IPL route. Flow cytometry data revealed a significant increase in the number of monocytes/macrophages in the lungs and spleen at all time-points studied (24-72 hrs). At the same time, we examined the kinetics of T lymphocytes in the spleen and lungs. We observed a significantly increased level of both $CD4^+$ and $CD8^+$ T lymphocytes in both lungs and spleen (except at the 72 hr time point in spleen) after mucosal delivery of CpG-ODN. The histological examination of the lung showed increased cellularity with monocytes/macrophages and lymphocytes infiltration following CpG-ODN mucosal delivery.

These flow cytometry and histological data strongly suggested the enrichment of these organs as immunological niches.

The occurrence of costimulatory molecules, such as CD40, CD80 and CD86, is a sign of macrophage activation and maturation (114). CD40 signalling is involved in the maturation of APCs such as macrophages and DCs (159) . DCs licensing via CD40 signaling facilitates $CD8^+$ T-cell priming (358) thereby induces protective $CD8^+$ cytotoxic T cell (CTL) immunity (255). In our recent study, we found upregulation of CD40 but not CD80 or CD86 in monocytes/macrophages following *in ovo* administration of CpG-ODN (136). Therefore, in this study we investigated the expression of CD40 on monocytes/macrophages in spleens and lungs of CpG-ODN treated and control groups. We found significant upregulation of CD40 in monocytes/macrophages in lungs throughout our study (24-72 hours). In contrast, CD40 expression in the splenic monocytes/macrophages of control and CpG-ODN treated groups was not different, even after 48 hours post CpG-ODN mucosal delivery when the greatest potential effect was anticipated. Nonetheless, at the 72 hrs, CD40 was significantly upregulated in the splenic monocytes/macrophages of CpG-ODN group. These data suggest that although monocytes/macrophages infiltration in spleen significantly increased throughout our study (24-72 hrs), monocytes/macrophages maturation, as evidenced by CD40 expression, was delayed in the spleen. The differences in CD40 expression kinetics between lungs and spleen could be explained by the fact that CpG-ODN was directly delivered to lung mucosa. Interestingly, these flow cytometry and cytokine data indicate that mucosal delivery of CpG-ODN micro droplets via the IPL route activates targeted tissue as well as influences distant secondary organs such as spleen, demonstrating a systemic effect. Lung acts as the most common site of entry for a majority of pathogens of young chicks before they disseminate in the body (336). The number of lung macrophages in control chicks was significantly low, which explains why these young chicks are so susceptible to infections during early neonatal life. A significant upregulation in the number of macrophages in the lungs of chicks after CpG-ODN exposure suggests that these young chicks are better equipped with sentinel cells to combat pathogenic insults in the barn environment. Thus, increasing the availability of antigen presenting cells in the lung through mucosal delivery of CpG-ODN plays a vital role in inducing antibacterial immunity.

A previous study reported that a major development of the avian spleen begins after hatching with the exposure to various antigens (182). Our data showed that mucosal delivery of

CpG-ODN stimulated and created immunological niches in the lung and the spleen thus accelerating the immunological development. In a human phase I trial, IM injection of CpG-ODN caused infiltration of T lymphocytes at the injection site wherein the secretion of chemokines by the activated APCs provided chemoattractant to bring more lymphocytes to the site (146). Our recent data (136) and a study by De Silva *et al.* reported the infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in to the lungs following *in ovo* delivery of CpG-ODNs (81). In our present study, we observed a significant upregulation of IL-1β mRNA in the lungs and spleens of the birds treated with CpG-ODN in the IPL route. Being a strong chemoattractant, IL-1β upregulation could support increased monocytes/macrophages and T lymphocytes infiltration in these organs. Moreover, we found rapid and significant upregulation of LITAF in lungs and spleens that can also potentially induce infiltration of immune cells, which is also supported by a previous study showing TNFα promotes infiltration of lymphocytes and other immune cells to the site of inflammation (21). A previous study reported that TNFα stimulates MHCII expression in macrophages (269). Our finding of enhanced MHCII expression in monocytes/macrophages in the lungs and concurrent significantly increased LITAF expression in the lungs suggest important roles of these cytokines in CpG-ODN-mediated antibacterial immunity.

In conclusion, our study has demonstrated for the first time that, mucosal delivery of CpG-ODN micro droplets via intrapulmonary route, protects against *E. coli* infections by enhancing multifunctional cytokine expression and by accelerating immunological development by enriching immunological niches not only locally in the target organ (lungs) but also at distant organs such as spleen, resulting in the augmentation of immunocompetence in neonatal chicks.

PREFACE TO CHAPTER 5

Metabolomics analysis is a novel tool in "omics" research that explores the changes occurring in the small molecular metabolite level in the body upon various insults such as infections, toxicity and immunization etc. (64,65,425). In fact, metabolomics profiling could assists us to identify the earliest markers of various bodily changes such as disease processes (65). At the same time, metabolomics analysis is currently being used to understand immune cell functions and predict immunological mechanisms. It has been recently identified that APC such as DC and macrophages undergo metabolic reprogramming during pro-inflammatory stimulation, by switching to glycolysis and away from oxidative phosphorylation in order to promote the activation of T lymphocytes (193). The different metabolic pathways chosen by the immune cells and the involvement of various metabolites have been found to influence the immunological outcomes of these cells (315). As discussed, CpG-ODN administration induced immunomodulatory changes particularly in the innate immune system that resulted in protective antibacterial immunity against a multitude of bacterial pathogens. We were able to prove this significantly protective antibacterial efficacy by administering CpG-ODN in IM (125), SQ (126), *in ovo* (137,382) and IPL (128) routes. Now that we have partially understood the cytokine gene expression profiles and the immune cell activation and infiltration profiles corresponding to this protection, thus, we were keen to apply metabolomics analysis tool to unravel novel mysteries on the small molecular metabolic markers that predict the immunological outcomes. Although few studies had viewed the normal metabolome of the chicken (232), there were no studies conducted on the immunometabolism in this species. As a result, we utilized the metabolomics analysis technique to explore the metabolomics profile changes resulting from immunostimulatory CpG-ODN administration in neonatal broiler chickens. Chapter 5 discusses the interesting findings of the CpG-ODN mediated immunometabolic profile in chickens investigated for the first time in the literature.

CHAPTER 5 THE METABOLOMIC LANDSCAPE OF ANTIMICROBIAL IMMUNITY IN NEONATAL BROILER CHICKS INDUCED BY CPG-ODN MOTIFS

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Contribution: Dr. Gomis conceptualized the idea to perform metabolomics analysis and I designed the experiments accordingly. All the members of the research groups assisted to collect samples. Dr. Mandal from The Metabolics Innovation Centre (TMIC) processed the samples and performed NMR analysis under the supervision of Dr. Wishart. Dr. Karu from TMIC conducted the metabolomics data analysis and produced the graphical illustrations and tables. I wrote the first draft of the manuscript and Dr. Ahmed assisted to add more details. Dr. Karu's feedback

was taken into consideration to make necessary changes. All the authors mentioned above contributed to the manuscript by providing their feedback.

5.1 Abstract

CpG-ODN has been identified as immunostimulatory in various species including chickens. Recently, we demonstrated that injection of CpG-ODN to18-days old embryonating eggs (*in ovo*) and IPL delivery to neonatal broiler chicks could induce immunity against lethal bacterial septicemia. The immunological events following CpG-ODN administration are mostly being studied by exploring cytokine and cellular responses. Metabolomics analysis is a novel tool in omics studies which can assist to understand fundamental immunological changes at the level of small metabolites. The immunomodulatory response happening at the metabolite level upon CpG-ODN mediated immunomodulation is largely unknown in any species. As a result, the following experimental study was designed to discover the metabolomics changes in serum and lungs upon CpG-ODN administration in neonatal broiler chicks. We administered CpG-ODN to day old broiler chicks via IPL and IM routes. A negative control group was administered IPL DW. In each group, chicks (n=40) were challenged with a lethal dose of *E. coli*, 2 days post CpG-ODN administration. CpG-ODN administered chicks had a significantly higher survival ($P<0.05$), significantly lower CCS ($P<0.05$) and lesser bacterial loads compared to the DW control group. Parellely, serum and lung metabolomics profiles were analysed 24 hours post-treatment from 20 chicks/group. The CpG-ODN treated birds could be distinctly distinguished from DW controls due to the change in metabolite levels. The variable importance in projection (VIP) plot showed that 17 metabolites contributed highest to the separation. The significant metabolites that contributed to the difference were L-proline, choline, L-threonine, cytidine, hypoxanthine, acetic acid, betaine, uridine, 2-hydroxy butyrate, L-lysine and myoinositol. We identified multiple strong associations between certain metabolites in serum and differences between groups indicating the effects of treatment and route of administration. Pathway enrichment analysis showed multiple metabolites differentiating between groups and key metabolites involved in energy metabolism. Projection of these metabolites into metabolic pathways indicated their levels changing distinctly upon CpG-ODN administration. Our study revealed that metabolomics analysis could be a valuable tool to understand immunological changes in chickens through the changing levels of small molecular metabolites.

