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Research paper

Characterization of responses elicited by Toll-like receptor agonists in cells of the bursa of Fabricius in chickens

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ABSTRACT

Toll-like receptors (TLRs) are an evolutionarily conserved group of pattern recognition receptors that play an important role in mediating host-responses to pathogens. Several TLRs have been identified in chickens and their expression has been detected in many immune cell subsets including in B cells. However, the mechanisms through which TLRs modulate B cell responses have not been well characterized in chickens. The aim of the present study was to elucidate the responses mounted by cells of the bursa of Fabricius to treatment with the TLR 3, 4 and 21 ligands, poly I:C, lipopolysaccharide (LPS) and CpG oligodeoxynucleotides (ODN), respectively. The relative expression of several immune system genes was quantified at 1, 3, 8 and 18 h post-treatment. The results show that all three ligands induced the up-regulation of interferon (IFN)-γ and interleukin (IL)-10 transcripts and promoted the up-regulation of transcripts associated with antigen presentation, namely CD80 and major histocompatibility complex (MHC) class II. Furthermore, the results indicated that LPS and poly I:C induced the greatest IFN-γ and IL-10 responses, respectively, while CpG ODN was the most efficacious inducer of CD80 and MHC-II expression. Future studies may be aimed at elucidating the mechanisms of TLR-mediated activation of chicken B cells. These mechanisms may be then exploited for the development of adjuvants with enhanced ability to stimulate B cell responses.

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1. Background

Toll-like receptors (TLRs) are an evolutionarily conserved group of pattern recognition receptors that play an important role in mediating host-responses to pathogens (Akira et al., 2001; Medzhitov, 2001). The ligands for TLRs are typically conserved structural motifs expressed by microbes, termed pathogen-associated molecular patterns (PAMPs). To date, several TLRs have been identified in chickens, each responding to different PAMPs. For example, TLR3 binds double-stranded RNA, a product of some viruses during their replication cycle, while TLR4 binds lipopolysaccharide (LPS), which is present in the cell wall of Gram-negative bacteria, and TLR21 binds unmethylated CpG DNA motifs found in the nucleic acids of certain bacteria and viruses (Leveque et al., 2003; Temperley et al., 2008; Keestra et al., 2010). TLRs have been identified in many cell subsets of both the innate and adaptive immune system including macrophages, heterophils, T cells and B cells (Iqbal et al., 2005).

Responses mediated by interactions between TLRs and their ligands typically include the production of cytokines and cellular activation (Hopkins and Sriskandan, 2005). In the case of mammalian B cells, treatment with TLR ligands induces the production of immunoglobulins and cytokines, such as interferon (IFN)-γ and interleukin (IL)-10 (Barr et al., 2007; Giordani et al., 2009). Moreover, TLR stimulation facilitates antibody isotype switching (Jegerlehner et al., 2007) and promotes the proliferation of B cells and enhances their antigen presentation capabilities (Jiang

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et al., 2007). To date, several TLRs have been identified in mammalian B cells as reviewed by Booth et al. (2010).

Although extensively studied in mammals, the TLR-mediated responses of B cells have not been well characterized in chickens. It has been previously shown that chicken B cells express TLRs 2, 3, 4, 5, 7 and 21 at the transcript level (Iqbal et al., 2005; Han et al., 2010), and they proliferate in response to CpG oligodeoxynucleotides (ODN) (Wattrang, 2009). Additionally, our group has previously found that chicken B cells up-regulate certain immune system genes in response to LPS (Sarson et al., 2007). Although these studies suggest that chicken B cells may express functional TLRs 4 and 21, the responses of chicken B cells to stimulation with these TLR ligands and others have not been well examined. The bursa of Fabricius in chickens is the primary site for development and differentiation of B cells and approximately 98% of bursal lymphocytes are B cells (Davison et al., 2008). Therefore, the present study was an attempt to elucidate and characterize the responses of bursal cells to treatment with the TLR3, 4 and 21 ligands, poly I:C, LPS and CpG ODN, respectively. The results show that all three ligands induced the transcriptional up-regulation of IFN-γ and IL-10, and also promoted the up-regulation of transcripts associated with antigen presentation, namely CD80 and major histocompatibility complex (MHC) class II.

