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Characterization of host responses induced by Toll-like receptor ligands in chicken cecal tonsil cells

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ABSTRACT

The innate responses of cecal tonsils against invading microorganisms are mediated by conserved pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs). TLRs expressed by mammalian and avian immune system cells have the capability to recognize pathogen-associated molecular patterns (PAMPs). Although, the role of TLR ligands in innate and adaptive responses in chickens has been characterized in spleen and bursa of Fabricius, considerably less is known about responses in cecal tonsils. The aim of the current study was to assess responses of mononuclear cells from cecal tonsils to treatment with the TLR2, TLR4 and TLR21 ligands, Pam3CSK4, lipopolysaccharide (LPS), and CpG oligodeoxynucleotide (ODN), respectively. All three ligands induced significant up-regulation of interferon (IFN)-γ, interleukin (IL)-1β, IL-6 and CxCLi2/IL-8, whereas no significant changes were observed in expression of IL-13 or the antimicrobial peptides, avian β-defensin (AvBD) 1, AvBD2 and cathelicidin 3 (CATHL-3). In general, CpG ODN elicited the highest cytokine responses by cecal tonsil mononuclear cells, inducing significantly higher expression compared to LPS and Pam3CSK4, for IFNγ, IL-1β, IL-6 and CxCLi2 at various time points. These findings suggest the potential use of TLR21 ligands as mucosal vaccine adjuvants, especially in the context of pathogens of the intestinal tract.

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1. Introduction

The gastrointestinal tract of animals is continuously exposed to food antigens and commensal bacteria as well as pathogens (Koutsos and Arias, 2006). The gut-associated lymphoid tissues (GALT) play a significant role in inducing or regulating immune responses locally and systemically in chickens (Oláh et al., 2013). Cecal tonsils constitute the major lymphoid tissue within the GALT in chickens (Yun et al., 2000) and harbour distinct populations of B and T cells mediating both cell-mediated and antibody-mediated immune responses (Befus et al., 1980). Moreover, cecal tonsils play a pivotal role in maintaining intestinal homeostasis through the production of pro-inflammatory and regulatory cytokines (Haghighi et al., 2008; Brisbin et al., 2010) as well as antimicrobial peptides (Akbari et al., 2008).

Innate responses of cecal tonsils against invading microorganisms are mediated in large part by conserved pattern recognition receptors (PRRs) (Janardhana et al., 2009; Brisbin et al., 2008). Toll-like receptors (TLRs), the best characterized PRRs (Bekeredjian-Ding and Jego, 2009), are transmembrane proteins expressed by various immune system cells of mammalian and avian species and have the capability to distinguish different classes of pathogens and selectively recognize pathogen-associated molecular patterns (PAMPs) (St. Paul et al., 2013a). In chickens, TLR1A, 1B, 2A, 2B, 3, 4, 5, 7, 15 and 21 are expressed by cells of the immune system as well as by epithelial cells (Iqbal et al., 2005; Chrząstek et al., 2014). The expression patterns of chicken TLRs are similar to those of their counterparts in mammalian species (Iqbal et al., 2005).

Interactions between PAMPs and TLRs result in a cascade of signaling events leading to activation of cellular responses and production of cytokines and other immunoregulatory molecules (St. Paul et al., 2013a). For example in chickens, the direct interaction of TLR4 and TLR2 with microbial LPS and lipoprotein, respectively, triggers expression of pro-inflammatory cytokines by heterophils (Kogut et al., 2005; Malek et al., 2004), macrophages (St. Paul et al., 2013a; Barjesteh et al., 2014), splenocytes (St. Paul et al., 2011) and cells of the bursa of Fabricius (St. Paul et al., 2012b).

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In mammals, responsiveness to non-methylated cytosine-guanosine (CpG) motifs in bacterial and viral nucleic acids is mediated by TLR9 (Hemmi et al., 2000). Chickens, however, do not express TLR9, but TLR21 binds DNA with similar affinity and specificity (Brownlie et al., 2009). Previous results have shown that class B CpG oligodeoxynucleotides (ODN) promote the up-regulation of interferon (IFN)-γ and IL-10 in the bursa of Fabricius (St. Paul et al., 2012b), IFN-γ, IFN-β, IL-1β, and interferon regulatory factor (IFR) 7 in chicken macrophages (Barjesteh et al., 2014), IFN-γ, major histocompatibility complex (MHC)-II, and IL-10 in chicken splenocytes (St. Paul et al., 2011), IL-1β, IL-6 and CxCLi2 in chicken thrombocytes (St. Paul et al., 2012a) and type I IFNs in chicken erythrocytes (St. Paul et al., 2013c).

Given the importance of TLR ligands in induction of immune responses against a wide variety of microbial pathogens, the immediate goal of the current study was to evaluate the expression of cytokines and antimicrobial peptides elicited by the TLR ligands LPS, Pam3CSK4 and CpG ODN in cecal tonsil mononuclear cells of commercial broiler chickens, with the ultimate goal of developing TLR ligands as vaccine adjuvants to enhance immune responses in the chicken gut.