5.2 Introduction

The innate immune system in the animals functions the first line of host defense against infectious agents. The host innate immunity rely on a set of PRR, such as TLRs, which sense pathogens by recognising PAMPs (215). Microbe-derived lipopeptides, lipoteichoic acid, LPS, proteins (flagellin, profilin) and nucleic acids [single stranded (ss) RNA, double stranded (ds) RNA, unmethylated CpG DNA] are some of the well-known PAMPs (TLR ligands). Following the PRR-PAMP interactions, cell signaling cascades ensue that mount immune responses, ultimately leading to the development of adaptive immunity against the invading pathogens (7). Several studies have used TLR agonists as immune modulators (260). CpG-ODNs are synthetic, short, single stranded oligodeoxynucleotides up to 22 base pairs (208). Their action is similar to their bacterial counterparts which are CpG motifs in the bacterial genome. They act as PAMPs to elicit a danger signal and thereafter an innate immune response generates upon binding to its specific TLR. TLR9 and TLR21 recognize CpG-ODNs in mammals and avians, respectively. Chicken TLR21 and human TLR9 recognize CpG-ODNs containing GTCGTT motifs and both have similar intracellular localization, signaling cascades, and cytokine induction patterns (46,47,149,192,219,434). CpG-ODNs have great potential as immunotherapeutic agents and vaccine adjuvants against infections and cancer (42,148,363,441). Several studies in human (4,271,421), mice (59), cattle and sheep (299), fish (185), and chickens (72,125,382) reported that CpG-ODNs initiate immune responses by activating immune cells and inducing cytokine secretion (148). Our laboratory reported first that standalone CpG-ODN treatment can provide protection against bacterial infections in chickens (126). We reported that CpG-ODN administration protects chickens against *E. coli* (125,128,137) and *S.* Typhimurium infections (381). Other studies have also demonstrated antimicrobial function of CpG-ODN against *S. enteritidis* infections (155,256). Regardless of recent advances, the mechanism through which CpG-ODN alone provides protection against bacterial infections remains poorly understood.

Several recent studies in human and mice have suggested that energy metabolism significantly regulates immune cell fate and functions (58,134,316). Macrophages and DCs (the sentinel cells of innate immunity) were shown to have increased glucose metabolism (181) and increased expression of the glycolytic enzymes glucose-6-phosphate dehydrogenase and hexokinase (294). Naïve resting T lymphocytes utilize oxidative phosphorylation for adenosine triphosphate generation, whereas, aerobic glycolysis and glutaminolysis are the main methods of

energy generation in activated T lymphocytes (90,111,375,416). It was reported that activation of TLR4 by bacterial LPS in neutrophils increases glucose consumption (143). Cells stimulated via PRR-PAMP interactions undergo intense metabolic changes, which is important not only for the signalling processes but also for biosynthesis and energy production (193). Despite the potential interest in the involvement of the metabolic changes in the effects of CpG-ODN, this is still a less explored area of research (193).

In the present study, we hypothesized that CpG-ODNs potentially regulate metabolic pathways in chicken which contribute to the development of antimicrobial immunity. Antimicrobial immunity in chickens as induced by IM administration of CpG-ODN has been the gold standard against several bacterial pathogens (125,126,137,382). We recently found that IPL delivery of CpG-ODN induces a dose dependent protection in chickens against bacterial infections (128). To gain greater insights into CpG-ODN-mediated antimicrobial immunity, we compared serum samples by hydrogen-1 nuclear magnetic resonance (^{1}H NMR) metabolomics analysis. Serum metabolic profiles in chicken treated by IM versus IPL delivery of CpG-ODN were compared to controls who received saline IPL.

5.3 Materials and methods

5.3.1 Housing and maintenance of experimental chickens

This work was conducted with the approval of the Animal Research Ethics Board, University of Saskatchewan, obeying the guidelines of the Canadian Council on Animal Care. Day-old broiler chickens were obtained from a commercial hatchery in Saskatchewan. Groups of chicks were allocated randomly into an animal isolation room at the ACU. Water and commercial broiler feed were provided *ad libitum*. Air from each room was exhausted through a high efficiency particulate filter and non-recirculated intake air was provided at a rate of 15-20 air changes/hr. Air pressure differentials and strict sanitation was maintained in this isolation facility. Broilers were raised at 32 C for the first week of life; thereafter the temperature was decreased 0.5 C per day until a room temperature of 27.5 C was reached. Light was provided for 24 hr/day during days 0 to 2 post-hatch. Darkness was introduced at 3 days post-hatch with 1 hr of dark added daily until 4 hr of darkness was achieved.

5.3.2 CpG-ODN intrapulmonary delivery

The CpG-ODN (TCGTCGTTGTCGTTTTGTCGTT (2007)) was free of endotoxin and produced with a phosphorothioate backbone (Operon Biotechnologies, Inc; Huntsville, AL, USA). Synthetic CpG-ODN was diluted in sterile, DW and delivered by the IPL route. Briefly, the CpG-ODN solution was aerosolized as micro-droplets (particle size of 0.5–5 µm) using a Compressor Nebulizer (705-470) unit (AMG Medical Inc; Montreal, QC, Canada) in a closed 0.036 m^3 acrylic chamber containing 60 birds for 30 min (6mg CpG-ODN/chamber). The control group of birds was aerosolized with DW for 30 min in the acrylic chamber using a similar compressor nebulizer. The temperature was maintained at 28-30 C in the acrylic chamber during administration of CpG-ODN or DW.

5.3.3 *E. coli* culture and animal model

In order to confirm the immune protection induced by CpG-ODN delivery, a parallel *E. coli* challenge study was performed to the birds that were not sampled. According to our previously established animal model, the challenge strain used in this study was a field isolate of *E. coli* from a septicemic turkey (126,128). The *E. coli* belonged to serogroup O2 was nonhemolytic, serum resistant, aerobactin producing and had K1 capsule with type I pili (126). Aliquots of the bacterial isolate were stored at -80 C in brain heart infusion broth (Difco, Detroit, Mich.) supplemented with 25% (wt/vol) glycerol (VWR Scientific Inc., Montreal, QC, Canada). In order to challenge the birds, bacteria were cultured on 5% Columbia sheep blood agar for 18-24 hr at 37 C. One colony of *E. coli* was added to 100 mL of Luria broth (Difco LB broth, Miller, Becton Dickinson and Company; Sparks, MD, USA) in a 250 mL capacity Erlenmeyer flask. The bacterial culture was grown at 37 C for 16-18 hr, shaking at 150 rpm. This stationary phase culture contained approximately $1x10^9$ CFU of bacteria per mL which was then further diluted into saline to the concentration of bacteria required to challenge birds. The *E. coli* challenge dose was confirmed by plating serial dilutions of the diluted culture in duplicate on 5% Columbia sheep blood agar plates, incubating for 18 hr at 37 C then counting the number of colonies.

The *E.coli* challenge study was performed according to the well-established animal model that we documented earlier (125,128). Briefly, on the second day post treatment, the birds in each group were challenged with $1x10^5$ CFU (n=20) or $1x10^6$ CFU of bacteria per bird, SQ in

the neck. They were closely monitored three times a day for the most critical period of 3 days post challenge and 2 times a day thereafter until seven days post challenge. Each bird was observed for clinical signs and a daily clinical score was assigned: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1= depressed, unwilling to move; 1.5 = unwilling to move, may take a drink of water and peck some; 2 = unable to stand or reach for food or water; and 3 = found dead. Birds that received a clinical score of 2 were euthanized by cervical dislocation. At the end of the trial, each bird was given a CCS as a sum of daily clinical scores as previously described (125,128).

When chicks were found dead or euthanized, they were necropsied immediately. Remaining birds were euthanized on day seven post challenge. Air sac swabs were obtained from dead or euthanized birds and cultured on 5% Columbia sheep blood agar following the quadrant streaking method. Bacterial growth on these cultures were recorded as 0 = no growth, few = less than 5 colonies, 1+ = growth of bacteria on quadrant 1, 2+ = growth of bacteria on quadrants 1 and 2, 3+ = growth of bacteria on quadrants 1, 2 and 3 and 4+ = growth of bacteria on quadrant 1, 2, 3 and 4 as reported previously (160).

5.3.4 Metabolomic analysis of serum

5.3.4.1 Sample collection

Twenty chicks from each group were euthanized 24 hr post treatment. Blood was collected into serum tubes by severing the necks of the chicks with a sharp pair of scissors. The blood samples were then centrifuged at 3000 rpm for 15 min and serum was separated into 1.5 mL micro centrifuge tubes. Serum samples were stored at -80 C, transported on dry ice to the metabolomics facility, and stored at -80 C until further analysis.

5.3.4.2 Sample preparation

Serum samples were defrosted on ice and prepared in two batches according to a randomization template. Immediately following the preparation of each sample batch, analysis was conducted.

5.3.4.3 NMR spectroscopy

Plasma and serum samples contain a significant concentration of large molecular weight proteins and lipoproteins which can seriously compromise the quality of ^{1}H-NMR spectra though the generation of intense, broad lines that interfere with the identification and quantification of lower abundance metabolites. De-proteinization can eliminate these peaks. De-proteinization of the serum samples was done by centrifugation and ultrafiltration using 3-kDa cut-off centrifuge filter units (Amicon Micoron YM-3; Sigma-Aldrich, St. Louis, MO), following a previously reported de-proteinization procedure (325). The de-proteinized serum samples (280 μL) were then transferred to a 1.5 mL micro centrifuge tube followed by supplementing of 70 μL standard NMR buffer solution (1 mM DSS (disodium-2, 2-dimethyl-2-silapentane-5-sulphonate), in 10% D_2O). These samples (a total volume of 350 μL) were then transferred to a 3 mm NMR tube for spectral analysis. All ^{1}H-NMR spectra were collected on a Bruker Avance III Ascend 700 MHz spectrometer with a 5 mm cryo-probe (Bruker Biospin, Rheinstetten, Germany). ^{1}H-NMR spectra were acquired at 25 C using the first transient of the noesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy (353). Spectra were collected with 128 transients using a 4 second acquisition time and a 1 second recycle delay.

