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Nitric Oxide Mediated Antiviral Response in Avian Cells

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Nitric Oxide Mediated Antiviral Response in Avian Cells

by

Siamak Haddadi

A THESIS

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ABSTRACT

LPS activates TLR4 signaling pathway eliciting antiviral host responses in mammals although information on such responses in avian species is scarce. Objectives of the work described in the thesis were to 1. characterize the LPS induced expression of LPS receptors and NO in two avian cell lines, LMH and MQ-NCSU, and 2. observe whether NO can elicit antiviral response against ILTV replication. We found that LPS was capable of inducing the expression of TLR4, CD14 and NO production only in MQ-NCSU cells. We also showed that TLR4 mediated NO production in MQ-NCSU cells confers antiviral response against ILTV in susceptible LMH cells. Using a selective inhibitor of inducible NO synthase and a NO donor as a source of NO, we confirmed that this effect is positively correlated with NO. Our data showed that LPS can be a potential innate immune stimulant that can be used against ILTV infection in chickens.

Key words: infectious laryngotracheitis virus, lipopolysaccharide, nitric oxide, TLR4

PREFACE

This thesis is based on the studies done at the Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada from September 2011 to August 2013.

Chapter two of this thesis contains materials that are published in the journal, 'Veterinary Immunology and Immunopathology': Haddadi, S., Kim, D.-S., Jasmine, H., van der Meer, F., Czub, M., Abdul-Careem, M.F. (2013). Induction of Toll-like receptor 4 signaling in avian macrophages inhibits infectious laryngotracheitis virus replication in a nitric oxide dependent way. *Veterinary Immunology and Immunopathology*, 155(4), 270-275.

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LIST OF SYMBOLS, ABBREVIATIONS, NOMENCLATURES

Abbreviations	Definitions
Ab	Antibody
AIV	Avian influenza virus
ANOVA	Analysis of variance
AP-1	Activator Protein 1
APC	Ag-presenting cell
BSA	Bovine serum albumin
CAMP	Cationic antimicrobial peptide
CD	Cluster of differentiation
CEF	Chicken embryo fibroblast
CpG	Cytosine guanine dinucleotide
d	Days; in formulae: deoxy or distilled
DAMP	Danger associate molecular patterns
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ED	Embryo day
FACS	Fluorescent-activated cell sorter
FBS	Fetal bovine serum
FcR	Fc receptor
FITC	Fluorescein isothiocyanate

Abbreviations	Definitions
<i>g</i>	<i>g</i> force
GPI	Glycosylphosphatidylinositol
h	Hour
HIV	Human immunodeficiency virus
HSV-2	Herpes simplex virus -2
HVT	Herpes virus of turkey
IBV	Infectious bronchitis virus
IFN	Interferon
Ig	Immunoglobulin
IKK	I κ -kinase
I κ B	Inhibitory κ B
IL	Interleukin
ILTV	Infectious laryngotracheitis
IRAK	IL-1 receptor-associated kinase 4 family proteins
IRF	Interferon regulatory factors
ISGs	IFN-stimulated genes
LBP	LPS-binding protein
LITAF	Lipopolysaccharide-induced TNF factor
LMH	Leghorn male hepatocarcinoma cell
LPS	Lipopolysaccharide
mAb	Monoclonal antibody

Abbreviations	Definitions
MAPK	Mitogen-activated protein kinas
MD-2	myeloid differentiation protein-2
MDV	Marek's disease virus
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mRNA	Messenger RNA
MyD88	Myeloid differentiating factor 88
NDV	Newcastle disease virus
NF	Nuclear factor
NO	Nitric oxide
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PFU	Plaque forming units
PRR	Pattern recognition receptor
SE	Standard error
SEM	SE of the mean
SMT	S-methyl-isothiourea
SNAP	S-nitroso-N-acetyl-DL-penicillamine

Abbreviations	Definitions
ss	Single stranded
TIR	Toll/IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TRAF6	Tumor necrosis factor receptor associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon- β

CHAPTER 1: INTRODUCTION

1.1 General introduction:

Understanding innate antiviral host responses in poultry species is essential for developing strategies for control of viral diseases. Information on potential antiviral mechanisms in poultry is scarce, yet understanding the antiviral mechanisms leading for formulation of control measures are critical, with a view of the negative impact of poultry viruses on poultry production and public health, and the limitations of current control methods available against these viral infections. In this thesis, the antiviral responses elicited by a male leghorn chicken hepatocarcinoma cell line (LMH), and one avian macrophage cell line, a Marek's disease virus (MDV) transformed chicken macrophage cell line (MQ-NCSU), against infectious laryngotracheitis virus (ILTV) were investigated. One of the vital components of the innate immune system that is responsible for activating antiviral response in invertebrate hosts to higher mammals is toll-like receptor (TLRs) and their activation culminate in producing a range of antiviral compounds including nitric oxide (NO). This chapter of the thesis provides an overview of TLRs, specially TLR4 signaling in avian, activation of antiviral mechanisms in this host species, cell lines used for the study and the biology of virus, ILTV used for demonstrating antiviral response in avian cell lines, research questions and hypotheses.

1.2 Innate immune system:

Host-pathogen interaction triggers a series of responses by the host. Host responses can be classified broadly into 'innate' and 'adaptive' components. Innate immune responses play

vital roles in invertebrates as well as in vertebrates. However, the adaptive immune responses are just important in vertebrates. In vertebrates, both innate and adaptive arms of the immune system function in a highly integrated way, and elicit a coordinated response against invading pathogens (Janeway, 2001; Janeway and Medzhitov, 2002). The discovery of functions and components of innate immune system have been recognized as critical contributions to the field of physiology or Medicine by awarding the 2011 Nobel Prize to Bruce Beutler and Jules Haffmann, for their discovery of activation of innate immunity, particularly TLR4 signaling, and Ralph Steinman, for his discovery of the dendritic cell, which is one of the innate immune cells that collaborate with adaptive arm of the immune system (O'Neill et al., 2013).

The immune system is made up of several types of cells and molecules that have various functions used to do in fighting pathogens. There are two major types of responses elicited against invading microbes. The first category is known as innate responses which are short, quick, broad spectrum and non-specific. However, due to lack of immunological memory, they happen to the same extent whenever the same infectious agent is reencountered, while adaptive responses are augmented on repeated exposure to the same infection (Aderem and Underhill, 1999; Fraser et al., 1998). The innate response is composed of phagocytic cells (neutrophils, monocytes, and macrophages), cells secreting inflammatory mediators (basophils, mast cells, and eosinophils) (Abraham and Arock, 1998; Wardlaw et al., 1995), natural killer cells (Biron et al., 1999) and dendritic cells (Bell et al., 1999). The molecular components of innate responses consist of complement system, acute-phase proteins, and cytokines like the interferons (IFNs) (Epstein et al., 1996; Ricklin et al., 2010; Steinstraesser et al., 2011; Zasloff, 2002). Adaptive responses include antigen-specific B and T cells, which become activated when their surface receptors bind to a specific antigen (Garcia et al., 1999; Novotny et al., 1983).

1.3 Pathogen associated molecular patterns:

Pathogen associated molecular patterns (PAMPs) including lipoproteins, lipopeptides, lipopolysaccharide (LPS), flagellin, double stranded (ds)RNA, single stranded (ss)RNA and unmethylated cytosine guanine dinucleotide (CpG) DNA motifs are recognized by the innate immune system through receptors known as pattern recognition receptors (PRRs) (Beutler et al., 2003). PRRs are divided into two groups; first, cell-associated PRRs that include TLRs, C-type lectin receptors (CLRs), scavenger receptors, nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs), retinoic acid inducible gene (RIG)-I- like receptors (RLRs), and N-formyl Met-Leu-Phe receptors. The second category includes, soluble recognition molecules including pentraxins, collectins, and ficolins (Akira, 2006; Akira and Takeda, 2004; Meylan et al., 2006). The ligands or PAMPs that bind to different chicken (ch) TLRs are summarized in the Table 1.

1.4 Toll-like receptors (TLRs):

TLRs, as a large group of PRRs, are highly conserved innate immune receptors (Akira, 2006; Akira and Takeda, 2004; Meylan et al., 2006). TLR family members are type 1 transmembrane molecules and comprised of an extracellular domain with leucine-rich repeats (LRR) flanked by C-terminal cap motifs, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain (Jin and Lee, 2008; Werling et al., 2009).

Table 1. Chicken toll-like receptors and their ligands (this table was adapted from St Paul and others (St Paul et al., 2013).

TLR	Structure recognized	Agonist	Reference
TLR1/TLR2 (except TLR2-1/ TLR1-2)	Bacterial cell wall components	diacyllipopeptides (Malp-2) and triacyllipopeptides (Pam3)	(Higuchi et al., 2008; Keestra et al., 2007)
TLR2-1/ TLR1-2	Bacterial cell wall components	Pam3, peptidoglycan	(Higuchi et al., 2008; Keestra et al., 2007)
TLR3	dsRNA	Poly I:C	(Liu et al., 2008; Pirher et al., 2008)
TLR4	Bacterial cell wall components	LPS	(Dil and Qureshi, 2002a; Farnell et al., 2003; Kogut et al., 2006)
TLR5	Bacterial flagellin	Flagellin	(He et al., 2006b; Kogut et al., 2005b)
TLR7	ssRNA	Imiquimod, Resiquimod	(Ahmad-Nejad et al., 2002; Heil et al., 2003; Matsumoto et al., 2003)

TLR	Structure recognized	Agonist	Reference
TLR15	virulence-associated fungal and bacterial proteases	Proteases from <i>Pseudomonas</i> <i>Aeruginosa</i> , <i>Trichosporon</i> spp., <i>Penicillium</i> spp. and <i>Mucor</i> spp.	(de Zoete et al., 2011)
TLR21	Microbial CpG DNA Motifs	CpG ODN	(Brownlie et al., 2009)
TLR15	virulence-associated fungal and bacterial proteases	Proteases from <i>Pseudomonas</i> <i>Aeruginosa</i> , <i>Trichosporon</i> spp., <i>Penicillium</i> spp. and <i>Mucor</i> spp.	(de Zoete et al., 2011)

TLRs are mainly expressed in tissues engaged in immune functions, like spleen and peripheral blood leukocytes, and mucosal surfaces which are exposed to the external environment including lung and the gastrointestinal tract. Their expression patterns vary among tissues and cell types. TLRs are expressed on the plasma membranes with the exception of TLR3, TLR7 and TLR9 which are found on the endosomal compartments (Nishiya and DeFranco, 2004) since these TLRs sense dsRNA, ssRNA and CpG motifs of pathogens which will be exposed intracellularly.

With the binding of ligands (PAMPs) to TLRs, TIR domain interacts with intracellular adapter molecules such as myeloid differentiation factor (MyD)88 , TIR domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon- β (TRIF), or Trif-related adapter molecule (TRAM) to initiate a complex signaling cascade that regulate the transcription of pro-inflammatory and immunomodulatory genes (Akira, 2003; Higuchi et al., 2008; Jin and Lee, 2008; O'Neill, 2002). TLR-mediated signal transduction also includes several intermediate molecules such as tumor necrosis factor receptor associated factor 6 (TRAF6), IL-1 receptor-associated kinase 4 family proteins (IRAK), Ik-kinase (IKK) family proteins, mitogen-activated protein kinase (MAPK), and transcription factors like nuclear factor (NF)- κ B (NF- κ B) family proteins, Activator Protein 1 (AP-1), and interferon regulatory factors (IRFs). The consequences of this signal transduction are the production of pro-inflammatory cytokines, chemokines, NO, anti-microbial peptides, adhesion molecules, up-regulation of major histocompatibility complex (MHC) molecules, and co-stimulatory molecules that facilitates antigen presentation to the adaptive immune system (Akira, 2003; Akira and Takeda, 2004; Beutler, 2005; Higuchi et al., 2008; Hu et al., 2002; Jin and Lee, 2008; O'Neill, 2002; Vandewalle, 2008; Verstak et al., 2009; West et al., 2006) . Generally, TLRs and their downstream signaling components are conserved in chickens (Lynn et al., 2003; Philbin et al., 2005), yet there are specific signaling differences found particularly related to chicken TLR4 signaling (Keestra and van Putten, 2008).

The chicken genome encodes the following TLRs; chTLR1-1 (forming an evolutionary cluster with mammalian TLR1, TLR6, and TLR10), chTLR1-2, chTLR2-1, chTLR2-2, chTLR3, chTLR4, chTLR5, chTLR7, chTLR15, and chTLR21 (Iqbal et al., 2005; Lynn et al., 2003; Philbin et al., 2005; Yilmaz et al., 2005). However, orthologs of mammalian TLR8 and TLR9

are absent in avian species (Cormican et al., 2009; Roach et al., 2005; Schwarz et al., 2007; Temperley et al., 2008).

1.5 Chicken TLR4 (ChTLR4):

TLR4 is expressed not only on the surface membrane of immune and non-immune cell types (Arpaia et al., 2011; Qureshi and Medzhitov, 2003) but also in the cytoplasm of intestinal epithelial cells (Cario et al., 2002; Hornef et al., 2002). The expression of chTLR4 varies among different chicken lines and genotypes (Abasht et al., 2009; Dil and Qureshi, 2002a), which determines the disease susceptibility to infectious agents. Also, the chTLR4 gene is polymorphic and determines the resistance or susceptibility of young chickens to pathogens such as *Salmonella enterica serovar Typhimurium* (Leveque et al., 2003).

TLR4 signaling pathway in chickens that leads to the induction of antiviral molecules is illustrated in Figure 1. One of the prerequisite for TLR4 and LPS (one of the most important ligands of TLR4) interaction is the binding of LPS to LPS-binding protein (LBP) (Kogut et al., 2005a), that transfers LPS monomers to CD14 which delivers the LPS to myeloid differentiation protein-2 (MD-2). MD2 is located bound to the extracellular domain of TLR4 (Palsson-McDermott and O'Neill, 2004; Shimazu et al., 1999). Binding of LPS delivered to MD2 hydrophobic pocket induces conformational change leading to transfer LPS to the TLR4 molecule (Kim et al., 2007; Ohto et al., 2007). Since chicken CD14 (chCD14) is a type I transmembrane protein rather than glycosyl phosphatidyl inositol (GPI)-anchored protein (Haziot et al., 1988; Wu et al., 2009) this receptor is less mobile. It has also been shown that due to the lack of a TLR4/TRAM/TRIF pathway in chicken cells, chickens are unable to produce type I IFNs via chTLR4 signaling (Keestra and van Putten, 2008). In fact, they have reported that

cloning and expression of recombinant chTLR4 and chMD-2 in HeLa 57A cells activate NF- κ B at low concentrations of LPS (100 pg/ml). Also, in comparison to the TLR3 agonist poly (I:C) that induces a strong IFN- β expression, LPS is not capable of triggering the TIRF-dependent expression of IFN- β in chicken cells (Keestra and van Putten, 2008). The aforementioned reasons may explain why, in comparison to mammalian species, avian species are more resistant to the toxic effects of LPS (Adler and DaMassa, 1979; Berczi et al., 1966; Iliev et al., 2005).

