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Bacterial and viral induction of chicken thrombocyte inflammatory responses

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ABSTRACT

Thrombocytes express Toll-like receptors (TLRs) that detect bacterial or viral pathogens to signal the release of cytokines and mediators. We examined inflammatory responses when thrombocytes were exposed to four TLR ligands. Treatment of thrombocytes with TLR ligands demonstrates differential effects on gene expression of interleukin (IL)-6. Among the TLR ligands examined, lipopolysaccharide stimulation led to the most significant up-regulation of the IL-6 gene and a significant amount of active IL-6 in thrombocyte culture media. Lipoteichoic acid stimulation led to only marginal up-regulation of IL-6 gene expression. Although gene expression of inducible nitric oxide synthase (iNOS) did not increase due to different ligand exposure, a low level constitutive expression of iNOS was observed in all cases. Only thrombocytes treated with polyinosinic–polycytidylic acid and thymidine homopolymer phosphorothioate oligodeoxynucleotides induced rapid, significant production of nitric oxide. We also observed that thrombocytes are able to respond faster upon TLR ligand exposure compared to MQ.NCSU macrophages.

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1. Introduction

The role of platelets as mediators and effector inflammatory cells has been a focus of research for the past few decades (Capron et al., 1987; Herd and Page, 1994; Klinger, 1997; Page, 1989), and evidence for nucleated thrombocytes having a significant role in avian immune responses is growing. Chicken thrombocytes have been shown to release β-thromboglobulin (Wachowicz et al., 1981), thrombocyte-derived growth factors (Horiuchi et al., 1990), lysosomal compounds (Carlson et al., 1968; Daimon and Uchida, 1982), chemotactic factors (Lam, 2002), platelet-derived growth factor-A and -B chains (Horiuchi et al., 2001, 2002) and some of the enzymes from the arachidonic acid cascade [cyclooxygenase-2 (COX-2), and prostaglandin D_2 (PGD_2), PGE_2, thromboxane A_2 synthases and leukotriene B_4] (Hitchcock, 2009; Jha et al., 2005; Scott and Owens, 2008; Scott lab unpublished). Our laboratory has demonstrated that chicken thrombocytes respond to lipopolysaccharide (LPS) (Ferdous et al., 2008; Scott and Owens, 2008), and this stimulation takes place through Toll-like receptor (TLR) 4 and mitogen-activated protein (MAP) kinase (ERK, MEK1 and p38 MAPK) and nuclear factor-κ light-chain-enhancer of activated B cell (NF-κB) pathways (Scott and Owens, 2008). Thrombocytes also express transcripts for TLRs 2–5, 7 and 21 (Ferdous, 2014; St Paul et al., 2012). Activation of the TLR4 pathway results in gene expression of pro-inflammatory cytokines interleukin (IL)-6, IL-1β, IL-8, and IL-12 (Ferdous et al., 2008; Hitchcock, 2009; Scott and Owens, 2008). According to another group of researchers, thrombocytes constitutively express transcripts for both pro- and anti-inflammatory cytokines for anti-viral responses and antigen presentation (St Paul et al., 2012). In a recent study, virus (i.e. H5N1 Avian Influenza) replicated to a high titer in chicken thrombocytes and caused up-regulation of TLR3 and several cell adhesion molecules (Schat et al., 2012).

Here, we have examined inflammatory responses when thrombocytes are stimulated *in vitro* with different TLR ligands {LPS from Gram-negative bacteria (TLR4 ligand), lipoteichoic acid (LTA) from Gram-positive bacteria (TLR-2 ligand), polyinosinic–polycytidylic acid [Poly (I:C)], a synthetic analog of double-stranded RNA (ds RNA) (TLR3 ligand), and thymidine homopolymer phosphorothioate oligodeoxynucleotides [Poly (dT)] a single-stranded RNA product (TLR 7/8 ligand)}. Chicken thrombocytes express TLRs and, therefore, should participate in inflammatory responses to bacterial and viral TLR ligands. Unanswered is whether or not thrombocytes respond to the same degree and magnitude as other immune cells due to stimulation by either bacterial or viral TLR ligands. We attempt to answer this question by examining gene expression in and product release from thrombocytes exposed to respective ligand types. Further, we have used MQ.NCSU cells, a well-established chicken macrophage cell line for examining possible differences in the innate immune response of a reference cell type.