5.3.4.4 NMR compound identification and quantification

Prior to spectral analysis, all free induction decays were zero-filled to 240 k data points and a line broadening of 0.5 Hz was applied. The methyl singlet of the added DSS served as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H-NMR spectra were processed and imported into the Chenomx NMR Suite 8.1 software (Edmonton, Canada). The Chenomx NMR Suite software allows for quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Specifically, the spectral fitting for metabolite was done using the standard Chenomx 700 MHz metabolite library. Most of the visible peaks are annotated with a compound name. Each spectrum was processed and analyzed by at least two experienced NMR spectroscopists to minimize compound mis-identification and mis-quantification.

5.3.5 Data processing and statistical analysis

Metaboanalyst 3.0 free software (427) was used for statistical analysis of the metabolomics data. Data were log transformed and pareto scaled prior to univariate and multivariate analysis. Principal Component Analysis (PCA) was performed for quality-control measures (data not shown). Partial Least Squares-Discriminant Analysis (PLS-DA) model was used to suggest potential biomarkers for treatment effect. Several univariate analysis tests were employed. ANOVA was conducted to compare metabolite levels between all three groups, with Tukey's HSD post-hoc analysis to indicate significant pairs. Comparison between two experimental groups in terms of fold-change and significance of metabolite difference was done using student's t-test. In all tests, *p* values were further corrected to multiple comparisons by applying false discovery rate (FDR), where a value of 0.1 was considered a significance threshold. Correlations between serum metabolites were tested by Pearson correlation analysis.

Pathway enrichment analysis was performed on log-transformed and auto-scaled data, against *Gallus Gallus* pathway database in Metaboanalyst software. It consisted of ANOVA test with the use of relative-betweeness centrality algorithm for pathway topology analysis.

5.4 Results

5.4.1 Immunoprotective efficacy of intramuscular vs intrapulmonary delivery of CpG-ODN against *E. coli* septicemia

Chicks that received CpG-ODN either through IM or IPL routes or saline controls were challenged with lethal doses (1×10^4 or 1×10^5 CFU) of a pathogenic strain of *E. coli*. During the 7 days of challenge experiments, chicks that received CpG-ODN were significantly protected compared to saline controls (Figure 5.1). We found that CpG-ODN delivery through IM route induced a higher survival against bacterial challenge compared to IPL CpG-ODN delivery. In our previous studies, we have consistently found that, IM CpG-ODN delivery as an efficient method of CpG-ODN delivery to induce protective immunity in chicks. We also calculated the CCS for each chick by summing the daily clinical scores through the seven-day observation period after the *E. coli* challenge. The birds that received CpG-ODN IM or IPL had significantly lower CCS values ($P<0.05$) compared to the DW control (Figure 5.2); the lowest CCSs values were in birds that received IM CpG-ODN. When bacterial isolations were analysed it showed that the CpG-ODN administered birds had visually lower bacterial load in contrast to the DW

control (Figure 5.3). Birds that succumbed to challenge or were euthanatized, had lesions such as pericarditis or airsacculitis or a combination of airsacculitis together with polyserositis or pericarditis.

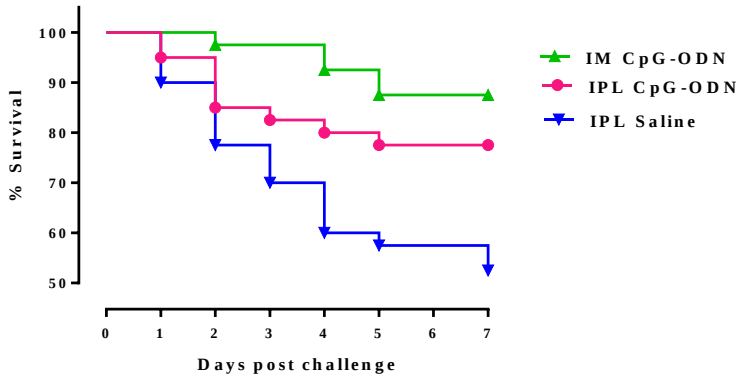

Figure 5.1 Survival percentages of the birds following lethal *E. coli* infection. Birds that received IM CpG-ODN and IPL CpG-ODN treatments showed significantly better survival than the DW control group (P<0.05) over seven days post challenge.

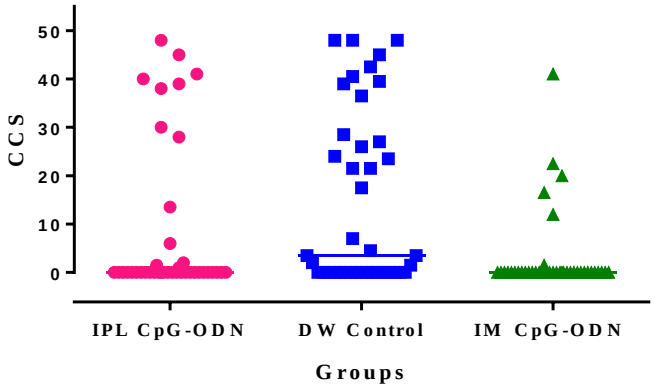

Figure 5.2 Cumulative clinical score values of birds at the end of the trial. Birds that received IM CpG-ODN and IPL-CpG-ODN had a significantly lower CCS value compared to the DW control group (P<0.05) by the end of 7 days post challenge.

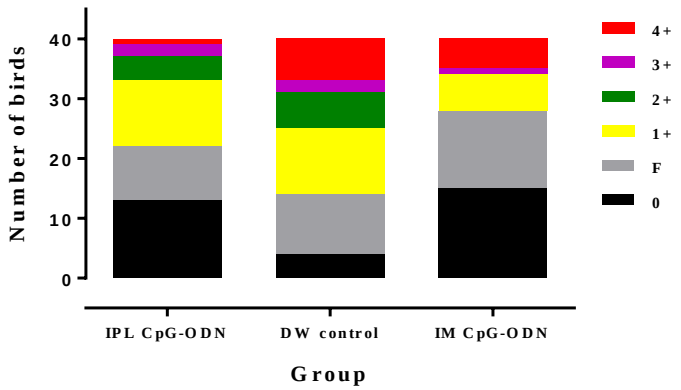

Figure 5.3 Bacterial isolation pattern in each group. IM CpG-ODN and IPL CpG-ODN groups show a visually low bacterial isolation compared to the DW control group.

5.4.2 Effects of CpG-ODN on serum metabolome and correlation of metabolomics shift with the level of antimicrobial protection in chicks

The CpG-ODN receiving birds could be distinguished from DW control birds by level of serum metabolites 24 hr after treatment. When combined, CPG-ODN treated groups (IM + IPL) and the DW control are projected by level of serum metabolites 24 hr after treatment, CpG-ODN group could be distinguished from the saline control on the PLS-DA plot. In the VIP plot, it showed that 17 metabolites contributed highest to the separation (VIP score >1). The significant metabolites that contributed to the difference were L-proline, choline, L-threonine, cytidine, hypoxanthine, acetic acid, betaine, uridine, 2-hydroxy butyrate, L-lysine and myoinositol ($P<0.1$) (Figure 5.4 and Table 5.1). Further, other than 2-hydroxybutyrate, all the significant metabolites were downregulated in the CpG-ODN treated groups 24 hrs post treatment.

Table 5.1 ANOVA and Tukeys HSD *post hoc* analysis displaying p values along with FDR

Metabolite	F value	ANOVA *p* value	FDR – adjusted *p* value	Tukey's HSD
Betaine	21.39	1.18E-07	5.41E-06	IM-DW; IPL-IM
L-Serine	16.23	2.64E-06	6.07E-05	IM-DW; IPL-IM
Tyrosine	15.54	4.1E-06	6.27E-05	IM-DW; IPL-IM
L-proline	15.1	5.45E-06	6.27E-05	IM-DW; IPL-DW
Choline	12.43	3.31E-05	3.04E-04	IM-DW; IPL-DW
L-Alanine	11.44	6.66E-05	4.92E-04	IM-DW; IPL-IM
Ketogenic AA	11.28	7.48E-05	4.92E-04	IM-DW; IPL-IM
L-Threonine	10.44	1.37E-04	7.87E-04	IM-DW; IPL-DW
Glycine	9.99	1.91E-04	9.79E-04	IM-DW; IPL-DW
Cytidine	8.52	5.78E-04	0.003	IM-DW; IPL-DW
TRP/LNAA	8.02	8.55E-04	0.004	IP-DW; IPL-IM
L-Glutamine	7.38	0.001	0.005	IM-DW; IPL-DW
L-Lysine	7.34	0.001	0.005	IM-DW; IPL-IM
Hypoxanthine	6.96	0.002	0.006	IM-DW; IPL-DW
Acetic acid	6.56	0.003	0.008	IM-DW; IPL-DW
Valine	6.07	0.004	0.012	IM-DW; IPL-IM
Methionine	5.69	0.006	0.015	IM-DW; IP-IM
D-Glucose	5.61	0.006	0.015	IM-DW
Creatine	5.6	0.006	0.015	IM-DW; IPL-IM
BCAA	5.33	0.008	0.017	IM-DW; IPL-IM
2Hydroxybutyrate	4.96	0.01	0.023	IPL-DW
Uridine	4.62	0.014	0.029	IML-DW;IPL-DW
Isoleucine	4.5	0.015	0.031	IPL-IM
Aspartate	4.2	0.02	0.038	IPL-DW
Citric acid	4.04	0.023	0.042	IPL-DW; IPL-IM
L-Leucine	3.87	0.027	0.047	IM-DW; IPL-IM
Formate	3.68	0.031	0.053	IPL-DW; IPL-IM
Myo-inositol	3.42	0.04	0.065	IPL-DW
Fumaric acid	3.22	0.047	0.075	IPL-DW
Tryptophan	3.17	0.049	0.076	IPL-DW