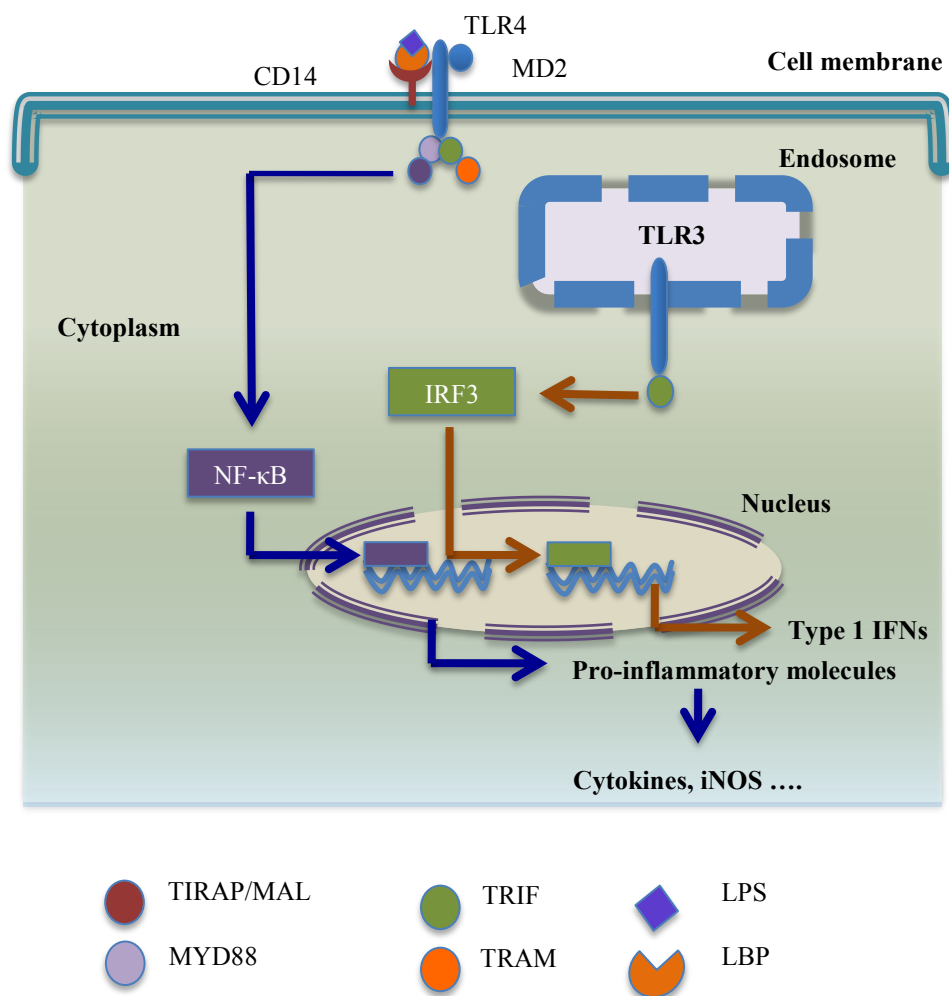


Figure 1. TLR4 signaling pathway in chicken that leads to the induction of antiviral molecules.

ChTLR4 and its co-receptors, in response to LPS, induce the expression of inducible NO synthase (iNOS), IL-1 β , IL-6, and IL-18 mRNA (Dil and Qureshi, 2002b; Farnell et al., 2003; He et al., 2006a). Furthermore, in chickens, instead of TNF- α , its counterpart named tumor necrosis factor (TNF) S15 has been identified (Takimoto et al., 2008) that is induced by the transcription factor named lipopolysaccharide-induced TNF factor (LITAF) (Hong et al., 2006). While both TLR4 and MD-2 genes are highly conserved among the orthologs found in mammals, birds, and some fish species (Iliev et al., 2005; Meijer et al., 2004), the interaction between chTLR4 and chMD-2 is species-specific. This could be due to differences between chickens and mammalian species in the amino acid composition that makes differences in the formation of complex between TLR4 and MD-2 (Keestra and van Putten, 2008). The LPS specificity is not only determined by MD-2, but also by TLR4 (Hong et al., 2004).

1.6 Toll-like receptors and antiviral response:

Therapeutic application of imiquimod (TLR7 agonist) against papilloma virus-induced genital warts in human has been in clinical use. Further, clinical trials of CpG against hepatitis C and poly (I:C) against HIV are in preclinical and phase II clinical trial status, respectively (Kanzler et al., 2007).

As molecules induced following TLRs stimulation, pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6, can elicit antiviral effects against HIV-I in macrophages including microglia (Lokensgard et al., 1997). Stimulation of TLR3 or TLR4 by Poly (I:C) or LPS respectively induces a potent anti-HIV effect in human microglia. Indeed, activation of TLR3 and TLR4 produces the same intracellular cell activation pathways and leads to the expression of the same sets of genes in microglia, including pro-inflammatory cytokines, chemokines and IFN-

stimulated genes (ISGs). However, PIC leads to the higher induction of many ISGs, and LPS gives rise to higher induction of many non-IRF3-dependent genes (Suh et al., 2009). In general, both TLR3 and TLR4 are the main innate antiviral immune receptors in human microglia (Suh et al., 2009).

In vitro and *in vivo* studies show that fimbriae (Fim)H, an adhesion portion of type 1 fimbriae available in most Enterobacteriaceae like uropathogenic *E.coli* (Ashkar et al., 2004; Krogfelt et al., 1990), in a TLR4-dependent way strongly elicits innate antiviral protection against herpes simplex virus-2 (HSV-2). This innate response is correlated with IFN- β production and depends on MyD88, TRIF, TLR4, IRF-3 and type I IFN signaling (Ashkar et al., 2004; Mian et al., 2010).

In chicken, antiviral innate response induced by TLR ligands has been shown towards reovirus (Pertile et al., 1996), MDV (Xing and Schat, 2000), infectious bronchitis virus (IBV) (Dar et al., 2009), Newcastle disease virus (NDV) (Tseng et al., 2009), and avian influenza virus (AIV) (St Paul et al., 2012). St. Paul et al have reported that pretreatment of chickens using TLR ligands including poly (I:C), LPS and CpG following challenge with AIV can reduce virus load and shedding (St Paul et al., 2012). In fact, this antiviral innate response can be highlighted about TLR3 ligand poly I:C mounting the greatest innate response against AIV. Moreover, Pertile et al (Pertile et al., 1996) have shown that LPS-pretreatment of chicken macrophage cell line HD11 can hamper replication of reovirus. Xing et al. (Xing and Schat, 2000) have revealed that the inhibition of MDV replication through co-stimulation of chicken embryo fibroblast (CEF) and chicken by recombinant ChIFN- γ and LPS is mediated by NO.

1.7 Macrophages:

Macrophages discovered by Élie Metchnikoff are considered as ‘sentinel’ cells that clear the interstitial environment of extraneous cellular material like apoptotic bodies via phagocytic receptors including scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors (Erwig and Henson, 2007). This homeostatic clearance process is done independent of other immune cells (Kono and Rock, 2008). On the other hand, clearance of necrotic bodies loaded with endogenous danger associated molecular patterns (DAMPs), such as heat-shock proteins, nuclear proteins, histones, DNA, other nucleotides, and extracellular matrix materials proteases by macrophages. This then leads to the activation of TLRs (Chen et al., 2007; Kono and Rock, 2008; Park et al., 2004), intracellular PRRs and the interleukin-1 receptor (IL-1R) (Chen et al., 2007) and consequently production of cytokines and pro-inflammatory mediators independent of adaptive immune responses (Kono and Rock, 2008; Zhang and Mosser, 2008). In general, macrophages detection of PAMPs (or DAMPs) by PRRs, in particular TLRs (Kaisho and Akira, 2002), consequently results in clearance of necrotic bodies from extracellular spaces, tissue homeostasis and host defense. These functions make macrophages a major sentinel in the host (Kono and Rock, 2008).

In addition to this innate activation process, macrophages can respond to signals provided by antigen-specific immune cells. Since these signals are more long-lasting than innate immune stimuli, they can make long-term changes in macrophages (Gordon, 2007). In response to certain extracellular signals, macrophages become mature antigen presenting cells and induce the activation of T helper cells (Th cells) affecting the overall immune responses (Iwasaki and Medzhitov, 2004). On the other hand, these long-lasting interactions between microbial components and macrophages can result in a state of hyporesponsiveness to the same stimulus or

related stimuli. This phenomenon is recognized as tolerance. Several mechanisms contribute to this process: down-regulation of TLR4 (Nomura et al., 2000); decreased expression of downstream signaling molecules (Li et al., 2000); alterations in the association between TLR5 and downstream signaling molecules (Mizel and Snipes, 2002); increased expression of proteins inhibiting TLR signal transduction (Kinjyo et al., 2002; Kobayashi et al., 2002; Nakagawa et al., 2002); and upregulation of the p50 and p52 subunits of the NF- κ B transcription factor leading to the interference of NF- κ B transcription (Karsten et al., 2009; Wedel et al., 1999). In practice, macrophage tolerance to the normal microbial flora, particularly in gastrointestinal tract, through inhibition of inflammation can maintain normal immune homeostasis (Erdman et al., 2001).

Circulating monocytes migrate into tissue and differentiate to macrophages to renew long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli (alveolar macrophages), central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Gordon and Taylor, 2005). Nevertheless, the local proliferation of tissue-resident colony-forming cells can directly produce populations of mature macrophages in the tissue, in particular the microglial cells of the central nervous system (Ajami et al., 2007).

Macrophages on the basis of the pathway of their activation (activated phenotype) are classified into M1 macrophages and M2 macrophages which are reserved for classically activated macrophages and alternatively activated macrophages (Gordon, 2003). However, according to fundamental functions, these cells can be grouped into classically activated macrophages, wound-healing macrophages, and regulatory macrophages. Further, macrophages are capable of evolving to different subsets with characteristics shared by more than one

macrophage population. As a result, this situation can lead to a 'spectrum' of macrophage subpopulations based on their functions (Edwards et al., 2006).

1.7.1 Avian macrophage cell lines:

In comparison to primary cell cultures, chicken macrophage cell lines give rise to a population of cells with approximately the same functional features. These cell lines are characterized as adherent, phagocytic, and express monocytic-related cell surface markers. Different transformation methods have been used to develop chicken macrophage cell lines. There are several avian macrophage cell lines have been established. The HD11 cell line is transformed by an avian myelocytomatosis (MC)29 retrovirus (Beug et al., 1979). The MQ-NCSU cell line, which is more active than the HD11 cells at many effector and regulatory functions, is transformed by MDV (Qureshi et al., 1994; Qureshi et al., 1990). Also, there are two monocytic chicken leukemia cell lines; IN24 established using a natural myelocytic leukemia virus (Inoue and Sato, 1988), and LSCC-NP1 originated from the bursa of Fabricius of a chicken infected with lymphoid leukosis virus (Inoue et al., 1992). These cell lines, in comparison to primary macrophages are convenient and permit greater experimental control and repeatability, but some disadvantages include the shedding of virus used for the transformation or functional anomalies in the cell lines might lead to some controversial results. Chicken macrophages are capable of producing cytokines with functions akin to mammalian pro-inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor (TNF)-like ligand 1A (equivalent of TNF α in mammals) (Bombara and Taylor, 1991; Klasing and Peng, 1987; Qureshi et al., 1994; Qureshi and Miller, 1991; Qureshi et al., 1993; Takimoto et al., 2008). Further, NO

and eicosanoids (including both the leukotrienes and the prostaglandins) are secreted by activated macrophages (Dietert and Golemboski, 1998).

1.7.2 Mouse macrophage cell lines:

The RAW 264.7 cell line originates from the ascites of a tumor induced in a male BAB/14 mouse by the intraperitoneal injection of the Abelson leukemia virus (A-MuLV). RAW 264.7 cells readily take up neutral red dye, synthesize and secrete lysozyme, phagocytose zymosan and latex beads, and mediate antibody dependent lysis and phagocytosis of sheep erythrocytes (SRBC). RAW 264.7 cells are not capable of secreting measurable virus particles. Also, this cell line is negative in the XC plaque formation assay and the fibroblast transformation assay for Abelson virus, yet it can produce Abelson virus after rescue by Moloney leukemia virus (Raschke et al., 1978).

The growth of RAW 264.7 cells are inhibited by trace amount (sub ng/ml) of LPS (Raschke et al., 1978). A similar situation occurs with other macrophage cell lines and a few B cell lines (Ralph et al., 1974) which are particularly sensitive to macrophage-activating factors such as LPS, mycobacterium strain Bacillus Calmette-Guerin (BCG), zymosan, tuberculin-purified protein derivative (PPD) and dextran sulfate. RAW 264 cells are sensitive to LPS (Raschke et al., 1978).

1.8 Lipopolysaccharide:

Endotoxins contribute to bacterial pathogenesis (Beutler and Rietschel, 2003), and they have been chemically characterized as LPS (Raetz et al., 2007; Raetz and Whitfield, 2002). LPS is comprised of three parts: lipid A, core sugars and O-antigen repeats. Lipid A is the

hydrophobic constituent of LPS which anchors into the outer leaflet of the outer membrane, whereas core sugars and O-antigen repeats are located on the surface of bacteria. Lipid A is recognized to mediate the toxic effects of infections with Gram-negative bacteria (Galanos et al., 1985). The biochemical structure of LPS varies from one bacterium to another, and this variation could affect the virulence of bacteria. In fact, all regions of LPS show diversity. Regarding the O-specific side chain, heterogeneity occurs due to the various amount of polymorphism (S-type), a single repeating unit (SR-type), and absence of a side chain (R-type). Further, incomplete core oligosaccharide (core-defective R-types) leads to more heterogeneity in different species of LPS. The outer region of the core oligosaccharide displays minimal or limited variations. On the other hand, the maximum compositional and structural diversity happens in the O-specific side chain (Wilkinson, 1996). The phosphate groups and the length and number of fatty acyl chains of lipid A are responsible for TLR4 activation (Persing et al., 2002; Rietschel et al., 1994).

In *S. enteritidis*, O-antigen side chain is comprised of repeating the following unit [$\rightarrow 2\text{-}\beta\text{-D}$ mannose-1 \rightarrow 4- $\alpha\text{-L}$ -Rhamnose-1 \rightarrow 3- $\alpha\text{-D}$ -Galactose-1 \rightarrow] with nonstoichiometric glucosylation at a much lower level in comparison to *S. typhi* (Hellerqvist et al., 1969). *Salmonella* type lipid A includes a $\beta\text{-1'}$, 6-linked disaccharide of glucosamine, with phosphates at the 1 and 4' positions and acylated at the 2, 3, 2', and 3' positions with R-3-hydroxymyristic acid (3-OH C14:0). This structure becomes more acylated with secondary laurate (C12:0) and myristate (C14:0) chains in acyloxyacyl linkage at the 2' and 3' positions (Raetz and Whitfield, 2002). Indeed, the myristate chain is required for bacterial growth in lysogeny broth (LB) medium and resistance to bile salts, CO₂, acidic pH, and high osmolarity (Karsten et al., 2009; Murray et al., 2001). Lacking the myristate chain also leads to reduced pro-inflammatory cytokines such as TNF- α and NO production (Khan et al., 1998; Low et al., 1999). Further,

Salmonella type Lipid A is modified further through the addition of small molecules, like phosphoethanolamine (PETN) moieties or 4-amino-4-deoxy-L-arabinose (L-Ara4N), or alterations in the fatty acid chains. The organism utilizes these strategies to modulate host innate immunity and protect itself in various microenvironment niches (Guo et al., 1997; Guo et al., 1998; Trent et al., 2001).

