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Research paper

In ovo delivery of toll-like receptor 2 ligand, lipoteichoic acid induces pro-inflammatory mediators reducing post-hatch infectious laryngotracheitis virus infection

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ABSTRACT

Toll-like receptor (TLR) ligands are pathogen associated molecular patterns (PAMPs) recognized by the TLRs resulting in induction of host innate immune responses. One of the PAMPs that binds to TLR2 and cluster of differentiation (CD) 14 is lipotechoic acid (LTA), which activates downstream signals culminating in the release of pro-inflammatory cytokines. In this study, we investigated whether *in ovo* LTA delivery leads to the induction of antiviral responses against post-hatch infectious laryngotracheitis virus (ILTV) infection. We first delivered the LTA into embryo day (ED)18 eggs *via in ovo* route so that the compound is available at the respiratory mucosa. Then the LTA treated and control ED18 eggs were allowed to hatch and the hatched chicken was infected with ILTV intratracheally on the day of hatch. We found that *in ovo* delivered LTA reduces ILTV infection post-hatch. We also found that *in ovo* delivery of LTA significantly increases mRNA expression of pro-inflammatory mediators in pre-hatch embryo lungs as well as mononuclear cell infiltration, predominantly macrophages, in lung of post-hatch chickens. Altogether, the data suggest that *in ovo* delivered LTA could be used to reduce ILTV infection in newly hatched chickens.

1. Introduction

Similar to mammals, the chicken immune system consists of innate immune cells expressing toll-like receptors (TLRs) that are responsible for recognizing pathogen associated molecular patterns (PAMPs) (Keestra et al., 2013). The interaction of the TLRs with PAMPs leads to the induction of pro-inflammatory or antiviral cytokines eliciting protective responses against invading pathogens (Akira and Takeda, 2004). Because of this role of the TLRs in host defence they have been studied as therapeutic targets against different infections (Horscroft et al., 2012; O'Neill et al., 2009). Among TLRs, TLR2 binds to peptidoglycan, lipoteichoic acid (LTA), and lipoproteins of Gram-positive bacteria and zymosan of yeast (Gantner et al., 2003). These receptor–ligand interactions result in downstream signalling *via* induction of an adaptor protein, myeloid differentiation primary response (MyD)88 leading to the production of pro-inflammatory mediators and recruitment of immune cells (Akira and Takeda, 2004; Opitz et al., 2001; Schwandner et al., 1999) leading to anti-microbial activities (Boehme et al., 2006; Compton et al., 2003).

In avian model, the LTA recognition by the TLR2 is well reported (Farnell et al., 2003) where the activation of TLR2

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using LTA on heterophils was shown to up regulate the expression of mRNA of the pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and IL-8 (Kogut et al., 2005). Induction of TLR2 signalling leading to antiviral responses against hepatitis B (HBV) virus (Zhang et al., 2012) and human cytomegalovirus (CMV) (Boehme et al., 2006; Compton et al., 2003) infections *in vitro* has been shown. TLR2-mediated antiviral activity was also shown against influenza virus induced pneumonia in mice (Tuvim et al., 2012) and parainfluenza virus infection in guinea pigs (Drake et al., 2013). Although it has been shown that *in ovo* delivery of LTA reduces pre hatch infectious laryngotracheitis virus (ILTV) infection (Haddadi et al., 2014), the efficacy of LTA against post-hatch ILTV infection is yet to be determined.

ILTV belongs to the family of *Herpesviridae*, subfamily of *Alphaherpesvirinae*, and genus of Iltovirus. ILTV infection leads to sporadic cases of infectious laryngotracheitis (ILT) in chickens and pheasant world-wide including North America and Europe. ILTV can remain latent in chickens after infection and due to stress, the virus can be reactivated and shed intermittently. In contrast to the mild form of ILT, deaths may occur within a short period in per acute and acute clinical manifestations (Ou and Giambrone, 2012). Vaccination against ILTV had been a very reliable means of disease prevention although vaccine breaks emerge due to the increased virulence of the virus (Coppo et al., 2013). One of the ways of addressing limitations of the current ILT control methods in chickens may be the development of novel disease control methods that could be used as alternatives or adjunct to the existing ILT control measures. One such approach may be the stimulation of innate immune system through synthetic or natural PAMPs.

We hypothesized that one of the PAMPs, TLR2 ligand (LTA) when delivered *in ovo* may induce antiviral response against ILTV infection encountered post-hatch and that is associated with the production of pro-inflammatory mediators and recruitment of macrophages. In this study, we investigated whether *in ovo* LTA delivery can mediate antiviral responses against ILTV *in vivo*. We showed that *in ovo* delivery of LTA reduces ILTV infection in lung of chickens post-hatch and that it is associated with mRNA expression of pro-inflammatory mediators pre-hatch and the expansion of macrophages in the respiratory tract post-hatch.

2. Materials and methods

2.1. Animals and virus

Specific Pathogen Free (SPF) eggs were purchased from Canadian Food Inspection Agency, Ottawa, Canada, incubated, hatched and chickens were maintained at the University of Calgary's Spyhill campus or Foothill campus. All procedures requiring the use of eggs, embryos and live chickens have been approved by the University of Calgary's Veterinary Sciences Animal Care Committee. The ILTV (strain N-71851) was purchased from the American Type Culture Collection (ATCC).

2.2. In ovo delivery of TLR2 ligand LTA or PBS

Purified LTA from *Staphylococus aureus* (InvivoGen, San Diego, CA, USA) was delivered *in ovo* with control eggs receiving PBS at embryo day (ED)18 SPF eggs. LTA delivery on ED18 was carried out manually as has been described previously (Abdul-Careem et al., 2008; Guo et al., 2003; Wakenell et al., 2002). Briefly, an 18-gauge needle was used to puncture the shell at the broad end of the egg. The delivery was done using a 23-gauge, 2.5 cm long needle inserted in its entire length through the hole targeting the amniotic cavity.

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

For the evaluation of efficacy of LTA treatment *in ovo* against post-hatch ILTV infection, eggs were injected with 50 μg LTA in 200 μl PBS per egg and control ED18 eggs received 200 μl PBS alone. The LTA dose was determined previously (Haddadi et al., 2014). The eggs from both groups were allowed to hatch. After hatch day old chicks were challenged with 5×10^4 plaque forming units (PFU) of ILTV per bird intratracheally. The birds were euthanized after 1, 3 and 5 days post-infection (dpi) to collect lungs for extraction of DNA to quantify the ILTV genome load in lungs. The experiment was repeated three times with 2–5 chickens per group per time point and the data were pooled for the purpose of analysis; 1 (n = 11–12 per group), 3 (n = 7 per group) and 5 (n = 11–13 per group) day post-infection.

2.4. Histological analysis of post-hatch lungs for cellular infiltration following in ovo LTA delivery

To study the LTA induced immune cell infiltration in lungs post-hatch, the lungs from one day old chickens that received *in ovo* LTA (n = 4) or PBS (n = 4) treatments were fixed in 10% formol saline for histological evaluation. The samples were submitted to the Histology Section of the University of Calgary Faculty of Veterinary Medicine (UCVM). The histological sections that were stained with haematoxylin and eosin (H & E) were examined and scored on a scale of 0–3 (no cellular infiltration = 0, <25% lungs with cellular infiltration = 1, 25–50% lungs with cellular infiltration = 2 and >50% lungs with cellular infiltration = 3) based on the extent of cellular infiltration (Kameka et al., 2014).

2.5. Evaluation of lung macrophages and NK cells in post-hatch lungs following in ovo LTA delivery

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