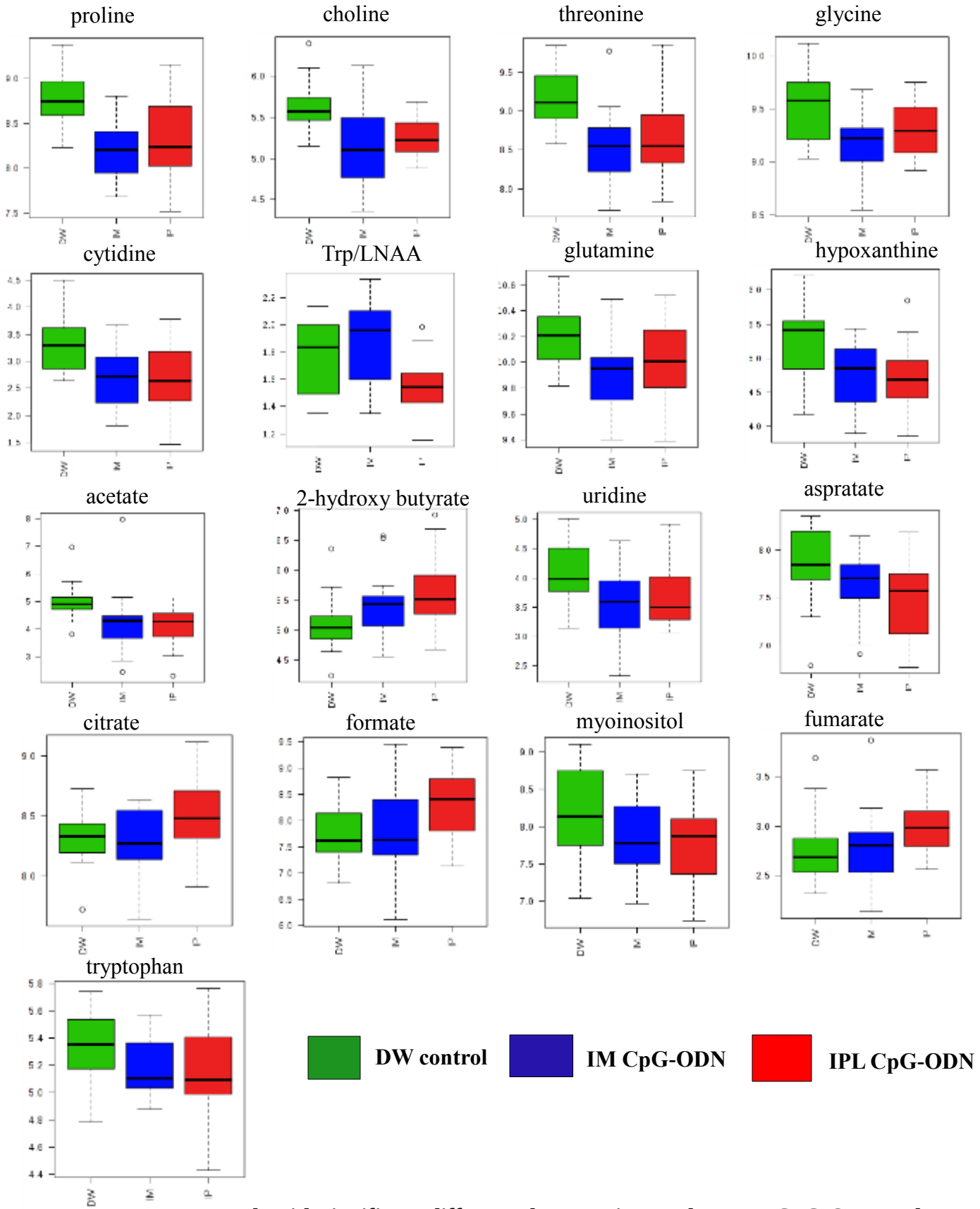

Figure 5.4 Compounds with significant difference between intrapulmonary CpG-ODN and distilled water by univariate analysis (ANOVA).

PLS/DA analysis comparing IPL CpG-ODN group with DW control showed a fine separation apart from two DW control samples (Figure 5.5A). However, the performance parameters of this model were only acceptable (*p* value of 0.15 for 1000 permutations; R2 of 0.7 yet Q2 of only 0.36). Here the major metabolites that contributed to the difference included common metabolites that were projected during the comparison of CpG-ODN treatment collectively with DW control which were acetic acid, cytidine, hypoxanthine, L-threonine, choline, L-proline, uridine and glycine. There were some unique metabolites that only changed between IPL CpG-ODN and DW control. myoinositol, aspartate, tryptophan/large neutral amino acids (TRP/LNAA) were down regulated in IPL CpG birds, while 2-hydroxybutyrate, formate, 3-hydroxybutyrate and fumaric acid were upregulated (Figure 5.5B). Table 5.2 summarizes the significant changes in metabolites between IPL CpG-ODN group and DW control by univariate analysis (*t*- test).

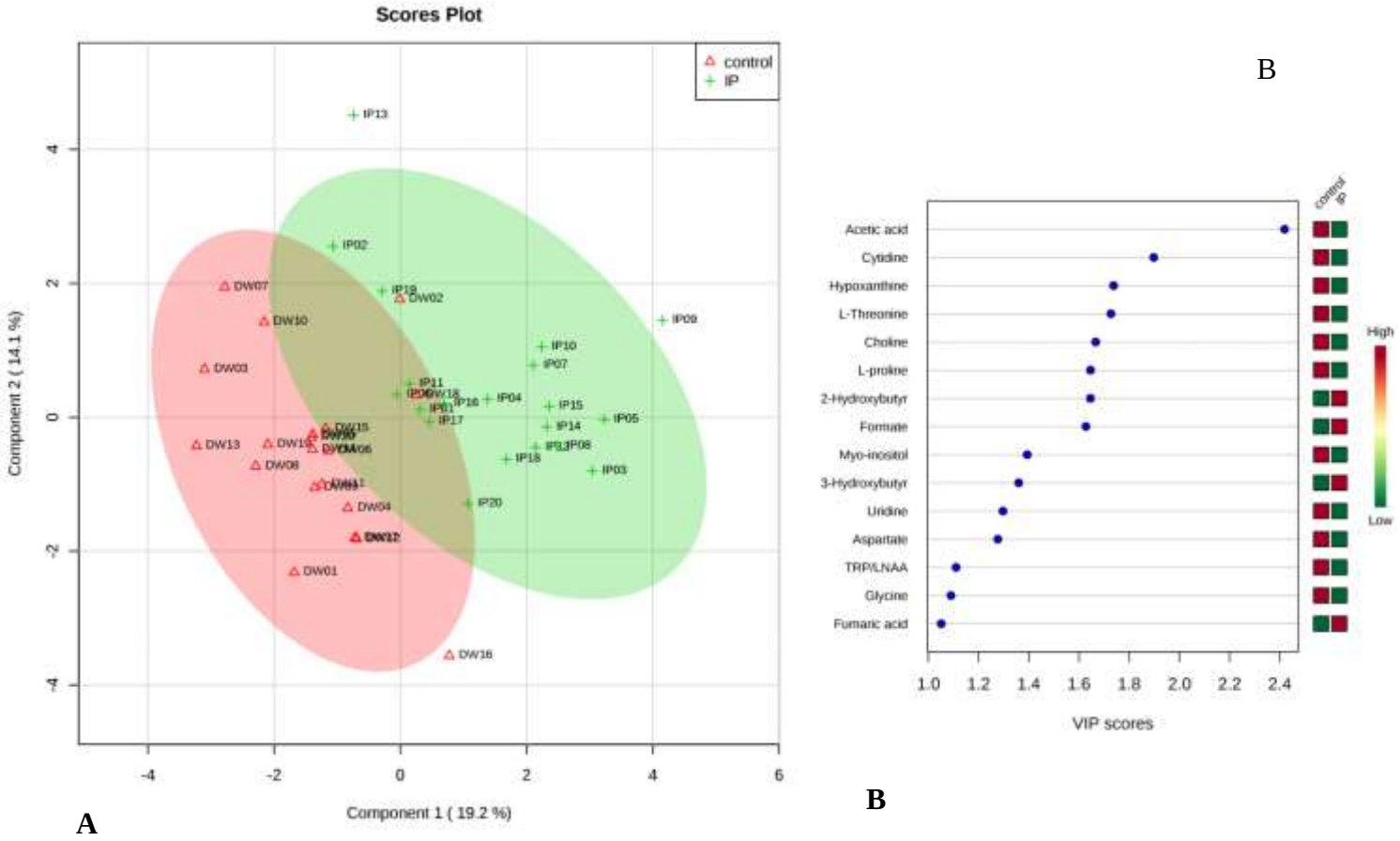

Figure 5.5 PLS/DA analysis of serum metabolites of intrapulmonary CpG-ODN and distilled water control groups (A). VIP score plot of the metabolites that contribute most to the separation between the classes. TRP/LNAA, Tryptophan / large neutral amino acids

Table 5.2 Summary of significant changes in metabolites between intrapulmonary CpG-ODN and distilled water control by univariate analysis.