The structure of *E.coli* Lipid A is similar to the *Salmonella* one except for the fact that two residues of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) which are covalently linked to the 6'-position (Hankins and Trent, 2009). The lipid A present in both *E. coli* and *Salmonella* is the most efficient in inducing pro-inflammatory responses via the Toll-like receptor 4 (TLR4)-MD2-CD14 pathway. Moreover, *E.coli* lipid As with fewer acyl chains, like tetra- or penta-acylated lipid A species, reveal markedly reduced immunostimulatory function (Brandenburg and Wiese, 2004). In contrast, the penta-acylated lipid A species in *Salmonella* are capable of activating the TLR4-MD2-CD14 pathway, whereas the tetra- or tri-acylated lipid A indicates lack of immunostimulatory activity (Rossignol and Lynn, 2005). Similar to the *Salmonella* type, the *E. coli* lipid A contains two phosphate groups and six acyl chains composed of 12 or 14 carbons, and acts as a strong activator of the innate immune system (Golenbock et al., 1991).

After synthesis, the structure of LPS can be modified. This modification is concentrated on the most conserved part lipid of A. Different structures of lipid A have been recognized in different bacteria. The most conserved part of lipid A is its backbone, disaccharide of glucosamine. The groups connecting to the backbone of lipid A could be modified. The modification of LPS can happen both in the hydrophilic polysaccharide region and the hydrophobic acyl chain domain. The structural modification of LPS might contribute to the bacterial resistance to the cationic antimicrobial peptides (CAMPs) produced by the innate

immune system, or evades recognition by TLR4 (Guo et al., 1997; Wang et al., 2006; Wilkinson, 1996). Through modification of the LPS structures, it would be possible to develop novel LPS immune adjuvants or antagonists (Hawkins et al., 2004; Persing et al., 2002; Stover et al., 2004), or augment the traditional Gram-negative bacterial live vaccines.

1.9 Nitric oxide (NO):

NO, a gaseous free radical, plays key roles in biological processes such as vascular regulation, neurotransmission, immune response, and pathogenesis of several diseases. This molecule is generated in a wide range of cell types and tissues by the concomitant conversion of L-arginine into L-citrulline via at least three distinct isoforms of the enzyme nitric oxide synthase (NOS) including neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Geller and Billiar, 1998; Marletta et al., 1998; Moncada et al., 1997; Nathan, 1992; Stuehr, 1997).

iNOS, with inducible expression, is independent of elevated intracellular Ca^{2+} , but nNOS and eNOS, with constitutive expression, are dependent on elevated intracellular Ca^{2+} for eliciting their functions. Generally, nNOS and eNOS expressions are constitutive, whereas the expression of iNOS is inducible. Further, both nNOS and eNOS contribute to physiological functions of NO in the healthy host, while iNOS is involved in inflammation and infection (Mannick et al., 1994; Xie et al., 1992).

Mechanism of antiviral effect of NO can be explained by the interaction of NO with target (macro) molecules by way of a rich redox and additive chemistry (Stamler, 1994). Additionally, NO reaction with oxygen, superoxide, and transition metals, gives rise to reactive nitrogen intermediates (e.g., peroxynitrite) that provide more nitrosative reactions. The greater

prevalence and reactivity of thiols in comparison to other nucleophiles facilitate the formation of S-nitrosothiol (Stamler, 1994, 1995; Stamler and Hausladen, 1998).

In fact, greater prevalence of reactive cysteine residues in the large number of virus-encoded and host proteins including; proteases, reverse transcriptase, ribonucleotide reductase, transcriptional factors, zinc-finger domains, glycoproteins and hemagglutinin, makes them adequate targets for NO. However, under stringent conditions, tyrosine and tryptophan residues and DNA can be considered as the secondary targets. The antiviral effect of NO has been substantiated for several DNA and RNA virus families, including *Picornaviridae*, *Flaviviridae*, *Coronaviridae*, *Rhabdoviridae*, *Reoviridae*, *Retroviridae*, *Parvoviridae*, *Herpesviridae*, and *Poxviridae* (Persichini et al., 1999). The outcome of antiviral response mediated by NO is determined by both the virus and the host cell types. In general, the antiviral effects of NO can be explained not only at the transcriptional scale through influencing the activity of transcription factors, but also at the post-transcriptional scale with the enzyme activity and protein assembly (Maeda and Akaike, 1998; Mannick, 1995; Peterhans, 1997; Powell and Baylis, 1995; Reiss and Komatsu, 1998).

1.10 Avian infectious laryngotracheitis virus:

ILTV belongs to the group I of double stranded DNA viruses, and is placed in the order of *Herpesvirales*, family of *Herpesviridae*, subfamily of *Alphaherpesvirinae*, and genus of *Iltovirus*. Based on current nomenclature ILTV species is renamed as *Gallid herpesvirus 1* (Bagust et al., 2000). Since most literatures cite the virus as ILTV, we used the old nomenclature of virus throughout the thesis in order to prevent any confusion.

ILTV consists of a DNA-containing core within an icosahedral capsid surrounded by a proteinaceous tegument layer and an outer envelope with incorporated viral glycoproteins (Cruickshank et al., 1963; Watrach et al., 1963). Although all capsids have diameters of 100 nm, particle sizes vary between 200 and 350 nm, since ILTV tends to incorporate huge amounts of tegument proteins (Granzow et al., 2001).

ILTV infection leads to sporadic cases of infectious laryngotracheitis (ILT) in chickens and pheasant world-wide. ILT varies from mild to per-acute, with mortality in per-acute outbreaks exceeding 50%. ILTV can remain latent in carriers after infection and due to stress the virus can be shed intermittently. The chicken is the primary host and reservoir host. In contrast to the mild form, death may happen quickly and with high mortality in peracute and acute disease (Bagust et al., 2000; Jones et al., 1993).

Propagation of ILTV is done on the chorioallantoic membrane of embryonated chicken eggs (Fuchs et al., 2007), and in primary kidney and liver cells of chickens or chicken embryos (Mayer et al., 1967). Furthermore, the LMH cell line derived from a chemically induced chicken liver tumor permits ILTV replication (Kawaguchi et al., 1987; Schnitzlein et al., 1994; Scholz et al., 1993). First, ILTV infection of permissive chicken cells gives rise to the formation of syncytia and nuclear inclusion bodies (Reynolds et al., 1968). After 3 to 5 days, lysis of the cells causes plaque formation.

During the replication cycle of ILTV, first infectious progeny viruses are formed 8 to 12 hours (h) post-infection, and reach a maximum within 24 to 30 h (Fuchs et al., 2000). Similar to other herpesviruses, ILTV has typical steps of morphogenesis (Granzow et al., 2001; Guo et al., 1993; Mettenleiter, 2002) and a cascade-like regulation of viral gene expression (Prideaux et al., 1992). In the nucleus, virus replication takes place and then the genome is packaged. After that,

the nucleocapsids are transported into the cytoplasm following envelopment and de-envelopment steps at the inner and outer leaflets of the nuclear membrane, respectively. Finally, the cytoplasmic capsids that are covered with an electron dense tegument are re-enveloped by a second budding event in the trans-golgi compartment which is followed by release of mature particles by exocytosis.

In chicken, protection against ILTV is based on cell-mediated immune responses (Fahey and York, 1990). Also, prevention of ILTV infection is done using attenuated live-virus vaccines delivered via eye drop or sometimes aerosol or drinking water (Han and Kim, 2003). Since reversion of the vaccine to the virulent strain might occur (Clarke et al., 1980; Guy et al., 1991), a genetically engineered stable gene deletion mutant lacking nonessential, but virulence determining genes could be a safe alternative to the current vaccine strains (Davison et al., 2006; Tong et al., 2001) but are still under investigation. Vaccination against ILTV has been a very reliable means of ILT prevention (Fuchs et al., 2005). However, vaccine breaks emerge as a common problem due to the increased virulence of the virus by recombination between multiple vaccine and or field strains (Lee et al., 2012). One of the ways of addressing these issues may be the development of novel approaches that could be used as alternatives or adjunct to the existing ILT control methods. One such approach may be the stimulation of innate immune system through synthetic or natural PAMPs. Engagement of PRRs with PAMPs leads to the induction of anti-viral, anti-bacterial, anti-parasitic and anti-fungal host mechanisms that, eventually, guide the adaptive arm of the immune system to generate long lasting immunity against invading pathogens.

In avian, NO mediated antiviral response has been studied using two *in vitro* models; HD11 cell line-reovirus and CEF-HVT/MDV (Pertile et al., 1996; Xing and Schat, 2000). It is

not known whether any other avian cell line is capable of activating antiviral response against any other avian virus. With a view of the considerable economic importance, it was of interest to delineate the role of TLR4 signaling pathway in inducing antiviral mechanisms against ILTV. The study of molecular mechanisms behind antiviral response induced by TLR4 ligands is important in the development of new strategies for ILTV control. In particular, we asked following research questions:

1.11 Research questions:

1. Does stimulation of ILTV susceptible avian hepatocyte cell line, LMH, using TLR4 ligand, LPS, increase NO production?
2. Does stimulation of avian macrophages using TLR4 ligand (LPS) increase NO production?
3. Does stimulation of avian macrophages using TLR4 ligand (LPS) increase the surface expression of its receptors (TLR4 and CD14)?
4. Does stimulation of avian macrophages using TLR4 ligand (LPS) induce antiviral response against an avian herpesvirus, ILTV?
5. Does inhibition of TLR4 mediated NO production in avian macrophages abolish antiviral effect induced by TLR4 ligand (LPS) against ILTV?
6. Does use of a NO donor as a source of NO confer antiviral effect against ILTV?

1.12 Hypotheses:

1. Treatment of ILTV susceptible avian hepatocyte cell line, LMH by TLR4 ligand (LPS) increases the expression of antiviral bioactive molecule, NO
2. Treatment of avian macrophages by TLR4 ligand (LPS) increases the expression of antiviral bioactive molecule, NO
3. Treatment of avian macrophages by TLR4 ligand (LPS) increases the expression of its receptors, TLR4 and CD14
4. Treatment of avian macrophages by TLR4 ligand (LPS) induces antiviral response against ILTV
5. Inhibition of NO production by avian macrophages abolishes antiviral response induced by TLR4 ligand (LPS) against ILTV
6. NO released by a NO donor confers antiviral effect against ILTV

CHAPTER 2: INDUCTION OF TOLL-LIKE RECEPTOR 4 SIGNALING IN AVIAN CELLS INHIBITS INFECTIOUS LARYNGOTRACHEITIS VIRUS REPLICATION IN A NITRIC OXIDE DEPENDENT WAY

2.1 Abstract:

LPS is one of the PAMPs that activates TLR4 signaling pathway eliciting antiviral host responses in mammals, although information on such responses in avian species is scarce. Our objectives were to characterize the LPS induced innate responses particularly the expression of LPS receptors (TLR4, CD14) and one of the downstream molecules, NO, in avian cells and observe whether TLR4 mediated induction of NO can elicit antiviral response against ILTV replication. We found that ILTV susceptible LMH cells express LPS receptors in very little number of cells and are not responsive to EC-LPS and SE-LPS treatments, as has been evaluated by production of NO. However, LPS was capable of inducing the expression of TLR4, CD14 and NO production in an avian macrophage cell line, MQ-NCSU. We also showed that TLR4 mediated NO production can lead to antiviral response against ILTV replication when MQ-NCSU cells were treated with LPS and the resultant supernatant was then transferred to ILTV replicating cells to assess antiviral activity. Antiviral activity of NO was blocked by a selective inhibitor, S-methylisothiourrea sulphate (SMT) that inhibits iNOS. Furthermore, using a NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP) as a source of NO, it was observed that antiviral response to ILTV replication in LMH cells depends on NO. These observations confirm that the antiviral activity is positively correlated with NO production. Our data show that LPS can be a potential innate immune stimulant that can be used against ILTV infection in chickens that require further evaluation in vivo.

2.2 Introduction:

Host-pathogen interaction activates early host responses that are mediated by innate immune system (Janeway and Medzhitov, 2002). Recognition of PAMPs by the innate immune system is mediated by a large group of innate immune receptors called PRRs (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). Among PRRs, TLRs play a predominant role in pathogen recognition. PAMPs recognition by cell associated TLRs leads to the activation of intracellular signaling pathways that trigger microbial killing mechanisms and numerous other functions relevant to host responses (Kobayashi et al., 2002; Nau et al., 2002).

TLRs and their downstream signaling components are mostly conserved in chickens (Lillehoj and Li, 2004; Lynn et al., 2003; Philbin et al., 2005). TLR4 is expressed not only on the surface membrane of immune and non-immune cell types but also in the cytoplasm of intestinal epithelial cells (Arpaia et al., 2011; Tang et al., 2008). One of the well-characterized ligands that bind with TLR4 is LPS derived from gram-negative bacteria such as *E. coli* (EC-LPS) and *S. enteritidis* (SE-LPS). LPS recognition by TLR4 requires the activation of co-receptors, such as CD14 and MD2. Unlike in mammals, induction of chTLR4 signaling does not lead to production of type I IFNs via TLR4/TRAM (TRIF-related adaptor molecule)/TRIF (TIR-domain-containing adaptor protein inducing IFN- β) pathway (Kestra and van Putten, 2008). Instead, chTLR4 and co-receptors, in response to LPS, induce pro-inflammatory mediators such as iNOS, IL-1 β , IL-6, and IL-18 (Dil and Qureshi, 2002b; Farnell et al., 2003; He et al., 2006a). NO, a gaseous free radical produced following iNOS activation, plays a critical role in immune response and pathogenesis of several diseases. This molecule is generated in a wide range of cell types and tissues by the simultaneous conversion of L-arginine into L-citrulline (Lillehoj et al., 2004; Nathan, 1992).

It has been shown that LPS-TLR4 signaling induce antiviral effects against viruses such as HIV, human cytomegalovirus, classical swine fever virus, and avian reovirus (Croen, 1993; Harwani et al., 2007; Knoetig et al., 2002; Pertile et al., 1996; Regev-Shoshani et al., 2013). NO mediated antiviral response also has been shown for mammalian viruses (Croen, 1993; Mehta et al., 2012; Regev-Shoshani et al., 2013) and avian viruses such as reovirus, herpes virus of turkeys (HVT) and MDV (Pertile et al., 1996; Xing and Schat, 2000).

It has been shown that EC-LPS and IFN- γ mediated NO production inhibit replication of MDV in chicken embryo fibroblasts (CEF) (Xing and Schat, 2000). LPS mediated NO production in avian macrophages, HD11 cells also has been shown to inhibit reovirus replication (Pertile et al., 1996). It is not known whether any other avian macrophage cell lines can elicit antiviral response similar to HD11 macrophages against other avian viruses when stimulated using EC-LPS and SE-LPS, with concomitant increase in TLR4 and CD14 expression and NO production.

