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Toll-like receptor 2 ligand, lipoteichoic acid is inhibitory against infectious laryngotracheitis virus infection *in vitro* and *in vivo*

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ABSTRACT

Lipoteichoic acid (LTA) is one of the pathogen associated molecular patterns (PAMPs) that activates toll-like receptor (TLR)2-cluster of differentiation (CD)14 signalling pathway. This recognition elicits antiviral responses that have been recorded against viruses of mammals although such responses have not been characterized adequately against avian viruses. In this investigation, we characterized the LTA induced antiviral responses against infectious laryntotracheitis virus (ILTV) infection *in vitro* and *in vivo*. We found that LTA is capable of up regulating mRNA expression of innate proteins in macrophages such as MyD88, iNOS and IL-1β and reduces the ILTV plaques *in vitro*. Similarly, we found that LTA treatment of embryonic day 18 (ED18) eggs can lead to the antiviral response against pre-hatch ILTV infection *in vivo* and is associated with expansion of macrophage populations and expression of IL-1β and MyD88 in the lung. The data highlight that LTA can be a potential innate immune stimulant that can be used against ILTV infection in chickens.

1. Introduction

Toll-like receptors (TLRs) and their downstream signalling molecules are generally conserved and less variable in chickens when compared with mammals (Lillehoj and Li, 2004; Lynn et al., 2003; Philbin et al., 2005). The chicken genome consists of the following TLRs; TLR1-1, TLR1-2, TLR2-1, TLR2-2, TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 (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). 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 respectively dsRNA, ssRNA and CpG motifs of pathogens which will be exposed intracellularly.

In mammals, TLR2 is expressed in a variety of immune (Cario et al., 2000; Flo et al., 2000; Matsuguchi et al., 2000; Muzio et al., 2000; Yang et al., 1998; Zhang et al., 1999) and non-immune cells (Chaudhary et al., 1998; Matsuguchi et al., 2000; Yang et al., 1998). TLR2 can act as a receptor for many pathogen associated molecular patterns (PAMPs) such as peptidoglycan (Schwandner et al., 1999; Yoshimura et al., 1999), lipoteichoic acid (LTA) (Schwandner et al., 1999), lipoarabinomannan (Means et al., 1999a), lipoproteins and lipopeptides such as Pam3Cys-Ser-(Lys)4, trihydrochloride (Aliprantis et al., 1999; Brightbill et al., 1999; Hirschfeld et al., 1999; Lien et al., 1999), and zymosan (Underhill et al., 1999), as well as many Gram-positive bacteria containing peptidoglycan, LTA and lipopeptides, mycobacteria, spirochetes, and mycoplasmas (Brightbill et al., 1999; Flo et al., 2000; Means et al., 1999b; Schwandner et al., 1999; Yoshimura et al., 1999). The binding of aforementioned ligands (PAMPs) to TLR2 activates a signalling cascade that involves the interaction of cytoplasmic Toll/IL-1 receptor (TIR) domain of the TLR2 with intracellular adapter molecules such as myeloid differentiation factor (MyD)88 leading to the regulation of the transcription of pro-inflammatory mediators particularly in innate immune cells (Akira, 2003; Higuchi et al., 2008; Jin and Lee, 2008; O'Neill, 2002). These pro-inflammatory molecules are able to elicit antiviral effects that have been shown in many host–viral models (Haddadi et al., 2013; Pertile et al., 1996; Xing and Schat, 2000).

In chickens, macrophages are an important innate immune cell in the responses against viral infections such as Marek's disease virus (Abdul-Careem et al., 2009) and infectious bronchitis virus infections (Kameka et al., 2014), so while some are potentially being used

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as a site of replication, others are responding normally to help clear the infection using number of effector molecules and stimulate the adaptive immune response against viral infections.

LTA recognition by TLR2 requires binding of the ligand to a co-receptor called CD14 as has been shown in mammals (Schwandner et al., 1999) as well as in avian hosts (Farnell et al., 2003). In chickens, activation of TLR2 on heterophils leads to an up regulation in the expression of mRNA of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and IL-8 (Kogut et al., 2005) and the same interaction on monocytes releases the gaseous free radical nitric oxide (NO) through inducible nitric oxide synthase (iNOS) activation (He et al., 2006). However, it is not known whether induction of chicken macrophages using TLR2 ligands leads to the activation of pro-inflammatory mediators.

TLR2 ligands such as LTA and synthetic triacylated lipopeptide (Pam3Cys-Ser-(Lys)4, trihydrochloride) have been shown to activate antiviral response against number of viruses that affect mammals. In humans, induction of TLR2 signalling leads to an anti-human cytomegalovirus (HCMV) effect in ectocervical tissues via the induction of IFNβ (Harwani et al., 2007). It has also been shown that TLR2 ligands induce antiviral response against hepatitis B virus *in vitro* (Thompson and McHutchison, 2009; Zhang et al., 2012). In mice, TLR2 activation also significantly contributes to the control of murine cytomegalovirus infection *in vivo* in an NK cell-dependent way (Szomolanyi-Tsuda et al., 2006). TLR2 ligands also elicit *in vivo* antiviral effect that has been recorded in a mouse model of influenza virus infection (Tan et al., 2012). In chickens, TLR2 ligand, lipopeptide (Pam 3) has been shown to possess partial adjuvant activity against avian influenza virus infection (St Paul et al., 2014). However, there is a lack of information available on the TLR2 ligand mediated antiviral activity against an avian virus *in vitro* or *in vivo*. We hypothesised that TLR2 ligand, LTA may induce antiviral response against a chicken herpesvirus, namely infectious laryngotracheitis virus (ILTV) infection and that is associated with the production of pro-inflammatory mediators. 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%.