Metabolite	IPL vs. DW	Fold change	t-test p value	FDR-adjusted *p* value
Choline	down	1.32	3.17E-05	0.001
Acetic acid	down	1.85	1.72E-04	0.004
L-proline	down	1.33	5.26E-04	0.008
L-Threonine	down	1.38	0.001	0.013
Cytidine	down	1.51	0.002	0.015
Hypoxanthine	down	1.48	0.002	0.018
Trp/LNAA	down	1.18	0.003	0.022
2-Hydroxybutyrate	up	1.47	0.004	0.022
Formate	up	1.49	0.006	0.032
Aspartate	down	1.26	0.010	0.041
3-Hydroxybutyric acid	up	1.33	0.011	0.041
Fumarate	up	1.18	0.015	0.054
Myo-inositol	down	1.4	0.019	0.058
Uridine	down	1.29	0.019	0.058
L-Glutamine	down	1.14	0.024	0.069
Citrate	up	1.15	0.035	0.089
Tryptophan	down	1.14	0.035	0.089
Ketogenic AA	down	1.13	0.047	0.11

Direct comparison of the delivery method as well as treatment (IM CpG-ODN vs. DW control) provided a better PLS/DA model (Figure 5.6A). From the VIP scores it is evident that there are common metabolites contributing to the difference between CpG-ODN treatment and DW control as mentioned in the previous comparison (Figure 5.6B). The unique metabolites that were only different between IM CpG-ODN and DW groups were betaine, L-carnitine, L-lysine, L-serine, L-alanine, tyrosine, L-glutamine (downregulated in IM CpG-ODN) and creatine (upregulated in IM CpG-ODN) (Table 5.3).

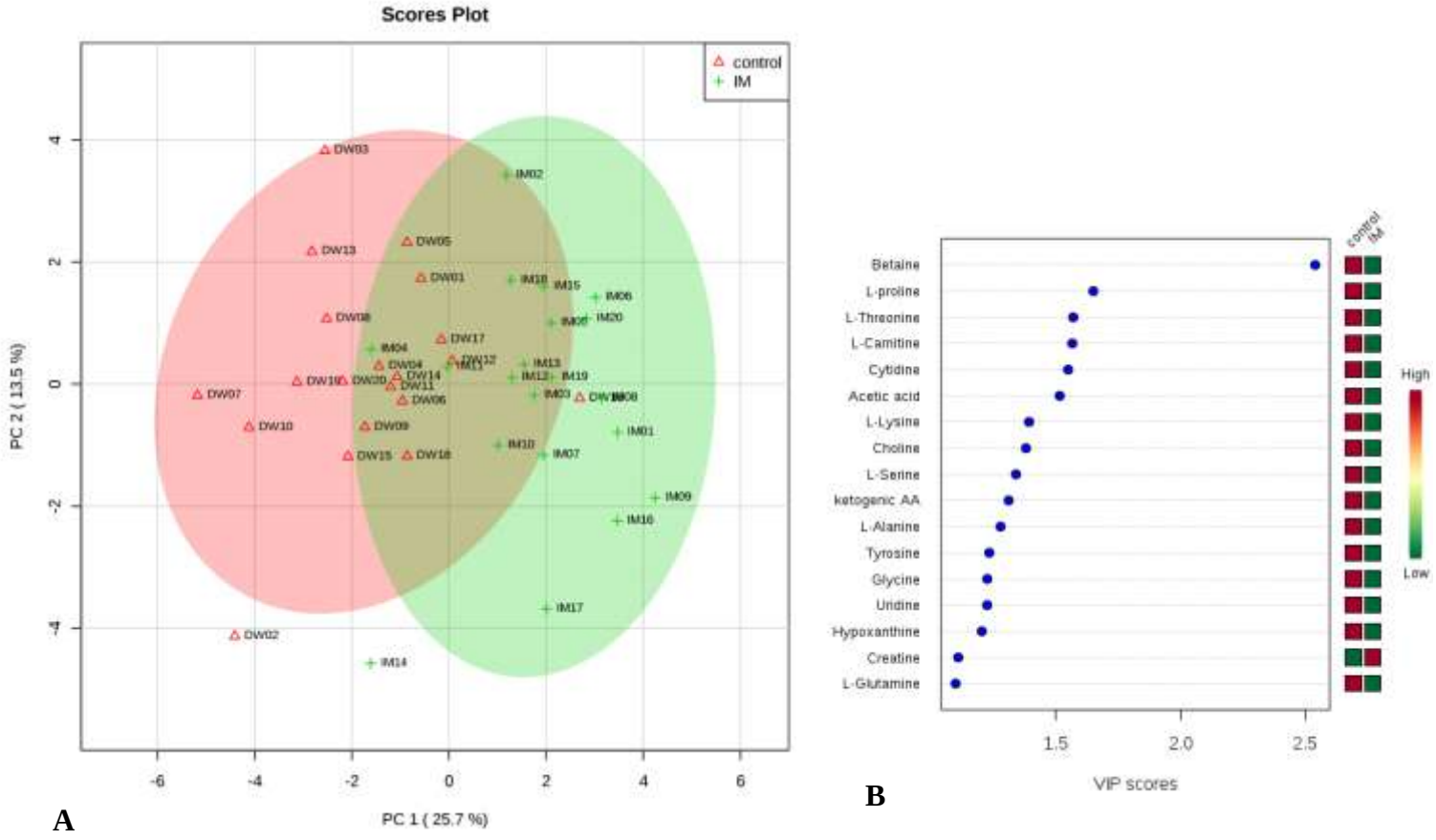

Figure 5.6 PLS/DA analysis of serum metabolite levels in intramuscular CpG-ODN and distilled water control groups (A). VIP score plot (B).

Table 5.3 Significant changes in metabolites between intramuscular CpG-ODN group and distilled water control summarized in the table by univariate analysis.

Metabolite	IM CpG-ODN vs. DW control	Fold change	t-test p value	FDR-adjusted *p* value
Betaine	down	2.42	3.36E-07	1.19E-05
L-proline	down	1.49	5.18E-07	1.19E-05
L-Serine	down	1.36	1.69E-05	2.58E-04
Ketogenic AA	down	1.33	2.64E-05	3.03E-04
L-Alanine	down	1.31	3.41E-05	3.14E-04
L-Threonine	down	1.51	5.29E-05	3.75E-04
Tyrosine	down	1.3	5.71E-05	3.75E-04
Glycine	down	1.32	1.46E-04	8.40E-04
Choline	down	1.38	1.65E-04	8.42E-04
L-Glutamine	down	1.24	1.98E-04	9.10E-04
Cytidine	down	1.59	3.62E-04	0.002
L-Lysine	down	1.49	0.001	0.004
Hypoxanthine	down	1.44	0.005	0.016
Acetic acid	down	1.21	0.008	0.022
Uridine	down	1.37	0.008	0.022
L-Leucine	down	1.25	0.019	0.044
Malonate	down	1.27	0.034	0.072
L-Glutamic acid	down	1.24	0.036	0.072
L-Carnitine	down	1.59	0.038	0.073
2-Hydroxybutyrate	up	1.26	0.043	0.077
Creatine	up	1.54	0.044	0.077

Pearson correlation analysis showed multiple strong associations between certain metabolites in serum and differences between groups indicating the effects of treatment and route of administration (Table 5.4).