We investigated whether ILTV susceptible LMH cell line and avian macrophage cell line, MQ-NCSU cells are able to respond following treatment with a range of EC-LPS or SE-LPS producing NO. We then examined whether EC-LPS and SE-LPS can increase the expression of LPS receptors, TLR4 and CD14, in MQ-NCSU cells in response to LPS. Finally, we evaluated whether NO in culture supernatants elicit antiviral effects against another avian viral infection other than reovirus, HVT, and MDV. We found that only avian macrophages, MQ-NCSU could be stimulated with EC-LPS and SE-LPS to produce significant amount of NO. This increase in NO was associated with increase in the expression of LPS receptors and antiviral response against ILTV.

2.3 Materials and methods:

2.3.1 Cells and virus:

Three cell lines were used in the experiments; the avian macrophage cell line, MQ-NCSU cells was a gift from Dr. Shayan Sharif (University of Guelph, Canada) and the murine macrophage cell line, RAW 264.7 cells was a gift from Dr. Robin Yates (University of Calgary, Canada). The LMH cell line (ATCC® CRL-2117™) and ILTV were purchased from ATCC.

2.3.2 Cell culture:

LMH cells were cultured in flasks or plates pre-coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA). The growth medium for LMH cells consists of Waymouth's MB 752/1 medium (Invitrogen, Burlington, ON, Canada) whereas RAW 264.7 cells were grown in DMEM (Invitrogen, Burlington, ON, Canada). The media were supplemented with heat-inactivated fetal bovine serum (FBS) 10% (Cellgro, Manassas, VA, USA), 100 units of penicillin and 100µg of streptomycin per ml (Invitrogen, Burlington, ON, Canada), and L-glutamine 1% (Invitrogen, Burlington, ON, Canada). The MQ-NCSU cells were cultured in LM HAHN medium comprising of Leibovitz L-15 medium (38.5 ml), McCoy's 5A medium (38.5 ml), Chicken serum (10.0 ml), L-Glutamine-200mM (1.0 ml), sodium pyruvate-100X (1.0 ml), 100 U of penicillin and 100 µg of streptomycin per ml, fungizone 250 µg/ml (0.2 ml), 2-mercaptoethanol 1.0 mM (1.0 ml) (Invitrogen, Burlington, ON, Canada), tryptose phosphate broth (5.0 ml) (Sigma-Aldrich, St. Louis, MO, USA), and FBS (8.0 ml) (Cellgro, Manassas, VA, USA) (Okamura et al., 2004). The cells were maintained in cell culture incubator (Binder GmBH, Tuttlingen, Germany) under 5% carbon dioxide (CO₂) at 37°C (LMH and RAW 264.7) or 40°C (MQ-NCSU).

2.4 Experimental design:

2.4.1 Propagation and titration of ILTV:

LMH cell monolayers were prepared in 6-well tissue culture plates and infected with 10-fold serial dilutions of ILTV with a titre of 1.27×10^6 plaque forming unit (PFU)/ml with uninfected controls. 2-day (d) and 5 d post-infection, ILTV infected and control 6-well plates were examined microscopically to see morphological changes produced by ILTV. At the same time, images were taken to document the cytopathic effects of ILTV infection in LMH cells. Five d following the infection, the plates were stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) to visualize the plaques.

2.4.2 Stimulation of LMH and RAW 264.7 cells with LPS for quantification of NO production:

LMH and RAW 264.7 cells were propagated and cell stimulation was done in 96 well plates (1×10^5 cells/well). The cells were cultured for 24 h, growth media removed, washed the monolayer with $1 \times$ hanks balanced salt solution (HBSS) (Invitrogen, Burlington, ON, Canada) and stimulated either with EC-LPS (Sigma-Aldrich, St. Louis, MO, USA) or SE-LPS (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 μ g/ml. Different concentrations of LPS were made in phenol red free growth media. The plates were incubated for 24, 48 or 72 h at 37°C with 5% CO₂ in a cell culture incubator before collection of culture supernatants for NO assay. Each treatment was done in triplicate.

2.4.3 Stimulation of macrophages (MQ-NCSU and RAW 264.7) with LPS for quantification of NO production:

MQ-NCSU and RAW 264.7 macrophage cell lines were propagated and cell stimulation was done in 96 well plates (1×10^5 cells/well). The cells were cultured for 24 hours (h), growth media removed, washed the monolayers with $1 \times$ hanks balanced salt solution (HBSS) and stimulated either with EC-LPS (Sigma-Aldrich, St. Louis, MO, USA) or SE-LPS (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and $0 \mu\text{g/ml}$. Different concentrations of LPS were made in applicable phenol red free growth media. The plates were incubated for 24, 48 or 72 h at either 37°C (LMH and RAW264.7) or 40°C (MQ-NCSU) in a CO_2 incubator before collection of culture supernatants for NO assay. Each treatment was done in triplicate or six replicates.

2.4.4 Detection of TLR4 and CD14 expression in LMH cells:

The expression of TLR4 and CD14 in LMH cells was quantified without any treatment to determine the presence of these receptors in LMH cells. LMH cells were cultured in T-75 flasks for 24 h, the cells were dislodged from flasks using TrypLE™ Express (Invitrogen, Burlington, ON, Canada) and 2×10^6 cells were stained for TLR4 and CD14 expression. The experiment was done in triplicate.

2.4.5 Stimulation of avian macrophages (MQ-NCSU) with LPS for quantification of TLR4 and CD14 expression:

The avian macrophage cells, MQ-NCSU responded similar to the RAW264.7 cells following LPS treatment. The lowest concentration of LPS ($0.1 \mu\text{g/ml}$) that produce the highest

amount of NO was chosen to study the expression of TLR4 and CD14 expression in MQ-NCSU cells following LPS treatment. MQ-NSU cells were cultured in T-75 flasks for 24 h and treated with either EC-LPS or SE-LPS with un-stimulated controls. 3, 6 and 12 h post-treatment, cells were dislodged from flasks using TrypLE™ Express (Invitrogen, Burlington, ON, Canada) and 2×10^6 cells from each treatment or control stained for TLR4 and CD14 expression. The experiment was done in triplicate.

2.4.6 Antiviral assay for culture supernatants produced following avian macrophage (MQ-NCSU) stimulation with LPS in vitro:

MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatments with 0.1 µg/ml EC-LPS, SE-LPS or PBS. 18 h post-treatment culture supernatants, (500µl/well) were collected and transferred to naive LMH cells. Then, LMH cells were infected with 10-fold serial dilution of ILTV with a titre of 1.27×10^6 plaque forming unit (PFU)/ml. 5 days (d) following the infection, the plates were stained with 1% crystal violet and number of plaques was counted. The experiment was done in triplicate.

2.4.7 Determining the antiviral effect of LPS mediated NO production in vitro:

To determine the optimal concentration of iNOS inhibitor, S-methylisothiourrea sulphate (SMT; Sigma-Aldrich, St. Louis, MO, USA), MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatment with 0.1 µg/ml EC-LPS with different concentrations of SMT (0, 10, 100, 500 and 1000µg/ml) for 18 h. Each treatment was done in triplicate.

Following determination of the optimal concentration of SMT, MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatments with 0.1 $\mu\text{g/ml}$ EC-LPS or SE-LPS with or without SMT, or PBS alone. 6 and 18 h post-treatment culture supernatants were collected and transferred to naive LMH cells in 6 well plates (4×10^6 cells per well). Then, LMH cells were infected with 100 PFU/well of ILTV. Five d following the infection, the plates were stained with 1% crystal violet and number of plaques was counted. The experiment was done in triplicate.

2.4.8 Determining the antiviral effect of NO using a NO donor in vitro:

To further confirm the antiviral response mediated by NO, we used a NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP, Invitrogen, Burlington, ON, Canada) as a source of NO. First, a concentration of SNAP that yields nitrite concentration near to what resulted with EC-LPS treatment (0.1 $\mu\text{g/ml}$) of MQ-NCSU cells for 18 h, was determined using a range of SNAP solutions (800, 400, 200, and 100 μM) made in HBSS. After 10 minutes (min) exposure to UV visible light to accelerate the NO release, NO production was measured using Griess assay. Each concentration of NO donor was done in triplicate. Following determination of the required concentration of SNAP, this concentration of SNAP was used in antiviral assay. The antiviral assay was done in 6 well plates cultured with LMH cell (2×10^6 cells per well). We included four groups: 1). control treated with HBSS, 2) conditioned media of MQ-NCSU cells without treatment transferred to LMH cells, 3) LMH cells treated with 0.1 $\mu\text{g/ml}$ EC-LPS for 18 h, and 4) 800 μM SNAP made in HBSS transferred to LMH cells. Then, treated or control LMH cells were infected with 100 PFU/well of ILTV. 5 d following the infection, the plates were

stained with 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and the number of plaques was counted. The experiment was done in triplicate.

2.5 Assay for endotoxin contamination:

The LPS concentrations in the used reagents such as PBS, HBSS, FBS, chicken serum, endotoxin free water, gelatin and all the growth media were determined using Limulus Amebocyte Lysate LPS detection assay (E-TOXATE™ kit; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

2.6 Assay for NO production:

Cell-free culture supernatants were assayed for nitrite, a stable metabolite of NO, using a Griess reagent system (Promega, Madison, WI, Canada) according to the manufacturer's recommendation. A detailed protocol of Griess assay is enclosed (Appendix 1). The optical density (OD) of the final colorimetric product resulted from the reaction of nitrite with 1% sulfanilamide and 0.1% N-1-naftylethyldiamin dihydrochloride was read at 548 nm using a SPECTRAMAX M₂ microplate reader (Molecular devices, Sunnyvale, CA). The concentration of nitrite was quantified using sodium nitrite as a standard.

2.7 Flow cytometry:

Standard flow cytometry procedures were used in the experiments. Briefly, the isolated LMH cells or MQ-NCSU cells were washed with 1% bovine serum albumin (BSA) fraction V (w/v; OmniPur, EMD, Darmstadt, Germany) made in PBS and centrifuged for 10 min at $211 \times g$

(4° C) and then resuspended in 100µl of 1% BSA with purified mouse IgG (10µg per one million cells) (Abcam, Ontario, Canada) about 10 min for Fc blocking. Following centrifugation once again, cells were resuspended in the dark with a final concentration of 0.02µg/µL mouse anti-human CD284 (TLR4) phycoerythrin (PE) mAb (eBioscience, San Diego, CA, USA), or mouse anti-human CD14 FITC mAb (eBioscience, San Diego, CA, USA), with respective isotype controls or 1% BSA (unstained controls) on ice for 30 min. Finally, cells were washed twice with 1% BSA. Flow cytometry samples were analyzed using a BD LSR II (BD Biosciences, Mississauga, ON, Canada) flow cytometer.

2.8 Data analysis

NO concentrations data were analysed using ANOVA test followed by Tukey's test (Minitab Inc., State College, Pennsylvania, USA) for mean separation in order to identify differences between groups. Before being tested, each set of data was analyzed using the Grubbs' test (GraphPad software Inc., CA 92037, USA) to identify outliers. Comparisons were considered significant at $P \leq 0.05$.

2.9 Results:

2.9.1 ILTV replicates and produces cytopathic effects on monolayer of LMH cells:

ILTV has been known to replicate and produce plaques in LMH cells (Schnitzlein et al., 1994). We confirmed that the ILTV replicates in LMH cells and results in visible plaques at the second passage of the virus. The microscopic images of infected and uninfected monolayers of LMH cells 2- and 5-day post-infection are illustrated in Figure 2-1. By 2-day post-infection, the

infected cells increased in size and became rounded but still attached to the cell tissue plates (Figure 2-1b). By 5 day post-infection, the infected cells detached from the plates and plaque formation became evident (Figure 2-1d). Furthermore, the representative stained LMH cells infected with 10-fold serial dilutions of ILTV are shown in Figure 2-2.

2.9.2 LMH cells are unable to produce NO following LPS stimulation compared to RAW

264.7 cells:

Since ILTV replicates in LMH cells producing appreciable plaque forming units, compared to RAW 264.7 cells, we next evaluated whether these cells could be stimulated with LPS to produce NO with goal of using LMH cells for LPS stimulation and evaluation of treatment effect on ILTV replication. NO production in LMH and RAW264.7 cells following stimulation with a range of concentrations of EC-LPS and SE-LPS is illustrated in Figures 2-3 and 2-4, respectively.

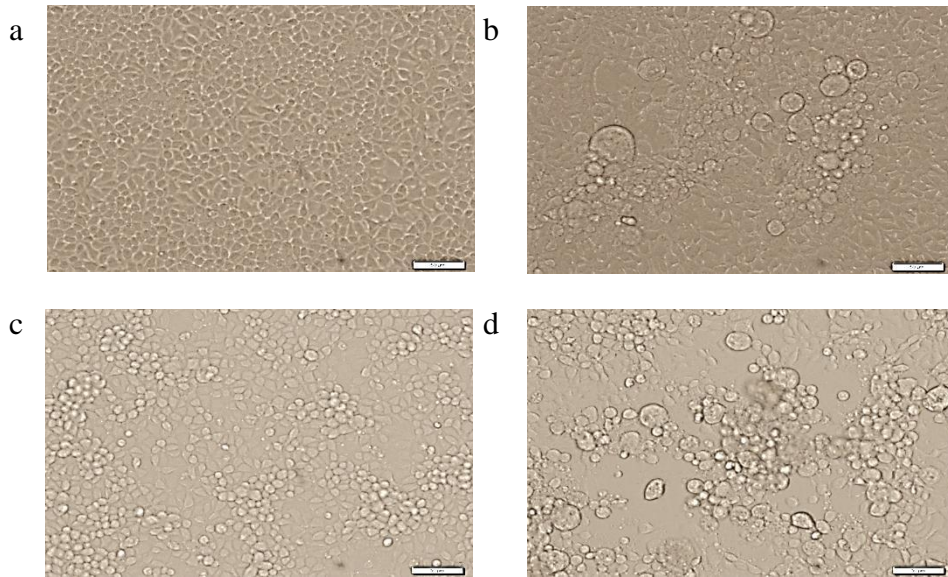


Figure 2-1: ILTV replicates and produces cytopathic effects on monolayer of LMH cells. LMH cells were cultured in 6-well tissue culture plates, and they were infected with 10-fold serial dilutions of ILTV with a titer of 1.27×10^6 PFU/ml with uninfected controls. 2- and 5-day post-infection ILTV infected and control 6-well plates were examined microscopically to observe morphological changes produced by the ILTV. a and c) uninfected monolayers of LMH cells at 2- and 5-day post-infection respectively, b and d) ILTV infected monolayers of LMH cells at 2- and 5-day post infection respectively. The scale bar represents 50 μ m.