2. Methods

2.1. Chickens

Four-week-old broiler chickens ($n = 6$) were procured from the Arkell Poultry Research Center, University of Guelph (Guelph, ON). This research was approved by the University of Guelph Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

2.2. B cell isolation

The bursa of Fabricius was collected from 6 chickens, minced and filtered through a 40 μm nylon cell strainer to obtain a single cell suspension. The suspension was overlaid onto a Histopaque-1077 (Sigma–Aldrich, Oakville, ON) gradient and centrifuged at 400 × *g* for 30 min, and the cells were harvested from the plasma-Histopaque interface and washed 3× in RPMI-1640 (Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin, 80 μg/mL streptomycin, 25 mg gentamicin, 10 mM HEPES buffer, 50 μM β-mercaptoethanol, and 2 mM L-glutamine. Bursal cells were seeded into 48-well plates at 1×10^7 cells/mL for in vitro stimulation with TLR ligands. As indicated before, 98% of lymphocytes in the chicken bursa of Fabricius are B cells (Davison et al., 2008), therefore, the cell cultures used in this study were highly enriched for B cells.

2.3. TLR ligands

Poly I:C and LPS from *Escherichia coli* 0111:B4 were purchased from Sigma–Aldrich-Canada (Oakville, ON), while synthetic class B CpG ODN 2007 [5′-TCGTCGTTGTCGTTTTGTCGTT-3′] and non-CpG ODN [5′-TGCTGCTTGTGCTTTTGTGCTT-3′] were purchased from Eurofins MWG Operon (Ebersberg, GER). All of the ligands used were re-suspended in sterile phosphate buffered saline (PBS, pH 7.4) and diluted to working concentrations in RPMI medium of the same formulation used to culture bursal cells.

2.4. Experimental design

Bursal cells from 6 chickens were stimulated with either a low or high dose of the TLR3 ligand poly I:C (5 μg/mL and 50 μg/mL), the TLR4 ligand LPS (0.5 μg/mL and 5 μg/mL) and the TLR21 ligand CpG ODN (0.5 μg/mL and 5 μg/mL), while control groups received either non-CpG ODN (5 μg/mL) or medium. These doses were selected as they have been shown to be immunostimulatory for several different chicken cell populations (Kogut et al., 2005; Keestra and van Putten, 2008; Villanueva et al., 2011; He et al., 2012). At 1, 3, 8 and 18 h post-stimulation, cells were harvested for RNA extraction.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from the bursal cells using TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and treated with DNA Free® (Ambion, Austin, TX) DNAse. Subsequently, 500 ng of purified RNA was reverse transcribed to cDNA using Superscript® II First Strand Synthesis kit (Invitrogen, Carlsbad, CA) and oligo-dT primers, according to the manufacturers recommended protocol. The resulting cDNA was subsequently diluted 1:10 in DEPC treated water.

2.6. Quantitative real-time PCR

Quantitative real-time PCR using SYBR Green was performed on diluted cDNA using the LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, GER) as previously described (Villanueva et al., 2011). Briefly, each reaction involved a pre-incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 min, 55–64 °C (T_A as per primer) for 5 s, and elongation at 72 °C for 10 s. Subsequent melt curve analysis was performed by heating to 95 °C for 10 s, cooling to 65 °C for 1 min, and heating to 97 °C. Many of these primers have been used in previous studies, and the new ones were designed using the NCBI Primer-Blast (St. Paul et al., 2011, 2012). Primers were synthesized by Sigma–Aldrich-Canada (Oakville, ON), and their specific sequences and accession numbers are outlined in Table 1.

2.7. Data analysis

Relative expression levels of all genes was calculated relative to the housekeeping gene β-actin using the LightCycler® 480 Software (Roche Diagnostics GmbH, Mannheim, GER), based on the formula developed by Pfaffl (2001). Data represent mean fold change from medium treated controls ± standard error. Statistical significance

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