2. Material and methods

2.1. Chickens and housing

Newly hatched commercial broiler chicks (n = 24) were obtained from Stratford Chick Hatchery (Stratford, ON, Canada) and housed at the Arkell Poultry Research Station (University of Guelph, Guelph, ON). This research was approved by the University of Guelph Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

2.2. TLR ligands

The TLR ligands used in the current study were LPS from *Escherichia coli (E. coli)* O111:B4 (an agonist of TLR4, Sigma–Aldrich, Oakville, ON), a synthetic triacylated lipopeptide Pam3CSK4 (agonist of TLR2/1 heterodimer; Invivogen, San Diego, CA), and synthetic class B CpG ODN 2007 [5′-TCGTCGTTGTCGTTTTGTCGTT-3′] (a TLR21 agonist, Sigma) with phosphorothioate backbone. These ligands were chosen for their ability to stimulate chicken mononuclear cells (St. Paul et al., 2011, 2013a). They were reconstituted in endotoxin-free water and diluted to working concentrations using RPMI-1640 medium.

2.3. Preparation of cecal tonsil single cell suspensions

Cecal tonsils were harvested from 24 (5-week-old) chickens and single cell suspensions of mononuclear cells were prepared as previously described (Brisbin et al., 2010). Briefly, cecal tonsils were aseptically removed and rinsed several times in 1× Hanks' balanced salt solution (HBSS) and then chopped finely with sterile scalpels. Tissue was disrupted by crushing with the flat end of a syringe plunger and strained through a 40 μm nylon cell strainer. The strained cells were overlaid onto Histopaque-1077 (Sigma) and centrifuged at 400*g* for 30 min. The mononuclear cells were harvested from the interface and washed three times with RPMI-1640 (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 80 μg/ml streptomycin, and 50 μg/ml gentamicin. Cell viability was checked using trypan blue and cell density was adjusted to 10^7 cells/ml in supplemented RPMI-1640. Cells from individual birds were cultured separately in 96-well round-bottom plates (10^6 cells/well in 100 ul).

2.4. In vitro stimulation with TLR ligands

Mononuclear cells were cultured and treated with two different concentrations (1 μg/ml and 5 μg/ml) of three different TLR ligands (LPS, Pam3CSK4, CpG ODN) and a control group received complete medium. After 2, 6, and 18 h incubation at 41 °C in a humidified 5% CO_2 environment, the cells were harvested and lysed in TRIzol reagent (Invitrogen). Subsequently, the cell lysates from two birds were pooled before RNA isolation to provide one biological replicate to evaluate the responses to the three ligands (resulting in total of 4 biological replicates at each time point).

2.5. RNA extraction and complementary single-stranded DNA (cDNA) synthesis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol with the addition of 10 μg glycogen (Invitrogen) as carrier to increase RNA recovery. Total RNA was treated with DNase (DNA-free kit, Ambion, Austin, TX) to eliminate genomic DNA and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Three hundred ng of total RNA was reverse transcribed using Superscript® II First Strand Synthesis kit (Invitrogen) following the manufacturer's instructions. cDNA was then diluted 1:10 in DEPC treated water.

2.6. Primers

Primers used in amplification are listed in Table 1.

2.7. Quantitative real-time PCR

Quantitative real-time PCR was conducted as previously described (St. Paul et al., 2011) in the LightCycler® 480 II system (Roche Diagnostics GmbH, Mannheim, DEU) using SYBR Green I Master mix (Roche Diagnostics); β-actin was used as a reference gene. Each reaction mixture consisted of a final volume of 20 μl, containing 10 μl master mix, 1 μl of 5 μM forward primer, 1 μl of 5 μM reverse primer, 3 μl PCR-grade water and 5 μl of target cDNA. The amplification conditions included a pre-incubation step at 95 °C for 10 min, 45–55 amplification cycles at 95 °C for 10 s, annealing as described in Table 1 for each of the primers and extension at 72 °C for 10 s. Melting curve analysis was performed by heating to 95 °C for 10 s, cooling to 65 °C for 1 min, and then heating to 97 °C.

2.8. Data analysis

All analyses were carried out using SAS version 9.3 (SAS, Cary, NC). The variable of interest in this experiment was the fold change in expression of individual cytokines by extracted cecal tonsil mononuclear cells. The expression of a cytokine by cells cultured in the presence of TLR ligands was expressed as a fold change relative to expression by cecal tonsil cells cultured in medium alone. Fold changes were logarithmically transformed to normalize their distribution before further analysis. Preliminary analyses were carried out using SAS Proc GLM (General Linear Model, a form of analysis of variance) to assess whether cytokine expression varied between the two concentrations of ligands used in the experiment. Expression data for the two different concentrations were pooled for all three ligands because cytokine responses did not differ significantly by concentration. The effects of ligand on cytokine expression at each separate time point were analyzed using Proc GLM, followed by Duncan's multiple range tests when significant differences were detected among ligands. Log transformed fold responses for each cytokine at each time point were tested for significance against the

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