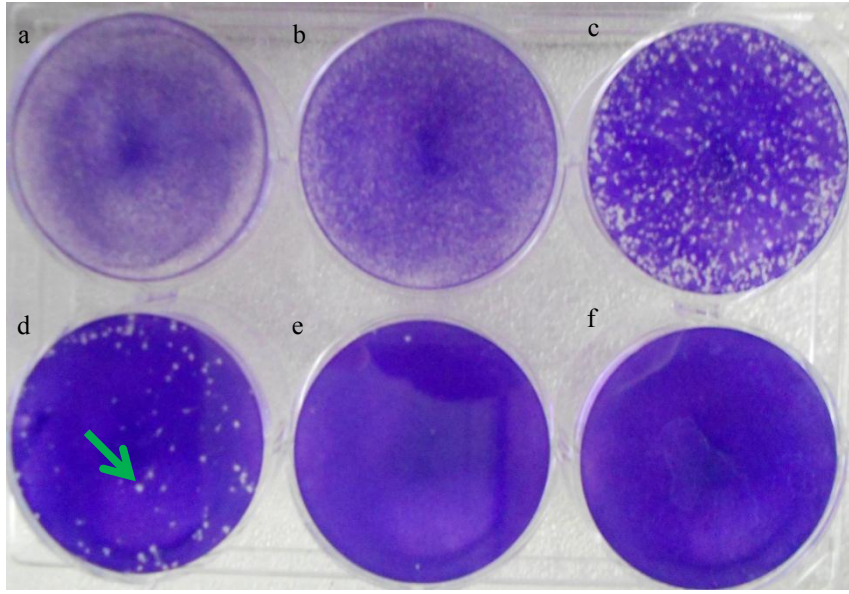


Figure 2-2: ILTV produces visible plaques in monolayers of LMH cells. LMH cell monolayers were prepared in 6-well tissue culture plates, infected with 10-fold serial dilutions of ILTV with a titre of 1.27×10^6 plaque forming unit (PFU)/ml with uninfected controls. 5 d post-infection infected and control 6-well plates were stained with 1% crystal violet to visualize plaques. The figure illustrates a representative ILTV infected 6-well plate. a) 10^{-1} ; b) 10^{-2} ; c) 10^{-3} ; d) 10^{-4} ; e) 10^{-5} ; f) 10^{-6} . The arrow points an ILTV plaque.

We discovered that compared to RAW 264.7, LMH cells were not responsive for stimulation with range of EC-LPS and SE-LPS concentrations (0.01-100 $\mu\text{g/ml}$) and did not produce a significant amount of NO when compared to the un-stimulated controls ($P > 0.05$). The viability of the cells at all different time courses (24, 48 and 72 h post-treatment) was more than 95% according to trypan blue exclusion.

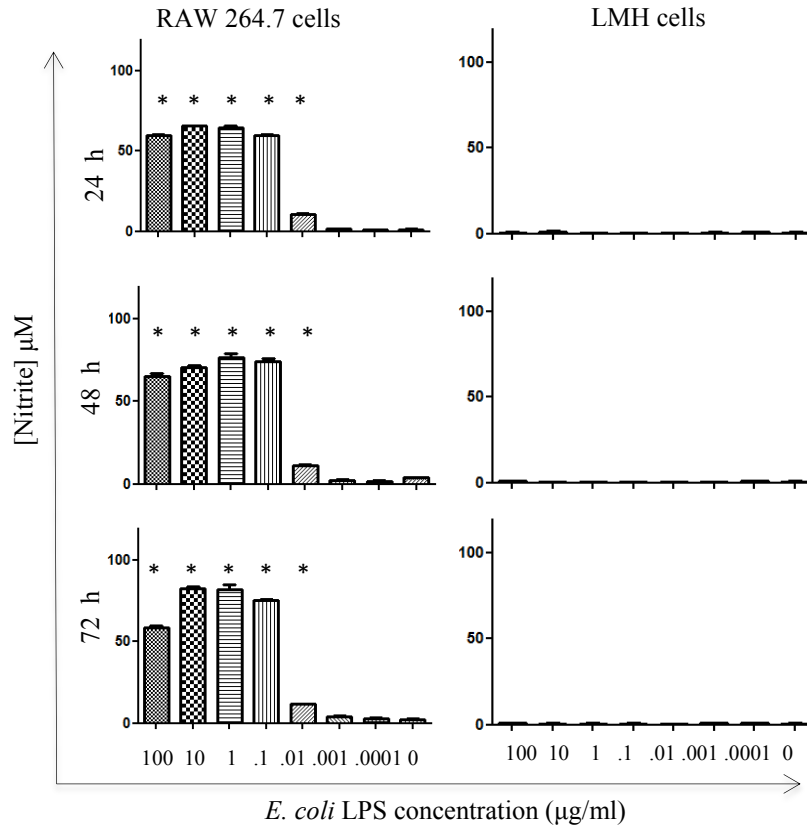


Figure 2-3: LMH cells are unable to produce NO following EC-LPS stimulation. LMH and RAW 264.7 cells were cultured in 96 well plates (1×10^5 cells/well) 24 h before treatments with 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 $\mu\text{g/ml}$ concentrations of EC-LPS. 24, 48 and 72 h post-treatment culture supernatants were collected and analyzed for NO production using Griess test. a-c) represent NO production in RAW 264.7 cells stimulated with EC-LPS 24, 48 and 72 h post-infection respectively whereas d-f) represent NO production in LMH cells stimulated with EC-LPS 24, 48 and 72 h post-infection respectively. Each bar represents mean values \pm SEM for 3 replicates for LMH cells. The data were analyzed using ANOVA test followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly higher when compared to the control.

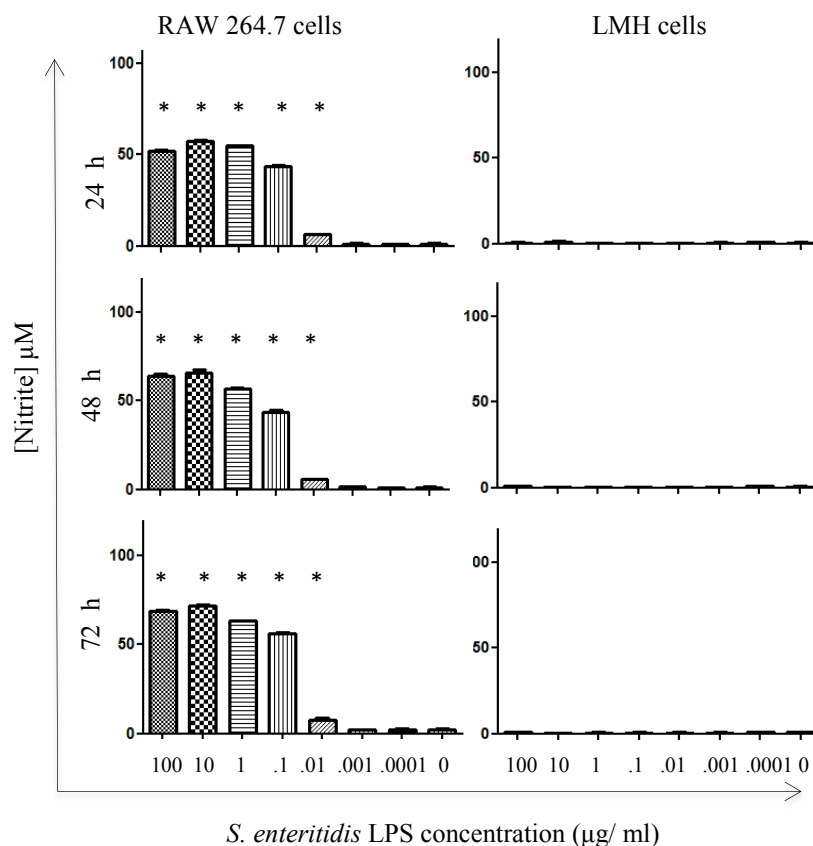


Figure 2-4: LMH cells are unable to produce NO following SE-LPS stimulation. LMH and RAW 264.7 cells were cultured in 96 well plates (1×10^5 cells/well) 24 h before treatments with 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 µg/ml concentrations of SE-LPS. 24, 48 and 72 h post-treatment culture supernatants were collected and analyzed for NO production using Griess test. a-c) represent NO production in RAW 264.7 cells stimulated with SE-LPS 24, 48 and 72 h post-infection respectively whereas d-f) represent NO production in LMH cells stimulated with SE-LPS 24, 48 and 72 h post-infection respectively. Each bar represents mean values \pm SEM for 3 replicates for LMH cells. The data were analyzed using ANOVA test followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly higher when compared to the control.

2.9.3 LMH cells express TLR4 and CD14 constitutively:

Since LMH cells are not responsive to different concentrations of EC-LPS and SE-LPS, we next evaluated whether LMH cells express LPS receptors such as TLR4 and CD14 receptors. As illustrated in the Figure 2-5, only a small percentage of LMH cells express TLR4 and CD14 receptors.

2.9.4 Avian macrophages (MQ-NCSU) produce NO following treatment with LPS similar to murine macrophages (RAW 264.7) in a dose dependent manner:

NO production in MQ-NCSU and RAW 264.7 cells following stimulation, with a range of concentrations of LPS from EC-LPS and SE-LPS, is illustrated in Figures 2-6 and 2-7. MQ-NCSU cells responded to stimulation with LPS producing a significant amount of NO when compared to the un-stimulated controls ($P \leq 0.05$). The pattern of NO production by MQ-NCSU cells was similar to that observed in murine macrophages, RAW264.7. Both EC-LPS and SE-LPS were capable of stimulating MQ-NCSU cells and RAW264.7 cells similarly. Of the tested LPS concentrations of EC-LPS and SE-LPS, the concentrations that ranged from 0.01-100 $\mu\text{g/ml}$ resulted in significantly higher NO concentrations in MQ-NCSU and RAW264.7 cells when compared to the controls following LPS stimulation at 24, 48 and 72 h post-stimulation ($P \leq 0.05$). Viability of the cells in all different time courses were more than 95% according to trypan blue exclusion.

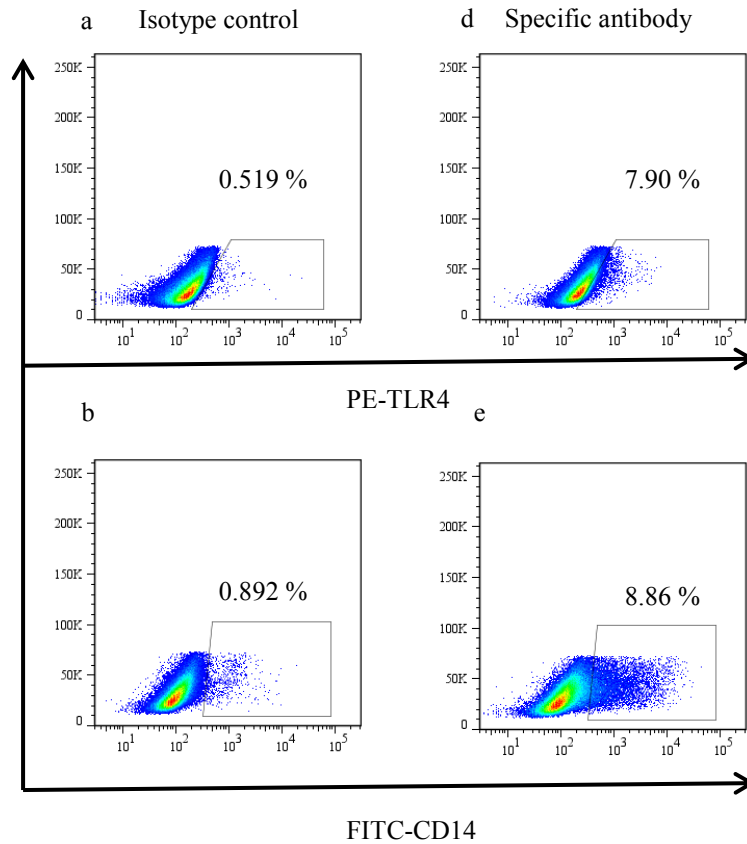


Figure 2-5: LMH cells express TLR4 and CD14 receptors constitutively. LMH cells were cultured in T-75 flasks for 24 h, cells were isolated and 2×10^6 cells were stained for TLR4 expression using PE labelled mouse anti-human TLR4 and CD14 expression using FITC labeled mouse anti-human CD14 mAbs for FACS analysis. The experiment was done in triplicate. a) a representative FACS plot of isotype control for PE labeled mouse anti-human TLR4 b) a representative FACS plot of isotype control for FITC labeled mouse anti-human CD14 c) a representative FACS plot showing TLR4+ LMH cells, d) representative FACS plots showing CD14+ LMH cells.

2.9.5 Avian macrophages (MQ-NCSU) increase the expression of TLR4 and CD14

following treatment with LPS:

Since MQ-NCSU cells were responsive to LPS, similar to murine macrophages, as has been indicated by the significant production of NO, MQ-NCSU cells were used for studying the expression of the LPS receptors, TLR4 and CD14. Although the concentration of the LPS used in this experiment was not the lowest concentration that produce significant NO production (0.01 µg/ml), we used 0.1 µg/ml as the lowest concentration of LPS that produced a substantial amount of NO over the controls.

Following treatment with EC-LPS, the expression of TLR4 and CD14 have been increased significantly in MQ-NCSU cells when compared to the controls at 6 h ($P \leq 0.05$) and 12 h ($P \leq 0.05$) post-treatment (Figure 2-8); whereas with SE-LPS, the MQ-NCSU cells expressing both TLR4 and CD14 increased significantly at 12 h post stimulation ($P \leq 0.05$) (Figure 2-9).

2.9.6 LPS treated culture supernatant of avian macrophages (MQ-NCSU) inhibits ILTV replication in vitro:

Since it has been shown that NO can elicit antiviral response against avian viruses such as HVT, MDV and reovirus (Pertile et al., 1996; Xing and Schat, 2000), we hypothesised that culture supernatant of MQ-NCSU cells rich in NO may inhibit ILTV replication in LMH cells. Culture supernatants of MQ-NCSU cells produced following EC-LPS and SE-LPS treatment were transferred to ILTV susceptible cell line (LMH), and inhibited the ILTV replication significantly ($P \leq 0.05$; Figure 2-10).

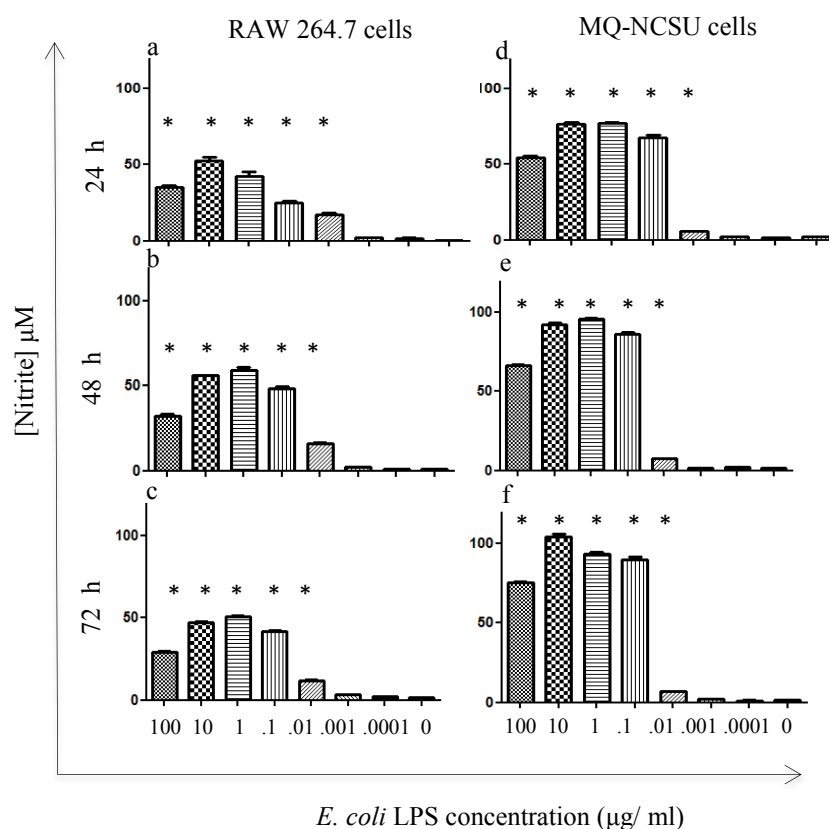


Figure 2-6. Avian macrophages (MQ-NCSU) produce NO following treatment with EC-LPS similar to mouse macrophages in a dose dependent manner. Murine (RAW 264.7) and MQ-NCSU were cultured in 96 well plates (1×10^5 cells per well) 24 h before treatments with 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 µg/ml concentrations with EC-LPS. 24, 48 and 72 h post-treatment culture supernatants were collected and assayed for NO production using Griess assay. Each bar represents mean values \pm SEM for 6 replicates for RAW 264.7 and MQ-NCSU cells. The data were analyzed using ANOVA test followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly higher when compared to the control.