Table 5.4 Pearson correlation analysis

Metabolite 1	Metabolite 2	DW only (n=20)	IPL CpG-ODN only (n=20)	IM CpG-ODN only (n=20)	All samples (n=60)
Glucose	Acetone	-0.42	-0.46	ns	-0.43
Glucose	BCAA	0.81	0.75	0.42	0.70
Glucose	Ketogenic amino acids	0.77	0.73	ns	0.69
Glucose	Glycerol	Ns	ns	-0.67	ns
Glucose	Glutamine	0.52	0.67	ns	0.61
Glucose	Leucine	Ns	0.62	0.52	0.70
Glucose	Citrate	0.64	ns	ns	0.47
Glucose/Pyruvate	Tryptophan	Ns	0.63	ns	ns
Glucose/Pyruvate	Lactate	-0.74	-0.42	-0.71	-0.57
Lactate/Pyruvate	Uridine	Ns	0.84	ns	ns
Lactate	Pyruvate	0.65	0.77	0.77	0.7
Citrate	Betaine	Ns	0.65	ns	ns
Citrate	Creatine	Ns	ns	-0.67	-0.42
Citrate	Glutamine	0.66	ns	ns	ns
Citrate	Alanine	0.81	0.51	ns	0.47
Citrate	Lactate	Ns	0.40	0.47	ns
Acetone	Proline	Ns	-0.68	ns	-0.47
Acetone	Leucine	Ns	-0.64	ns	-0.44
Acetone	Alanine	Ns	ns	-0.68	-0.48
Acetone	Malonate	-0.57	ns	ns	ns
3-OH butyric acid	Citrate	0.48	0.52	-0.54	ns
3-OH butyric acid	Fumarate	Ns	0.59	-0.55	ns
3-OH butyric acid	Succinate	Ns	ns	-0.70	ns
3-OH butyric acid	Ketogenic amino acids	0.60	ns	0.66	ns
3-OH butyric acid	Lysine	0.76	ns	0.44	ns
3-OH butyric acid	Isoleucine	0.60	ns	0.72	0.58
3-OH butyric acid	Inosine	-0.71	ns	ns	ns
3-OH butyric acid	Glucose	0.77	ns	ns	ns

Carnitine	Lysine	0.56	ns	ns	ns
Carnitine	Citrate	0.55	0.49	ns	ns
Carnitine	Succinate	-0.53	ns	ns	ns
Carnitine	Betaine	Ns	0.55	ns	ns
Myo-inositol	Dimethylamine	Ns	-0.62	-0.42	ns
Trp/LNAA	BCAA	-0.64	ns	-0.75	-0.56
Serotonin	Ketogenic amino acids	Ns	0.51	ns	ns
Serotonin	Acetic acid	Ns	0.53	-0.50	ns
Serotonin	Fumarae; succinate	Ns	ns	-0.54	ns
Serotonin	Succinate	Ns	ns	-0.57	ns
Serotonin	Lactate	Ns	ns	0.44	ns
Serotonin	Pyruvate	0.48	ns	ns	ns
Serotonin	3-OH butyric acid	Ns	ns	0.44	ns

Pathway enrichment analysis displayed leading serum metabolites differentiating between the experimental groups, including key metabolites involved in energy production and expenditure; glucogenic vs. ketogenic; citrate cycle vs. fatty acid oxidation (Table 5.5). When the metabolites were projected on metabolic pathways, certain metabolite levels related to the energy metabolism pathways were seen changing upon CpG-ODN administration (Figure 5.7). With reference to IPL CpG-ODN administration, we identified an increased formate level while acetate level decreased in the glucogenic pathways. Indicating an impact on the ketogenesis, 3-OH butyrate level increased upon IPL CpG-ODN delivery. Considering metabolites of the citrate cycle, citrate and fumarate levels were increased. Amino acid metabolites such as proline, aspartate, glutamine, uridine and cytidine reduced upon CpG-ODN delivery.

Table 5.5 Pathway enrichment analysis for serum.

Compared classes	Pathway	Hits	P value	FDR	Impact
Control vs. all CpG	Glycine, serine and threonine metabolism	7/33	5.48E-6	1.59E-4	0.59
Control vs. all CpG	Arginine and proline metabolism	6/38	8.58E-6	1.59E-4	0.18
Control vs. all CpG	Pyruvate metabolism	4/23	1.94E-4	0.0014	0.23
Control vs. all CpG	Alanine, aspartate and glutamate metabolism	7/23	8.08E-4	0.0025	0.55
Control vs. all CpG	D-Glutamine and D-glutamate metabolism	2/5	9.67E-4	0.0027	1.0
Control vs. CpG IM	Glycine, serine and threonine metabolism	7/33	8.97E-7	3.32E-5	0.59
Control vs. CpG IM	Arginine and proline metabolism	6/38	9.06E-6	1.11E-4	0.18
Control vs. CpG IM	Alanine, aspartate and glutamate metabolism	7/23	2.89E-4	0.0011	0.55
Control vs. CpG IM	D-Glutamine and D-glutamate metabolism	2/5	5.13E-4	0.0017	1.0
Control vs. CpG IM	Phenylalanine, tyrosine and tryptophan biosynthesis	2/4	0.0012	0.0025	1.0
Control vs. CpG IM	Pyruvate metabolism	4/23	0.0015	0.0027	0.23
Control vs. CpG IPL	Arginine and proline metabolism	6/38	2.83E-4	0.005	0.18
Control vs. CpG IPL	Glycine, serine and threonine metabolism	7/33	4.41E-4	0.005	0.59
Control vs. CpG IPL	Pyruvate metabolism	4/23	0.0010	0.0098	0.23
Control vs. CpG IPL	Glycolysis or Gluconeogenesis	3/26	0.0084	0.0347	0.13
Control vs. CpG IPL	Alanine, aspartate and glutamate metabolism	7/23	0.0130	0.037	0.55
Control vs. CpG IPL	D-Glutamine and D-glutamate metabolism	2/5	0.0276	0.057	1.0
Control vs. CpG IPL	Citrate cycle (TCA cycle)	4/20	0.057	0.095	0.20

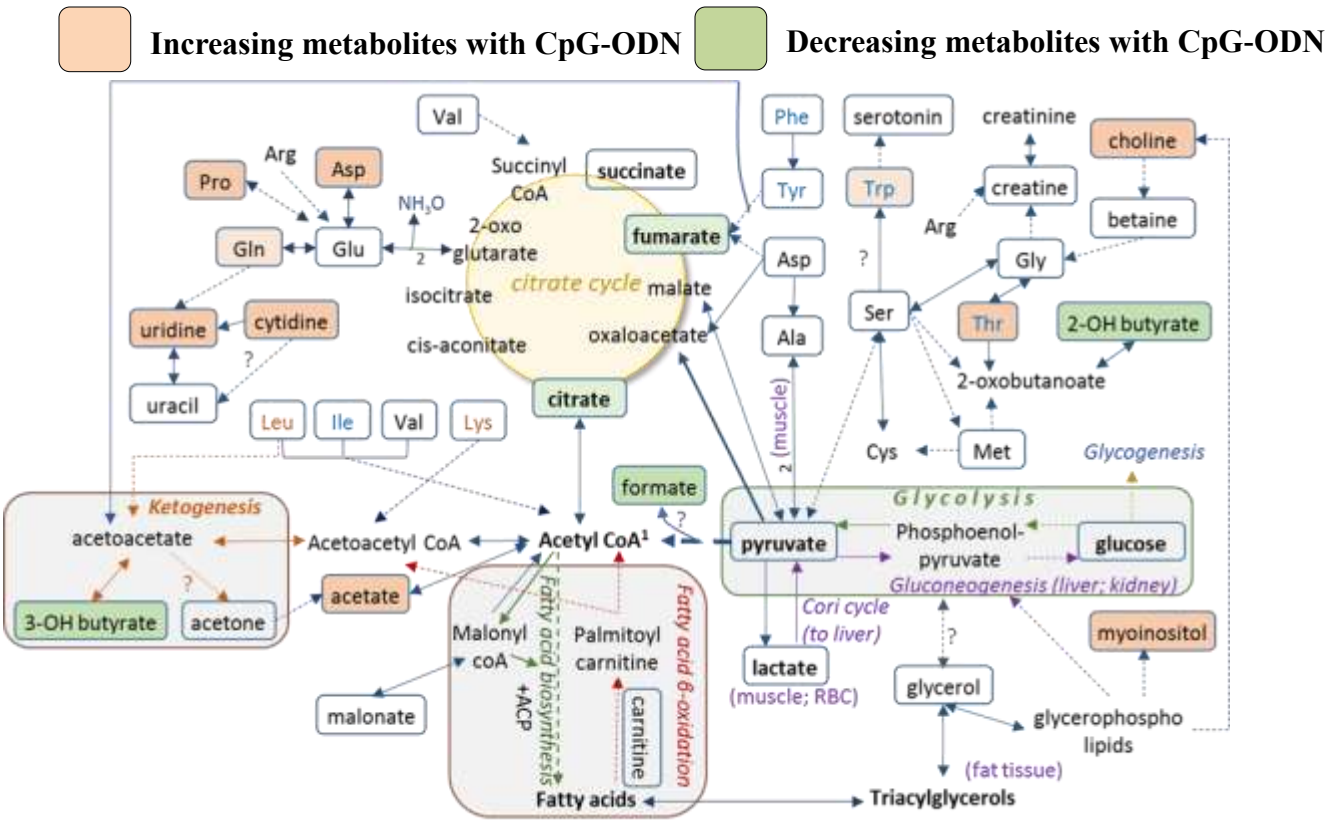

Figure 5.7 Differences in serum metabolites between intrapulmonary CpG-ODN administered birds and distilled water control group

5.5 Discussion

In the present study, we used NMR-based metabolomic approaches to explore the potential of immune-metabolic interactions in the orchestration of CpG-ODN-induced antimicrobial immunity. Thereby we attempted to identify key molecules or pathways associated with immunoprotective phenotypes based on their characteristic serum metabolite profiles. To our best knowledge, this is the first report on serum metabolic profiling for CpG-ODN induced antimicrobial immunity in chickens. A recent metabolomics characterization study provided valuable information about metabolome in various tissues of normal healthy chicken (179,232). However, no data was available related to CpG-ODN induced immune responses in chickens. The present study suggests that immune-metabolic interactions are involved in CpG-ODN-mediated immunity. The study revealed a wide array of differential metabolic signatures in CpG-ODN treated chickens. Significant metabolic changes were found in multiple cellular pathways, including glycolysis, lipid metabolism, and amino acid metabolism. The metabolic network of potential biomarkers is presented in Figure 5.7, which provides an overview of the metabolic pathways changed in chickens following CpG-ODN treatment.