We investigated whether LTA can increase the expression of its receptors, TLR2 and CD14 and mRNA expression of MyD-88, IL-1β, iNOS, interferon (INF)-α and INF-β, as well as NO production by avian macrophages. Then we evaluated whether LTA can mediate antiviral response against ILTV *in vitro* and *in vivo*. We showed that avian macrophages, MQ-NCSU cells could be stimulated with LTA to increase the expression of LTA receptors and mRNA expression of pro-inflammatory mediators. We also observed that the induction of pro-inflammatory mediators following LTA stimulation of avian macrophages lead to antiviral response against ILTV *in vitro*, which is not dependent on NO production. Finally we could confirm that *in ovo* delivery of LTA leads to inhibition of ILTV replication in chicken embryos and that it is associated with the expansion of macrophage populations and expression of mRNA of pro-inflammatory molecules *in vivo*.

2. Materials and methods

2.1. Animals

Specific pathogen free (SPF) eggs were purchased (Canadian Food Inspection Agency, Ottawa, Canada) and incubated at the Health Research Innovation Center or Veterinary Science Research Station (VSRS), University of Calgary. All procedures have been approved by the University of Calgary's Veterinary Sciences Animal Care Committee.

2.2. Cell lines and virus

Two cell lines were used in the experiments. Avian macrophage cell line, MQ-NCSU cells were a gift from Dr. Shayan Sharif (University of Guelph, Canada). Leghorn male hepatoma cell line (LMH) and ILTV (strain N-71851) were purchased from the American Type Culture Collection (ATCC).

2.3. Cell culture

Avian macrophage cell line, MQ-NCSU cells were cultured in LM HAHN medium comprising of Leibovitz L-15 medium (39.5%), McCoy's 5A medium (39.5%), chicken serum (10%), L-glutamine (1%), sodium pyruvate (1%), 100 units of penicillin and 100 μg of streptomycin per ml, fungizone (250 μg/ml), two-mercaptoethanol (1.0 mM) (Invitrogen, Burlington, ON, Canada), tryptose phosphate broth (1%) (Sigma-Aldrich, St. Louis, MO, USA), and foetal bovine serum (8%) (Cellgro, Manassas, VA, USA) (Okamura et al., 2004). Furthermore, LMH cells that were used for the titration of ILTV (Schnitzlein et al., 1994) were cultured in 0.1% gelatine (Sigma-Aldrich) coated flasks or plates. The growth medium for LMH cells consists of Waymouth's MB 752/1 medium (Invitrogen, Burlington, ON, Canada) supplemented with heat inactivated FBS 10% (Cellgro, Manassas, VA, USA), 100 units of penicillin and 100 μg of streptomycin per ml (Invitrogen), and L-glutamine 1% (Invitrogen). The cells were maintained at 37 °C (LMH) or 40 °C (MQ-NCSU) under 5% carbon dioxide (CO_2) in a cell culture incubator.

2.4. Stimulation of MQ-NCSU cells with LTA for quantification of CD14 expression

Ten μg/ml purified LTA from *Staphylococus aureus* (InvivoGen, San Diego, CA, USA) in phenol red free RPMI 1640 containing 10% FBS, 2.0 mM L-glutamine, and no antibiotics was used to study the expression of CD14 in MQ-NCSU cells following LTA treatment. MQ-NSU cells were cultured in T-75 flasks overnight and treated with LTA with controls recieving media alone. At 1, 3, 6 and 12 hours post-treatment, cells were dislodged from flasks using TrypLE™ Express (Invitrogen) and 2×10^6 cells from each treatment or control stained for CD14 expression. The experiment was done in triplicate. For stimulation of MQ-NSCU with LTA in order to quantify the mRNA expression of TLR2 and the relevant downstream molecules, MyD88, iNOS and IL-1β, MQ-NSU cells were cultured in T-75 flasks overnight and treated with LTA (10 μg/ml) with only media used for controls. At 1, 3, 6, 12 and 24 hours post-treatment, cells were dislodged from flasks using TrypLE™ Express (Invitrogen) and RNA was extracted from 4×10^6 cells from each treatment or control. The experiment was done in triplicate.

2.5. Determination of NO production by MQ-NCSU cells in response to LTA

MQ-NCSU cells were propagated and cell stimulation was done in 96 well plates (10^5 cells/well). The cells were cultured overnight and after removing the growth medium, washed with 1 × Hanks balanced salt solution (HBSS) (Invitrogen) and stimulated with LTA at 10 μg/ml in phenol red free RPMI 1640 (Invitrogen) containing 10% FBS, 2.0 mM L-Glutamine, and no antibiotics. The plates were incubated for 24 hours at 40 °C under 5% CO_2 before collection of culture supernatants for NO assay. Each treatment was done in six replicates.