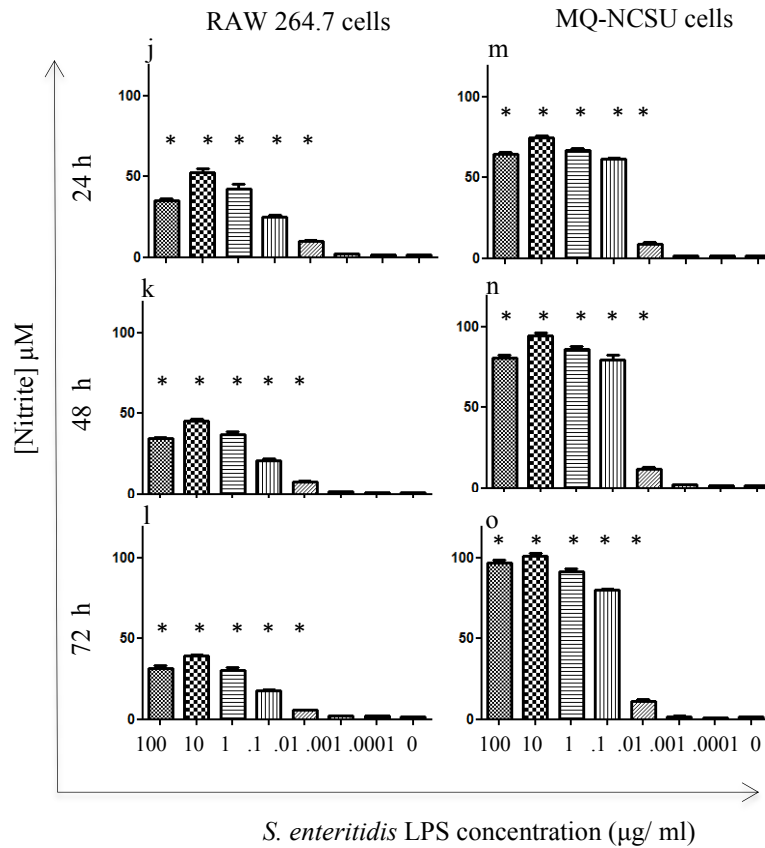


Figure 2-7. Avian macrophages (MQ-NCSU) produce NO following treatment with SE-LPS similar to mouse macrophages in a dose dependent manner. Murine (RAW 264.7) and MQ-NCSU were cultured in 96 well plates (1×10^5 cells per well) 24 h before treatments with 100, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0 µg/ml concentrations with SE-LPS. 24, 48 and 72 h post-treatment culture supernatants were collected and assayed for NO production using Griess assay. Each bar represents mean values \pm SEM for 6 replicates for RAW 264.7 and MQ-NCSU cells. The data were analyzed using ANOVA test followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly higher when compared to the control.

2.9.7 NO produced by avian macrophages (MQ-NCSU) following LPS treatment inhibits ILTV replication in vitro:

Since the culture supernatants of MQ-NCSU cells that were produced following EC-LPS and SE-LPS treatment inhibited ILTV replication significantly (Figure 10), we then hypothesized that the inhibition of ILTV replication may be associated with increased NO production in MQ-NCSU cells.

First we determined the dose of SMT to be used for inhibition of NO production. We found that 1 mM concentration of SMT inhibits LPS induced NO production similar to the unstimulated control group (Figure 2-11).

Treatment of MQ-NCSU cells with EC-LPS and SE-LPS with an iNOS inhibitor significantly reduced the NO production when compared to MQ-NCSU cells treated only with EC-LPS and SE-LPS at 6 and 18 h post-treatment ($P \leq 0.05$; Figure 2-12 a and b). The viability of the cells in both time courses were more than 95% according to trypan blue exclusion. Culture supernatants produced by MQ-NCSU cells 6 h post-treatment with EC-LPS with an iNOS inhibitor abrogated the inhibitory effect of ILTV replication when compared to MQ-NCSU cells treated with EC-LPS alone ($P \leq 0.05$; Figures 2-12 c and 2-13). Culture supernatants produced by MQ-NCSU cells 18 h post-treatment with EC-LPS or SE-LPS with an iNOS inhibitor abrogated the inhibitory effect of ILTV replication when compared to MQ-NCSU cells treated with EC-LPS and SE-LPS alone ($P \leq 0.05$; Figure 2-12 d and 2-13).

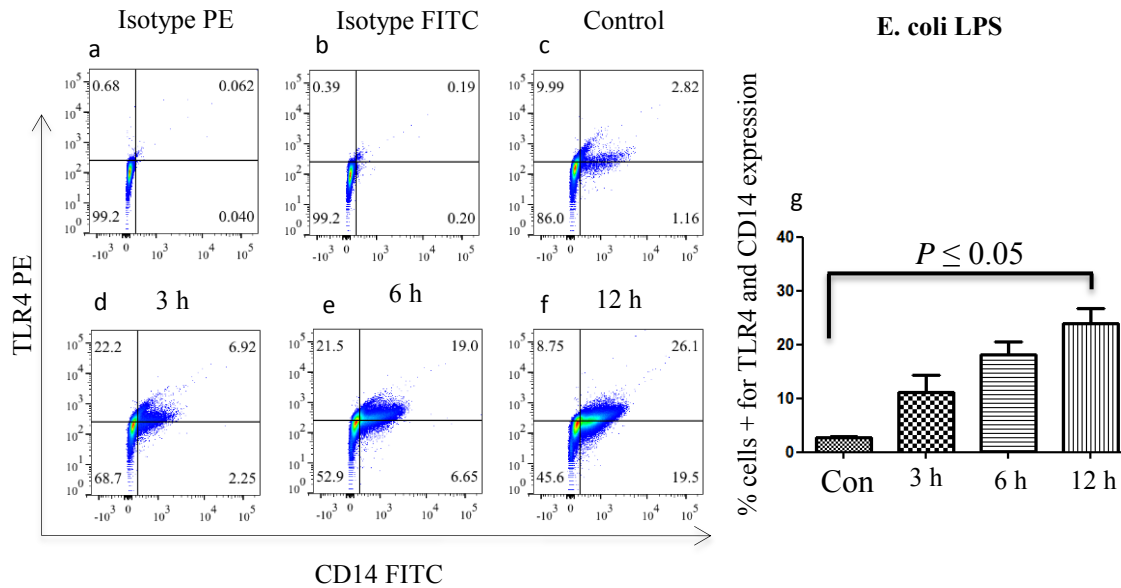


Figure 2-8. Avian macrophages (MQ-NCSU) increase the expression of TLR4 and CD14 following treatment with EC-LPS. MQ-NSU cells were cultured in T-75 flasks for 24 h and treated with 0.1 $\mu\text{g/ml}$ EC-LPS with un-stimulated controls. 3, 6 and 12 h post-treatment, the cells were isolated and 2×10^6 cells from each treatment or control stained for TLR4 expression using PE labelled mouse anti-human TLR4 and CD14 expression using FITC labeled mouse anti-human CD14 mAbs for flow cytometry analysis. The experiment was done in triplicate. Figure 2 a) a representative FACS plot of isotype control for PE labeled mouse anti-human TLR4 Ab, b) a representative fluorescent activated cell sorter (FACS) plot of isotype control for FITC labeled mouse anti-human CD14 Ab, c) a representative FACS plot showing TLR4+ CD14+ macrophages, d-f) representative FACS plots showing TLR4+ CD14+ macrophages at 3, 6 and 12 h post-treatment with EC-LPS respectively, g) illustrates the percentage of TLR4+ CD14+ macrophages 3, 6 and 12 h post-treatment with EC-LPS. To identify differences between groups ANOVA test was performed followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 .

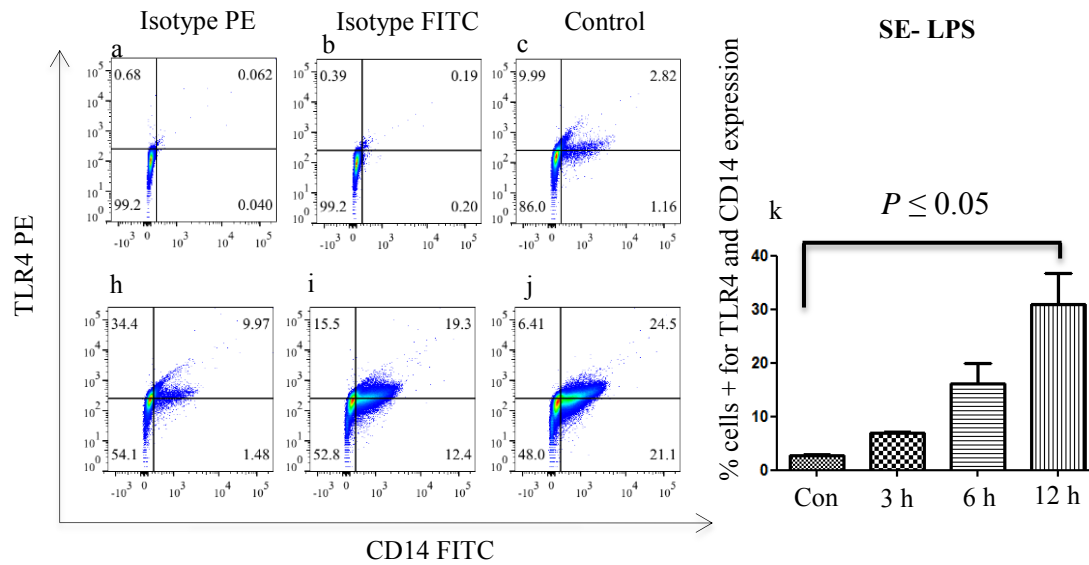


Figure 2-9. Avian macrophages (MQ-NCSU) increase the expression of TLR4 and CD14 following treatment with SE-LPS. MQ-NSU cells were cultured in T-75 flasks for 24 h and treated with 0.1 $\mu\text{g/ml}$ SE-LPS with un-stimulated controls. The experiment was conducted as has been indicated in the Figure 2.1 legend. Figure 2 a) a representative FACS plot of isotype control for PE labeled mouse anti-human TLR4 Ab, b) a representative FACS plot of isotype control for FITC labeled mouse anti-human CD14 Ab, c) a representative FACS plot showing TLR4+ CD14+ macrophages, h-j) representative FACS plots showing TLR4+ CD14+ macrophages at 3, 6 and 12 h post-treatment with SE-LPS respectively, k) illustrates the percentage of TLR4+ CD14+ macrophages at 3, 6 and 12 h post-treatment with SE-LPS. To identify differences between groups ANOVA test was performed followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 .

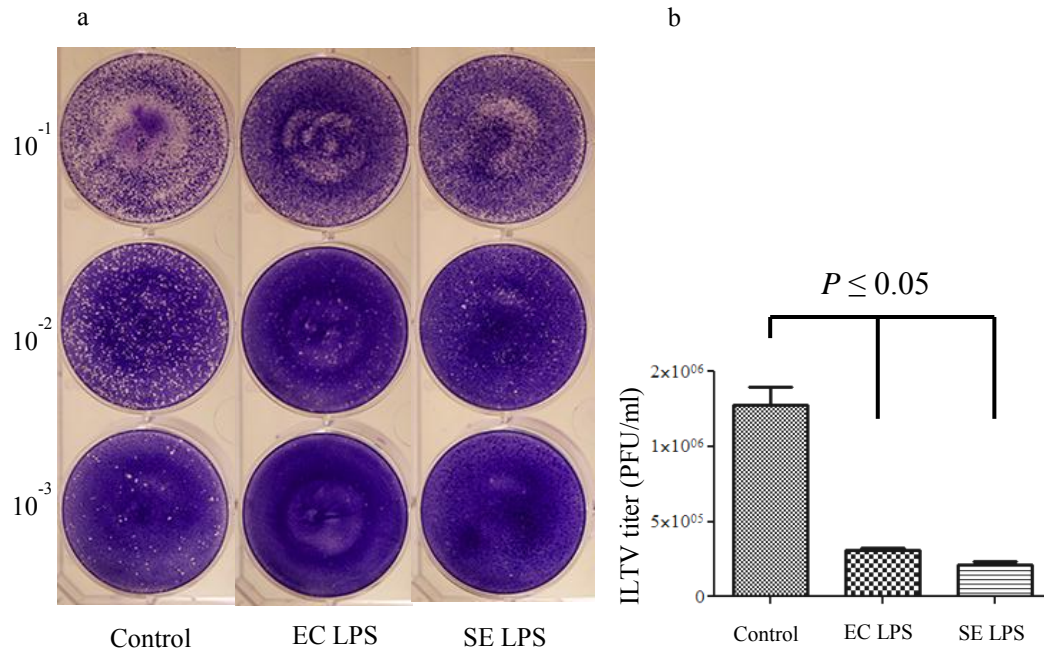


Figure 2-10. LPS treated culture supernatant of avian macrophages (MQ-NCSU) inhibits ILTV replication in vitro. MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatments with 0.1 μ g/ml EC-LPS, SE-LPS or PBS. 18 h post-treatment culture supernatants (500 μ l/well) were collected and transferred to naive LMH cells. Then, LMH cells were infected with 10-fold serial dilution of ILTV with a titer of 1.27×10^6 PFU/ml. 5 d post-treatment the plates were stained with 1% crystal violet and PFU was counted. a) illustrates ILTV titers in EC-LPS and SE-LPS treated groups when compared to the control, b) illustrates representative ILTV titration plates from EC-LPS and SE-LPS treated groups compared to the control. Data represent mean values \pm SEM. To identify differences between groups ANOVA test was performed followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 .

2.9.8 NO produced spontaneously by SNAP inhibits ILTV replication *in vitro*

We found that 800 μ M of SNAP gives rise to about 45 μ M of nitrite which is similar to the nitrite concentration produced by MQ-NCSU cells treated with 0.1 μ g/ml EC-LPS for 18 h (Figure 2-14). This concentration of SNAP was made freshly in HBSS and exposed for 10 min to UV visible light and then transferred to the naïve LMH cells to be infected with ILTV. The antiviral assay was done in 6 well plates cultured with LMH cells (2×10^6 cells per well). We included four groups: 1) control LMH cells treated with HBSS, 2) conditioned media of MQ-NCSU cells without treatment transferred to LMH cells, 3) LMH cells treated with 0.1 μ g/ml EC-LPS for 18 h, 4) 800 μ M SNAP made in HBSS transferred to LMH cells. All four groups were infected with 100 PFU ILTV/well. 5 d post-infection, the plates were stained with 1% crystal violet and PFU was counted. All experiments were done in triplicate. We found that the SNAP transferred group inhibits ILTV replication similar to what we observed for conditioned media of MQ-NCSU cells treated with 0.1 μ g/ml EC-LPS for 18 h. However, the other groups showed no antiviral response compared with unstimulated LMH cells (Figure 2-15).