In the present study, chickens treated with intramuscular CpG-ODN were better protected against bacterial infection followed by intrapulmonary group. Interestingly, CpG-ODN treatment regardless of the route of administration caused certain metabolites to change particularly to reduce in serum. However, the level of changes in most of the metabolites was more pronounced in IM group compared to IPL group. It is well known that CpG-ODN causes stimulation of the immune system resulting in pro inflammatory cytokine secretion (313) and activation of immune cells such as monocytes, macrophages and lymphocytes. We recently reported an increased number of mononuclear cells infiltrating into the lungs within 24 hours of IPL CpG-ODN treatment (128). Given that activated immune cells depend on the nutrients (such as amino acids) present in the extracellular environment, and thus due an increased cellular demand during activation serum levels would potentially decrease (253). Since the immune cell activation is certainly energy demanding, thus we hypothesize that the metabolomics changes in serum that we observed in the present study could be the result of increased uptake by cells during immunological responses. In our study, we observed more dramatic decrease in serum amino acids level following intramuscular CpG-ODN treatment, which may suggest an increased utilization by activated cells.

Several studies have reported that amino acids are critical in immune cell proliferation and functions (133). A recent study in mice demonstrated that serine and glycine plays important role T cell proliferation and function (253). In our study, we found significant decrease in the level of amino acids such as serine, glycine, tryptophan, L-lysine etc. and metabolites like betaine and L-carnitine. Tryptophan can be converted into 5-hydroxytryptophan, a serotonin precursor (132,165). Studies have shown that serotonin regulates macrophages function, cytokine secretion, neutrophil recruitment, and T cell proliferation (158). Remarkably, in the present study we found reduced serum tryptophan but increased serum serotonin levels in chicks treated with intramuscular CpG-ODN, indicating that tryptophan has been catabolized resulting in increased serotonin levels. Given the important roles for serotonin in immune modulation (158) and our data of an increased serum level of serotonin in chickens with an enhanced antimicrobial immunity strongly suggest important role for immuno-metabolic interactions in CpG-ODN-mediated immunity.

In our study, we observed that acetate levels in serum were lowered upon the administration of CpG-ODN. Acetic acid, as one of the simplest carboxylic acids, is an integral

member of metabolism. The acetyl group derived from acetic acid bound to co-enzyme A gives rise to acetyl-CoA which is an important metabolite of carbohydrate and fatty acid metabolism (449). Fatty acid oxidation provides energy to the cellular activities when in need through β-oxidation in mitochondria or in peroxisomes. When fatty acid uptake is increased in the liver, it results in ketogenesis where fatty acids undergo incomplete oxidation (295). As observed in the pathways analysis (Table 5.6 and Figure 5.7), lower serum acetate and malonate levels resulting from CpG-ODN administration must be indicating that cells had a sudden need for extra energy so that fatty acids have been started to undergo oxidation to provide that extra energy. At the same time, intrapulmonary CpG-ODN has resulted in increased levels of 3-OH butyrate which indicates ketogenesis to a certain extent. It could be indicating that IPL route is causing a higher energy demand resulting in higher fatty acid oxidation and ketogenesis.

In this study, two important metabolites; choline and betaine, were highlighted throughout this analysis. Choline was reduced in the CpG-ODN treated birds and betaine was particularly low in the birds that received intramuscular CpG-ODN treatment. Choline is a precursor in the synthesis of acetylcholine and phosphoryl-choline, and is an important intermediate of phospholipid metabolism. There is increased consumption of phosphorylcholine under severe oxidative and systemic inflammatory condition (135). Betaine on the other plays an important role in protein and energy metabolism in cells. Choline is oxidized to betaine and both are linked to the folate-dependent one-carbon metabolism (400). Reducing choline levels in our study could also be contributing to the low betaine levels.

Overall, CpG-ODN induced metabolomics data generated by the present study provided a unique resource to identify regulatory molecules or pathways that impart protective immunity in chickens against bacterial infections. The metabolites highlighted in the present analysis are related to energy production pathways that are possibly linked to the immunomodulatory events occurring as a result of CpG-ODN administration. We hypothesize that these findings will enable future targeted studies to better distinguish antimicrobial metabolomics profiles and pinpoint the key molecules or pathways contributing to immunity. Furthermore, there were multiple strong associations between certain metabolites based on the CpG-ODN treatment and the route of administration.

CHAPTER 6 DISCUSSION

According to the CFC and Egg Farmers Canada, the Canadian poultry industry has 3,859 farmers and 182 processors which help sustain 104,800 jobs, pay $2.25 billion in taxes and contribute $6.97 billion to Canada's gross domestic product. AMU is important in the poultry industry to maintain health and welfare but there are growing concerns of emergence of AMR strains of bacteria that may eventually adversely affect animal and human health and the environment. Chicken meat is the most consumed meat protein for Canadians, according to CFC, the per capita consumption of chicken meat was 33.1 kg in 2017, which included a 0.6 kg increase from the previous year (450). Although the Canadian poultry industry is growing to fulfil consumer demand, bacterial infections of poultry, especially bacterial infections of neonatal broiler chickens, remain the major challenge associated with increased mortality, chronic infections and condemnations. Of bacterial infections, *E. coli* infections of neonatal broiler chickens remain the major economic burden due to increased mortality with septicemia and chronic infections (433). *E. coli* infections result in chronic infections such as arthritis, osteomyelitis, pericarditis, airsacculitis and polyserositis leading to slower growth and loss of uniformity of broiler flocks (301). These chronic infections further lead to increased condemnations and down grading at the time of processing. The poultry industry has been using antibiotics to reduce bacterial infections as a prophylactic measure in the past by delivering antibiotics by the *in ovo* or subcutaneous routes to newly hatched birds (161), but these practices could potentially lead to the emergence of antibiotic resistant bacteria (403). Because of these reasons, CFC voluntarily withdrew the use of category I antibiotics in 2014 and category II antibiotics in 2018. CFC is anticipating to discontinue the use of category III antibiotics as prophylactic measures by the end of 2020 (447). CFC has identified prudent use of antimicrobials as a priority for the Canadian poultry industry (450) and are also investing in the development of alternative strategies of disease control in poultry while ensuring food safety and public health.

Synthetic CpG-ODNs have been identified as immunostimulatory molecules in mammals (79,164,202,405,407,431), fish (44,188,233) and poultry (61,76,114,126,382). CpG-ODN administration via parenteral routes such as IM and SQ as well as *in ovo* delivery in 18-day-old hatching eggs enables protection of neonatal broiler chickens against lethal bacterial infections (125,126,137,382). Although, CpG-ODNs can be delivered by parenteral routes, industry-

feasible techniques such as *in ovo* delivery remain a practical solution for the poultry industry. Since the immunoprotective action of CpG-ODN lasts only for 6-7 days, *in ovo* delivery can protect neonatal broiler chickens only for 2-4 days following hatch. Yolk sac infections and bacterial septicaemias are very common in the first week of life of broiler chickens hence; it would be ideal to deliver an immunoprotective agent that could last for the entire first week of the life. In the second chapter of this thesis, we discussed an innovative technique of delivering CpG-ODN by the IPL route as micro-droplets using a compressor nebulizer and a closed acrylic chamber under experimental conditions to prove proof of concept. We were able to demonstrate IPL delivery of CpG-ODN for 30 minutes in newly hatch broiler chickens results in significant protection against a lethal *E. coli* challenge. Furthermore, we were able to demonstrate that we could achieve this immunoprotection by delivering CpG-ODN for a short period of 15 minutes under laboratory conditions. Interestingly we found that this immunoprotection began within 6 hours of IPL delivery of CpG-ODN and lasted up to 5 days following delivery at a statistically significant level.

Thereafter our goal was to apply the laboratory level findings to a commercial setting by expanding IPL delivery of CpG-ODN under field conditions to a large number of birds. We developed a CSPN with a chick enclosure accommodating 8,000 birds, collaborating with engineers at the University of Saskatchewan and an engineering firm in Saskatoon, Canada. The efficacy of IPL delivery of CpG-ODN in this CSPN was tested through a series of large scale field experiments in Saskatchewan and British Columbia. It was evident that the CSPN could successfully deliver CpG-ODN by the IPL route to neonatal broiler chicks when the chick enclosure of the CSPN was maintained with a humidex of 28 and RH 40-60%. The chicks sampled from different locations of the chamber demonstrated significant immunoprotection against a lethal *E. coli* challenge indicating efficient distribution of CpG-ODN as aerosolized micro-droplets in the entire chick enclosure of CSPN. Furthermore, the chicks treated with CpG-ODN by the IPL route in the CSPN demonstrated a significantly better clinical outcome (*i.e.* CCS) compared to broiler chickens not exposed to CpG-ODN. Furthermore, broiler chickens exposed to CpG-ODN by the IPL route had a lower bacterial load in their body compared to the birds not exposed to CpG-ODN. Our field studies were conducted during different seasons of the year in both provinces with distinctly different climatic conditions, but the CSPN was able to process outside air to maintain a humidex of 28 and RH 40-60%. Hence, broiler chickens in the

CSPN were protected against a lethal *E. coli* infection as under laboratory conditions. Moreover, CO_2 levels in the CSPN did not go above 8,000 ppm during the 30 minute CpG-ODN delivery, which was a safe level of CO_2 for neonatal broiler chickens. In summary, we were able to design, develop and test an industry-feasible prototype CSPN to deliver CpG-ODN by the IPL route with an ability to induce protective immunity against bacterial septicemia in neonatal broiler chicks under field conditions.