2.9.9 Endotoxin concentration in reagents:

Endotoxin concentrations of all the tested reagents were below 0.06 EU/ml.

2.10 Discussion:

Our investigation described in this thesis led to four major findings. First, ILTV susceptible LMH cells express LPS receptors, but they are not responsive to EC-LPS and SE-LPS treatments as has been evaluated by production of NO. Second, EC-LPS and SE-LPS are

capable of inducing avian macrophage cell line (MQ-NCSU) similar to murine macrophages, leading to significant increase in LPS receptor expression and NO production in a dose dependent manner. Third, EC-LPS and SE-LPS elicit antiviral response against ILTV replication *in vitro*, and this protection was dependent on the increase in the expression of TLR4 and CD14 expression. Finally, the inhibition of ILTV replication mediated by EC-LPS and SE-LPS was critically dependent on the NO production by MQ-NCSU macrophages.

We showed that the expression of TLR4 and CD14 proteins on the cell surface of small portion of LMH cells was constitutive. Furthermore, LMH cells were not responsive to stimulation with a range of EC-LPS and SE-LPS as assessed by the production of NO. These findings are in agreement with the findings of Scott and colleagues who have shown that the mouse primary hepatocytes possess low amounts of the TLR4 receptor, and they are less responsive to LPS treatment (Scott et al., 2009; Shiratori et al., 1998). However, our observations contrast with the work of others who have shown that the murine hepatocytes are capable of producing NO in response to LPS treatment (Curran et al., 1989; Wood et al., 1993). The discrepancy between our results and the work of Curran et al. and Wood et al. could potentially be due to (1) species difference, (2) LMH cells gene rearrangements leading to inactivation of iNOS gene or other downstream molecule in TLR4 signaling pathway, and (3) the lack of downstream signaling molecules in LMH cells

We provided evidence that MQ-NCSU cells were responsive, as determined by NO production, to LPS treatment similar to murine macrophages. We used murine macrophages (RAW 264.7) as a positive control since previous studies have shown that murine cells could produce NO in response to LPS treatment (Nathan and Hibbs, 1991). Production of NO by MQ-NCSU cells in response to EC-LPS and SE-LPS treatment has been shown previously (Dil and

Qureshi, 2002a, b; Lillehoj and Li, 2004). In agreement with the previous studies, we also observed that MQ-NCSU cells produce NO in response to EC-LPS and SE-LPS.

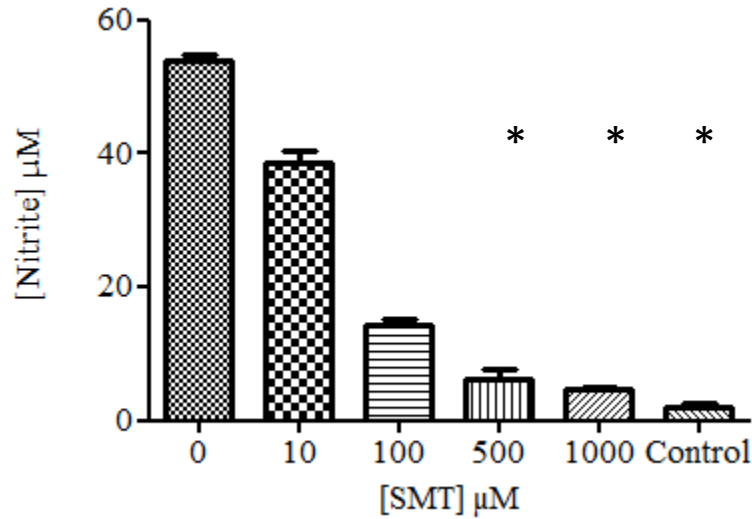


Figure 2-11. NO produced by avian macrophages (MQ-NCSU cells) following EC-LPS treatment with different concentration of SMT. MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatments with 0.1 $\mu\text{g/ml}$ EC-LPS with various concentrations of SMT (0, 10, 100, 500, 1000 μM). 18 h post-treatment, culture supernatants were collected and assayed for NO production using Griess assay. The control represents the group without LPS or SMT treatment. Data represent mean values \pm SEM. To identify differences between groups ANOVA test was performed followed by Tukey's test for mean separation. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly decreased when compared to the control.

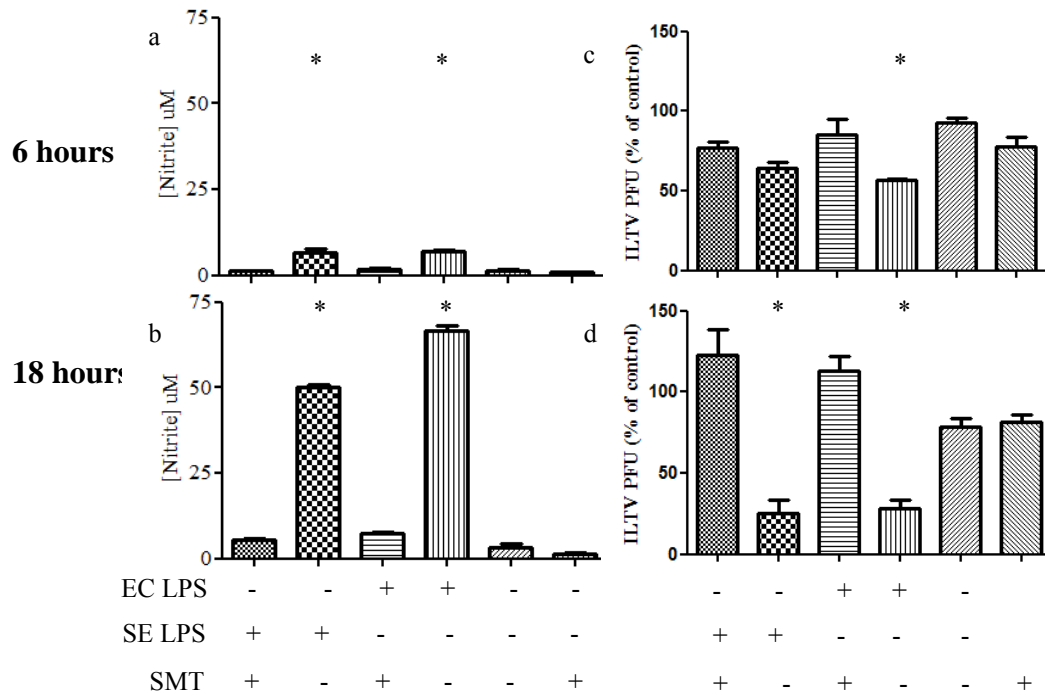


Figure2-12. NO produced by avian macrophages (MQ-NCSU) following LPS treatment inhibits ILTV replication *in vitro*. MQ-NCSU cells were cultured in 6 well plates (2×10^6 cells per well) 24 h before treatment with 0.1 $\mu\text{g/ml}$ EC-LPS or SE-LPS with or without NO inhibitor, SMT (1mM) or PBS. 6 and 18 h post-treatment culture supernatants were transferred to naive LMH cells. Then, LMH cells were infected with 100 PFU/culture of ILTV. The ability of SMT to inhibit production of NO was evaluated by Griess assay at 6 and 18 h. MQ-NCSU cells were incubated with or without SMT followed by treatment with LPS derived from EC-LPS or SE-LPS. a) represents the concentration of NO in cell culture supernatants at 6 h post-treatment, b) represents the concentration of NO in cell culture supernatants at 18 h post-treatment, c) shows the ILTV titers as a percentage following transfer of 6 h conditioned media from MQ-NCSU cells, d) shows the ILTV titers as a percentage following transfer of 18 h conditioned media from

MQ-NCSU cells. Data represent mean values \pm SEM. Significance was measured at p value of ≤ 0.05 . * Indicates that the NO production is significantly higher, or virus titer is significantly decreased when compared to the control.

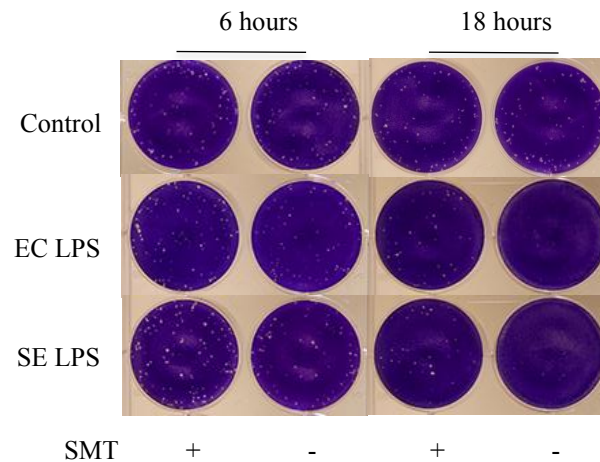


Figure 2-13. NO produced by avian macrophages (MQ-NCSU) following LPS treatment inhibits ILTV replication *in vitro*. The experiment was conducted as has been indicated in the Figure 2-12 legend. This figure illustrates representative ILTV titration plates from treatment and control groups.

It has been shown that the expression of avian CD14 and TLR4 can be quantified using anti-human mAbs against these two receptors, as they are components of the highly conserved innate immune system (Dil and Qureshi, 2002b; Janeway and Medzhitov, 2002). In agreement with this observation, we would also be able to quantify the expression of CD14 and TLR4 in MQ-NCSU cells using the same anti-human mAbs. Furthermore, we observed that MQ-NCSU cells express TLR4 and CD14 constitutively, but in a low number of cells, and both EC-LPS and SE-LPS were able to increase the expression of these two receptors significantly. These results

are in agreement with previous studies that have shown that LPS-induced up-regulation (both transcriptionally and translationally) of CD14 and TLR4 in different cell types such as human monocytes and gingival fibroblasts (Landmann et al., 1996; Tabeta et al., 2000), and avian macrophages (Dil and Qureshi, 2002a, b). Moreover, since increased expression of LPS-related receptors gives rise to stronger signal transduction and finally higher iNOS expression and activity, a large number of LPS receptors on the surface of MQ-NCSU cells is in accordance with the greater inducible NO production (Dil and Qureshi, 2002b). Not only are TLR4 and CD14 inducible in response to LPS in chicken cells such as MQ-NCSU cells, but also there is a correlation between the inducible number of LPS receptors and LPS-stimulated NO production (Dil and Qureshi, 2002b).

The TLR4 ligand, LPS, has been shown to be inhibitory against HIV, human cytomegalovirus, classical swine fever virus and avian reovirus (Croen, 1993; Harwani et al., 2007; Knoetig et al., 2002; Pertile et al., 1996; Regev-Shoshani et al., 2013). In agreement with these observations, we observed that culture supernatants collected following LPS treatment of avian macrophage cell line, MQ-NCSU could reduce ILTV replication in susceptible LMH cells.

One of the more important explanations of the antimicrobial activities of macrophages is production of NO (MacMicking et al., 1997; Stuehr and Marletta, 1987). The antiviral properties of NO have been shown to be effective against mammalian viruses (Croen, 1993; Mehta et al., 2012; Regev-Shoshani et al., 2013). Xing and Schat (2000) have shown that the inhibition of MDV and HVT replication in CEF stimulated by LPS following recombinant ChIFN- γ is mediated by NO, and not any other bioactive agents; including reactive oxygen species, IL-1 β and IL-6 like molecules produced following the treatment (Xing and Schat, 2000). Pertile et al. (1996) have also shown that a NO-mediated antiviral effect against avian reovirus in HD11 cells

treated with spleen cell conditioned-medium or LPS (Pertile et al., 1996). We showed that LMH cells are suitable for ILTV replication leading to plaque formation, but they are not responsive to LPS stimulation as has been shown by low production of NO. We also investigated to determine whether the NO produced by MQ-NCSU cells treated by EC-LPS and SE-LPS was inhibitory to ILTV replication in the avian LMH cell line. Our data indicated that EC-LPS and SE-LPS induced NO in MQ-NCSU cell elicited antiviral activity against ILTV.

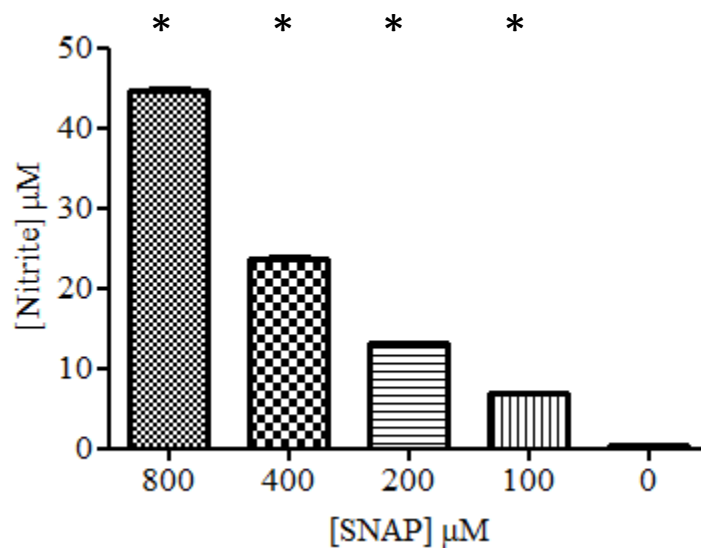


Figure 2-14. NO produced spontaneously by a range of concentrations of SNAP. Different concentrations of SNAP (800, 400, 200, 100 μM) were made in HBSS. Then, each concentration was assayed for NO production using the Griess assay. To identify differences between groups, ANOVA test was performed followed by Tukey's test for mean separation. Data represent mean values \pm SEM. Significance was measured at p value of ≤ 0.01 . * Indicates that the NO production is significantly higher.