Although CpG-ODN has been used in neonatal and adult poultry to prevent bacterial infections, the mechanism of immunoprotection associated with CpG-ODN is poorly understood. This led us to study the innate immune modulatory mechanisms of lymphocytes and macrophages following the exposure of CpG-ODN by the IPL route in neonatal broiler chickens. *In vitro* and *in vivo* studies in mice, chickens and humans have demonstrated that CpG-ODN elicits a Th1 type immune response (61,63,349,444). Patel *et al*. has demonstrated the upregulation of IL1β, IL6, IL8, IL10, IL18, IFNγ cytokine genes in the spleens of neonatal chickens following IM administration of CpG-ODN (312). Recently we have demonstrated that *in ovo* delivery of CpG-ODN in eighteen-day-old embryonating eggs upregulated pro-inflammatory cytokines together with LITAF in spleens and lungs leading to immunoprotection against lethal *E. coli* septicemia (136). In order to study cytokine responses following mucosal IPL delivery of CpG-ODN, we conducted experiments as discussed in chapter 4. It was evident that 24 hours following CpG-ODN delivery, pro-inflammatory cytokine genes (IL-1β, IL-6, LITAF, and IL-18) were expressed at a significantly high level both in the lungs and the spleen compared to birds not exposed to CpG-ODN. IL-1β was significantly upregulated by many fold in the lungs compared to the spleen, peaking at 12-24 hours following CpG-ODN delivery by the IPL route. LITAF expression was elevated 24-48 hours following CpG-ODN delivery by the IPL route both in lungs and spleen. IL-18 gene expression was upregulated 12-48 hours following CpG-ODN delivery by the IPL route. Here we demonstrate that both Th-1 type cytokine genes (IFN-γ, IFN-α) and Th-2 type cytokine genes (IL-4 and IL-10) were expressed more in the lung compared to the spleen, peaking at 12 hours post mucosal delivery of CpG-ODN. Our findings from chapter four added novel perspectives to the CpG-ODN mediated immunological mechanisms, highlighting the contribution of IL-1 β and LITAF in the launch of antimicrobial immunity upon mucosal IPL delivery of CpG-ODN.

Beyond exploring the cytokine gene expression profiles, we were interested in investigating the downstream cellular responses following CpG-ODN delivery induced cytokine and chemokine secretion. In a recent study conducted by our group, we have demonstrated that *in ovo* delivery of CpG-ODN in 18-day-old embryonating eggs resulted in a marked increase of APC (monocytes/ macrophages) in the spleen and lungs with a notable increase in co-stimulatory molecules (CD40) at 72 hours post CpG-ODN delivery. Furthermore, CD4+ and CD8+ T lymphocytes were markedly elevated in the spleen and lungs of chicks administered CpG-ODN . In the spleens, CD4+ cells were observed in higher number while the lung had more CD8+ T lymphocytes (136). Another recent study conducted by administering CpG-ODN by the *in ovo* route in birds infected with infectious bronchitis virus, demonstrated increased macrophages, CD4+ and CD8+ T lymphocytes in the trachea and lungs (81). As discussed in chapter four, when CpG-ODN was administered by the IPL route, we found that APC as well as CD4+ and CD8+ T lymphocytes significantly increased in the lungs and spleens of neonatal broiler chicks as early as 24 hours following administration. Unlike in the spleen, co-stimulatory molecule CD40 was highly upregulated in the APC of the lung indicating the maturation of these cells. However, by 72 hours post CpG-ODN administration, splenic macrophages/monocytes were not only elevated in number but also highly expressed maturation marker CD40. Likewise, in the lung, APC were increased in number by 72 hours after CpG-ODN treatment, as well the maturation marker CD40 and antigen-presenting molecule MHCII were highly expressed in these cells. It was evident that the lung APCs were maturing readily at the site of CpG-ODN delivery; the lung, preparing for efficient uptake and presentation of potential pathogens. In summary, it was interesting to discover that the local administration of CpG-ODN via the mucosal IPL route to the lung could induce not only a rapid infiltration of immune cells (APC and T lymphocytes) locally in the lung but also systemically as evident in the spleen. The macrophages/monocytes particularly were both elevated in number and equipped with maturation as well as antigen presenting molecules indicating their functional efficacy in combating potential pathogens.

As described in chapter five, our approach was focused on discovering and understanding the immunological mechanisms provoked by CpG-ODN administration in broiler chicks using a metabolomics approach. Metabolomics analysis allowed us to discover metabolic landmarks of various physiological events. In fact, metabolic changes related to immunological phenomena;

the immunometabolism is an area of research packed with curiosity and unknowns (315). We were curious how this technique could guide us to understand the cellular and molecular immunological outcomes in chickens following CpG-ODN administration. NMR analysis has been helpful in characterizing metabolic markers in normal chicken tissues and bio-fluids (232) as well as in distinguishing metabolic signatures in human diseases such as Lupus nephritis (135). Consequently, we planned an experiment to explore the changes occurring in the metabolome upon CpG-ODN delivery using NMR analysis as our metabolomics analysis tool. Our results indicated that CpG-ODN administration, regardless of the route of administration, distinctly changed the serum metabolome within 24 hours of CpG-ODN administration. The metabolites that significantly contributed to the change were L-proline, choline, L-threonine, cytidine, hypoxanthine, scetic acid, betaine, uridine, 2-hydroxy butyrate, L-lysine and myoinositol. We found that the route of administration (IPL or IM) affected different metabolites in significant levels. Pathway enrichment analysis displayed leading serum metabolites differentiating between the experimental groups, including key metabolites involved in energy production and expenditure; glucogenic vs. ketogenic; citrate cycle vs. fatty acid oxidation. When the metabolites were projected on metabolic pathways, certain metabolite levels related to the energy metabolism pathways were seen changing upon CpG-ODN administration. To our knowledge, this was the first metabolomics analysis study conducted to understand the immunometabolomic changes resulting from CpG-ODN administration.

Summarizing the findings of this study, it is clearly understood that mucosal delivery of synthetic CpG-ODN via IPL route is a practical technique that is feasible in the commercial poultry industry, particularly adaptable to use in commercial poultry hatcheries. With the efficacy in inducing significant protective immunity against *E. coli* septicemia as early as 6 hours after treatment and lasting up to 5 days, this technique ensures that neonatal broiler chicks are protected during the most vital first week of life. With the treatment exposure time as short as 15 to 30 minutes, it is a delivery system that has the potential to adapt to the commercial scale. The CSPN developed as part of this study proved efficient to deliver CpG-ODN under field conditions. The clinical protection of neonatal broiler chicks against lethal bacteremia was attributed to the upregulation of inflammatory, Th-1 and Th-2 cytokine genes following the administration of CpG-ODN. Further, activation and maturation of APC in lungs and spleen indicated that these sentinel cells were getting equipped to fight potential pathogens. Both the

local lung tissue and the systemic immune organs such as the spleen were infiltrated with large numbers of APC and T lymphocytes that could fight upcoming pathogenic infections. Not only that, CpG-ODN administration changed the levels of certain metabolites in serum; particularly those involved in energy consumption and production pathways, representing numerous cellular and molecular immunomodulatory events.

This project has opened a number of future avenues in terms of CpG-ODN formulation and delivery by the IPL route for the poultry industry. IPL delivery is industry-feasible since it's applicable to a large number of birds at commercial poultry hatcheries. Since we have demonstrated that CpG-ODN-mediated protective efficacy lasts only for 5 days following IPL administration without formulation of CpG-ODN, it will be interesting to use novel delivery systems to better schedule the activity of CpG-ODN in the lung and spleen of neonatal broiler chickens. We have previously demonstrated immunopotentiation of CpG-ODN by formulating with liposomes for *in ovo* delivery (137). It will be prudent to use nanoparticle formulations of CpG-ODN by the IPL route to enhance its efficacy.

In the immunological perspective, it is clear that IPL CpG-ODN administration is stimulating a large number of innate cells. Particularly APC, such as macrophages, are being activated, matured and infiltrated into the lung and immune organs of the body. Using histology, we have observed that the number of heterophils have markedly increased in the lungs and spleen as a response to CpG-ODN. Literature states that heterophils are vital warriors of antibacterial defense. Their antimicrobial effect is based on non-oxidative mechanisms which are cationic antimicrobial peptides (41). It would be advantageous to study the role of heterophils in CpG-ODN-mediated antimicrobial immune defense. Since we have demonstrated changes in the serum metabolome of neonatal broiler chickens following CpG-ODN administration, it would be beneficial to identify whether specific immune cell functions lead to changes in the metabolome of neonatal broiler chickens. With these proposed new research approaches, the utility of CpG-ODN in the poultry industry can be further improved at a significant level to improve the health and wellbeing of poultry while enhancing food safety and public health.

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