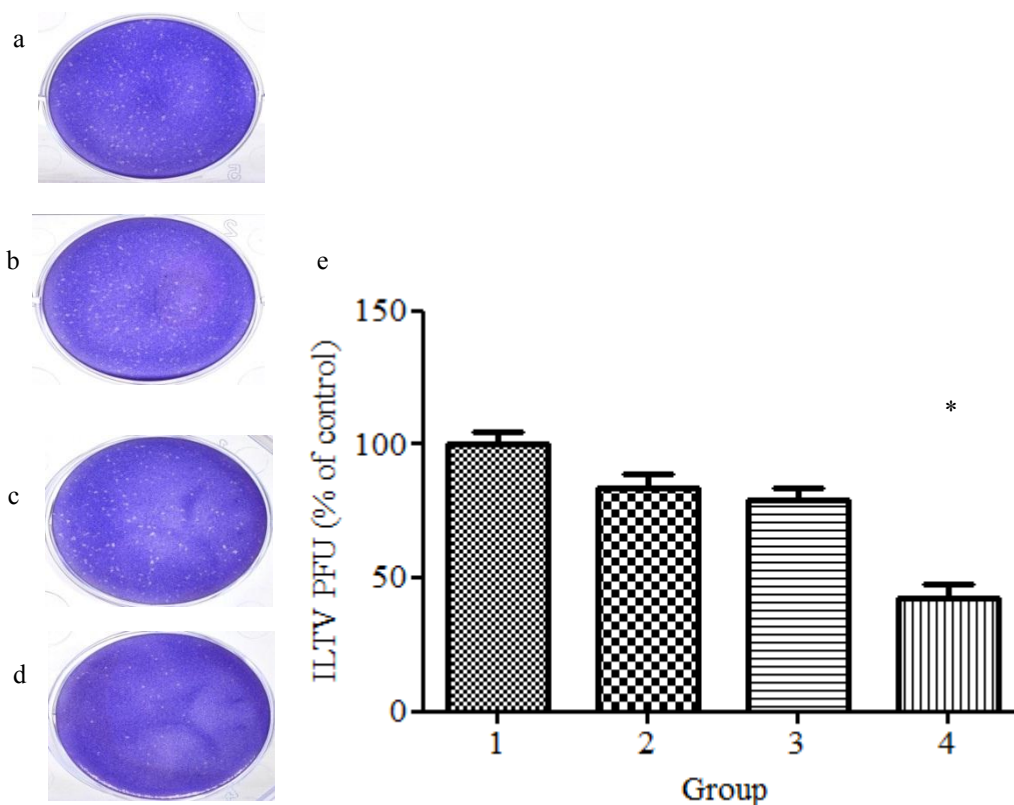


Figure 2-15. NO produced spontaneously by SNAP inhibits ILTV replication *in vitro*. The antiviral assay was done in 6 well plates cultured with LMH cells (2×10^6 cells per well). We included four groups: 1) control LMH cells treated with HBSS, 2) conditioned media of MQ-NCSU cells without treatment transferred to LMH cells, 3) LMH cells treated with $0.1 \mu\text{g/ml}$ EC-LPS for 18 h, 4) $800 \mu\text{M}$ SNAP made in HBSS transferred to LMH cells. All four groups were infected with 100 PFU ILTV/well. Five d post-infection, the plates were stained with 1% crystal violet and PFU was counted. All experiments were done in triplicate. a) representative ILTV infected monolayer of LMH cells with HBSS; b) representative ILTV infected monolayer of LMH cells transferred with 18 h untreated MQ-NCSU cell culture media; c) representative ILTV infected monolayer of LMH cells treated with $0.1 \mu\text{g/ml}$ EC-LPS for 18 h; d) representative

ILTV infected monolayer of LMH cells treated with 800 μ M SNAP; e) shows the ILTV titers as a percentage of the untreated control group. Data represent mean values \pm SEM. Significance was measured at p value of ≤ 0.01 . * Indicates that ILTV titer is significantly decreased when compared to the control.

CHAPTER 3: GENERAL DISCUSSION

Current control of respiratory viruses such as ILTV in chickens is based on vaccination with modified live attenuated vaccine strains originating from embryo or tissue culture, which is dispensed via eye drop, aerosols or drinking water (Han and Kim, 2003). While ILTV vaccines limit the development of clinical disease, the vaccine virus can undergo latency and reactivation similar to the field virus (Bagust, 1986) and as a result, ILT outbreaks are encountered in vaccinated flocks (Hughes et al., 1991). Bird to bird spread of vaccine viral strains and a resultant increase in virulence also has been a concern in ILT control (Guy et al., 1991). These disadvantages of the current vaccines demand studies with a view of developing novel approaches that could be used as alternatives or adjunct to the existing means of control. One such approach may be stimulation of innate immune system through TLR ligands. TLR ligands, due to their immunostimulatory properties have been used therapeutically and prophylactically to confer immunity against several pathogens in mammals. In humans, ligands for TLR 3, 7 and 9 are the subject of numerous clinical trials against viral infections (Kanzler et al., 2007). In fact, poly I:C, LPS and CpG ODN can protect mice against lethal influenza virus challenges (Shinya et al., 2012; Wong et al., 2009). One of the TLR ligands, CpG which interact with chicken TLR21 has been shown to elicit antibacterial effects and protection against *E.coli* and *Salmonella Thypimurium* septicemias in chickens (Gomis et al., 2004; Taghavi et al., 2008). The immunostimulatory properties and antiviral effects of TLR3 ligands (dsRNA), LPS, and CpG against avian influenza virus infection in chickens have been shown (St Paul et al., 2012).

In the present research, I investigated antiviral activity of a TLR4 ligand, LPS against ILTV under *in vitro* conditions. Our investigation described in this thesis led to three major

findings. First, EC-LPS and SE-LPS are capable of inducing only one of the two tested avian cell lines (MQ-NCSU), similar to murine macrophages by significantly increasing the TLR4-CD14 expression and NO production in a dose dependent manner. Secondly, EC-LPS and SE-LPS induce innate response towards ILTV replication *in vitro*, and this protection was correlated with TLR4 and CD14 expression. Finally, the inhibition of ILTV replication induced by the TLR4 pathway was critically dependent on the NO production by MQ-NCSU macrophages.

The long term goal of this research was to identify TLR ligands as antiviral compounds to be used for the control of ILTV, and other respiratory viruses in commercial and backyard poultry production systems. In recent years, much research has been directed towards the study of TLR ligands as antiviral compounds, and as adjuvants for the control of poultry viruses such as influenza, infectious bronchitis virus, reovirus and MDV (Dar et al., 2009; Pertile et al., 1996; St Paul et al., 2012; Xing and Schat, 2000). Before our studies were undertaken, little was known about the antiviral effects of LPS against poultry viruses (Pertile et al., 1996; Xing and Schat, 2000). Notably, these previous studies revealed that 1) LPS induced antiviral effect in HD11 avian macrophages against reovirus are dependent on NO production, and 2) IFN γ plus LPS induced antiviral effect in CEF cells against MDV is dependent on NO production. Although these studies provided evidence indicating that there is a NO dependent antiviral response in chickens against economically important poultry viruses, LPS mediated NO dependent antiviral response against avian respiratory viruses has not been reported. Our findings of antiviral response against ILTV are consistent with previous studies that recorded antiviral effect against reovirus and MDV (Pertile et al., 1996; Xing and Schat, 2000). Thus, the current observation and previous two observations might indicate that the LPS is indeed a useful antiviral agent that could be used *in vivo* for poultry viral diseases control. However, the exact mechanism of

antiviral response of NO against avian viruses as well as in vivo activity of LPS against avian viruses are still not defined adequately.

Although, there are limitations in the use of LPS in mammals, the chickens are less sensitive to toxic effects of LPS. The fact that chickens are more tolerant towards detrimental effects of LPS (Berczi et al., 1966) could be explained by the lower mobility of chicken CD14 in contrast to mammalian CD14, as it is a trans-membrane molecule rather than GPI-anchored molecule (Wu et al., 2009), and in particular by the lack of TRAM/TRIF pathway in chicken TLR4 signaling (Keestra and van Putten, 2008). Our group also has conducted some studies to elucidate whether LPS treatment of chicken embryos at embryo day (ED) 18 effect the hatchability of the embryonated eggs. The observation showed that delivery of LPS to ED18 embryo (up to 30µg/ embryo) could be tolerated without compromising the hatchability (unpublished data).

Despite the novel aspect of our work on the characterization of LPS-mediated NO-dependent antiviral effect against ILTV, there were limitations to these studies. Firstly, my experiments were not extended to see whether the antiviral response we observed against ILTV was dependent on TLR4 pathway. We observed that LPS increases the expression of LPS receptors, TLR4 and CD14 significantly and we would have conducted LPS receptor blocking studies using anti TLR4 and anti-CD14 antibodies to show that the antiviral response elicited against ILTV is dependent on TLR4-CD14 signaling. Secondly, although we have convincingly shown that antiviral effect induced by LPS against ILTV was dependent on NO, it is of interest to see whether LPS induces other innate antiviral molecules in avian macrophages and whether those molecules possess antiviral effects against ILTV. Thirdly, it would have been interesting to conduct a study to elucidate the mechanism of NO-mediated antiviral effect against ILTV. The

possible mechanisms of the NO-mediated antiviral effect that has documented for other viruses include direct DNA damage (Nguyen et al., 1992; Wink et al., 1991), disruption of metal ions in viral proteins (Rice et al., 1993), inhibition of ribonucleotide reductase encoded by herpesviruses (Kwon et al., 1991), and interference with intracellular signaling pathways affecting virus life cycle (Mannick et al., 1994). Fourthly, we need to gather accurate evidence to show that the NO-mediated antiviral effect against ILTV is elicited either extracellularly, intracellularly, or both. Finally, our work should be extended as an *in vivo* study to show the applicability of our work in the poultry production system. Taking the above limitations into account, future studies should be directed to extend our study. Over all, the findings of the studies presented here revealed the antiviral effect of LPS and NO against ILTV. Moreover our findings may lead to the development of novel strategies that effectively stimulate innate responses against ILTV.

CONCLUSIONS:

1. ILTV susceptible LMH cells express LPS receptors in less than 10% of cells and are not responsive to EC-LPS and SE-LPS treatments, as has been evaluated by the production of NO.
2. MQ-NCSU avian macrophages produce NO and increase LPS receptor expression in response to EC-LPS and SE-LPS treatments.
3. Cell culture supernatants of MQ-NCSU avian macrophages produced in response to EC-LPS and SE-LPS treatments are inhibitory to ILTV replication *in vitro*.
4. Inhibition of NO production in MQ-NCSU avian macrophages decreases the SE-LPS and EC-LPS induced NO production consequently, abrogating the ILTV inhibition.
5. The inhibition of ILTV replication by NO in LMH cells was further confirmed using a NO donor, SNAP, as a source of NO.

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APPENDICES

Appendix 1: NO measurement using Griess assay

The NO measurement was done using Griess test (Promega, Catalog Number: G2930). This test detects nitrite (NO_2^-) as a measure of NO present in a variety of biological matrices including cell culture supernatants. The limit of detection is $2.5\mu\text{M}$ nitrite. The assay is based on the chemical reaction that nitrite present in our samples reacts with sulfanilamide to form a compound which reacts with N-1-napthylethylenediamine dihydrochloride in water (NED Solution) to form a final azo compound, which can be detected using spectrophotometry. The reagents, sulfanilamide and NSED solutions are provided in the kit.

1. Generation of standard curve

Preparation of a nitrite standard reference curve is required for each assay to ensure accurate quantitation of nitrite.

- Prepared 1ml of a $100\mu\text{M}$ nitrite solution by diluting the 0.1M nitrite standard 1:1,000 in the matrix used for the experimental samples (for example, phenol red free cell culture media)
- The assay was performed in flat bottomed 96 well plates and the standards included two-fold serial dilutions of sodium nitrite ranged from 0 to $100\mu\text{M}$

2. Assay procedure

- 1) The reagents, sulfanilamide solution and NED solution were equilibrated to room temperature (15–30 minutes)
- 2) Added $50\mu\text{l}$ of each experimental sample to wells in duplicate or triplicate

- 3) Added 50µl of the sulfanilamide solution to all experimental samples and wells containing standards
- 4) Incubated for 5–10 minutes at room temperature in dark
- 5) Added 50µl of the NED solution to samples and standards
- 6) Incubated at room temperature for 5–10 minutes in dark
- 7) Measured the absorbance within 30 minutes in a microplate reader (SpectraMax M₂, Molecular Devices, California, United States) at a wave length of 548nm

3. Nitrite quantification

- A nitrite standard reference curve was generated based on average absorbance value of each concentration of the nitrite standard as a function of "Y" with nitrite concentration as a function of "X"
- Concentrations of each experimental sample were quantified by comparing to the nitrite standard reference curve generated

Appendix 2. Chemical reagents and kits

Reagent Name	Company	Cat. No.	Storage
TISSUE CULTURE MEDIA			
Dulbecco's modified eagle's medium (DMEM)	Invitrogen	11885-092	4°C
Leibovitz's L-15 medium	Invitrogen	11415-114	4°C
McCoy's 5A (modified)	Invitrogen	16600-082	4°C
Waymouth's MB 752/1	Invitrogen	11220-035	4°C
TISSUE CULTURE REAGENTS			
Gamma-irradiated fetal bovine serum (FBS)	Cellgro	35-070- CV	-20°C
Chicken serum	Invitrogen	16110-082	-20°C
Sodium pyruvate 100 mM	Invitrogen	11360-070	4°C
<u>2-Mercaptoethanol 55 mM</u>	Invitrogen	21985023	4°C
Fungizone	Invitrogen	15290018	-20°C
TrypLE™ select (1X)	Invitrogen	12563-029	RT
Penicillin (10000 Units/mL) & streptomycin (10000 µg/mL)	Invitrogen	15140-122	-20°C
L-Glutamine-200mM	Invitrogen	25030-081	-20°C
Endotoxin-free water	Sigma- Aldrich	95289-1L	RT

Reagent Name	Company	Cat. No.	Storage
Phosphate buffered saline (PBS)	Lonza	17-516F	RT
1× hanks balanced salt solution (HBSS)	Invitrogen	14175095	RT
Tryptose phosphate broth	Sigma-Aldrich	T8159- 100ml	4°C
Gelatin 2%	Sigma-Aldrich	G1393- 100ML	4°C
FLOW CYTOMETRY			
Mouse anti-human TLR4 PE (Clone: HTA125)	eBioscience	12-9917	4°C
Mouse IgG2a, kappa PE	eBioscience	12-9917	4°C
Mouse anti-human CD14 FITC (Clone: M5E2)	BD Biosciences	555397	4°C
Mouse IgG2a, kappa FITC	BD Biosciences	555397	4°C
BSA	OmniPur	2960	RT
Mouse IgG	abcam	Ab37355	4°C
pH measurement			
pH electrode storage solution	VWR	14002-828	RT
SympHony buffer 4	VWR	87000-094	RT
SympHony buffer 7	VWR	87000-096	RT

Reagent Name	Company	Cat. No.	Storage
MISCELLANEOUS			
<u>Dimethyl sulfoxide (DMSO) (1X)</u>	Sigma-Aldrich	C6295- 50ML	RT
S-methylisothiurea hemisulfate salt	Sigma-Aldrich	M84445- 100G	RT
S-nitroso-N-acetyl-DL-penicillamine	Invitrogen	N-7892	-20°C
Lipopolysaccharides from <i>Escherichia coli</i> 0127:B8	Sigma-Aldrich	L4516- 1MG	4°C
Lipopolysaccharides from <i>Salmonella enterica</i> serotype Enteritidis	Sigma-Aldrich	L7770- 1MG	4°C
1% Crystal violet	Sigma-Aldrich	V5265- 500ML	RT

Kit name	Company	Cat. No.
Griess reagent system	Promega	G2930
E-TOXATE™ kit	Sigma-Aldrich	ET0200-1KT

Appendix 3: Letter of permission to use the manuscript as a part of MSc thesis

As co-authors contributed to the paper entitled ‘Induction of Toll-like receptor 4 signaling in avian macrophages inhibits infectious laryngotracheitis virus replication in a nitric oxide dependent way’ (Veterinary Immunology and Immunopathology 2013 Aug 27. pii: S0165-2427(13)00236-5. doi: 10.1016/j.vetimm.2013.08.005), we permit using materials in this paper as part of Siamak Haddadi’s MSc thesis entitled ‘Nitric Oxide Mediated Antiviral Response in Avian Cells’ that will be submitted to the Faculty of Graduate Studies at University of Calgary in September 2013.

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