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2. Materials and methods

2.1. Chickens

Female Single Comb White Leghorn (SCWL) chickens (between 16 and 26 weeks old) were used for preliminary studies and this study. In all instances, six chickens per experiment were randomly selected for blood collection. The chickens were housed at the Clemson University Morgan Poultry Center, Clemson, SC, which is an Institutional Animal Care and Use Committee (IACUC) approved animal facility operating under standard management practices adhering to the Association for Assessment and Accreditation of Laboratory Animal Care International criteria.

2.2. Thrombocyte isolation and in vitro stimulation

Syringes fitted with needles were used to collect 3 mL of whole blood from the wing vein of each chicken into 0.1 mL of 10% ethylenediaminetetraacetic acid (EDTA) solution. The collected blood samples were stored on ice until brought back to the laboratory. Thrombocytes were isolated as previously reported by Scott and Owens (2008). Briefly, each blood sample was diluted (1:1) with calcium- and magnesium-free Hank's balanced salt solution (HBSS) (Cambrex Bio Sciences Walkersville Inc., Walkersville, MD). Diluted blood samples were then layered on a lymphocyte separation medium (Density 1.077–1.080 g/mL, Mediatech Inc., Herdon, VA) and centrifuged at 1700 × *g* for 30 min at 23 °C to collect the thrombocyte-rich band. Thrombocyte enrichment using this cell isolation process was assessed by fluorescent microscopy, an image-based cytometer, and flow cytometry. Over 99% of cells in the isolated cell suspensions were positive for thrombocyte specific marker, CD41/CD61 (Ferdous, 2014). Trypan blue solution (0.4% w/v in normal saline) was used for quantification of viable cell numbers on a SPolite® Hemacytometer (Baxter Healthcare, McGaw Park, IL) with the aid of an upright light microscope.

The isolated thrombocytes from each chicken were incubated with 1 μg/mL of ultra pure LPS from *Salmonella minnesota*, 400 μg/mL of purified LTA from *Staphylococcus aureus*, 400 μg/mL of Poly (I:C) and 50 μM of Poly (dT) (InvivoGen, San Diego, CA). The specific dosages of the TLR ligands were determined from preliminary experiments conducted in our laboratory. The control samples were incubated with only HBSS and no TLR ligands. The cell suspensions and TLR ligands were incubated in sterile 1.5 mL microcentrifuge tubes (1×10^7 cells per tube) on a rocking platform (VWR, Suwanee, GA) at 41 °C for 10 and 60 min.

2.3. MQ.NCSU culture and stimulation

The MQ.NCSU cell line was a generous donation from Dr. Matthew Koci's laboratory (NCSU Prestage Department of Poultry Science, North Carolina State University, Raleigh, NC). These cells were cultured at 41 °C and 5% CO_2 with LM Hahn medium made with a combination of Leibovitz' L-15 medium (Corning CellGro, Mediatech, Inc., Manassas, VA) and McCoy's 5A medium (Corning Cellgro, Mediatech, Inc., Manassas, VA), tryptose phosphate broth (Sigma Aldrich, St. Louis, MO), bovine fetal serum (HyClone®, Logan, Utah), chicken serum (Sigma-Aldrich, St. Louis, MO), glutamine, sodium pyruvate (CellGro®, Mediatech Inc, Manassas, VA), 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and a mixture of penicillin, streptomycin, and fungizone (Lonza Walkersville Inc., Walkersville, MD) (Qureshi et al., 1990). The MQ.NCSU macrophages (1×10^7 in 1 mL) were incubated with 1 μg/mL of LPS and 400 μg/mL of Poly (I:C) as described above for *in vitro* thrombocyte stimulation.

2.4. RNA isolation and quantification

For RNA isolation after thrombocyte stimulation, cells were centrifuged at 5000 × *g* for 2 min to pellet. The pellets were stored in 100 μL of RNAlater™ (Qiagen Inc., Valencia, CA), a RNA stabilizing solution. After 24 hr at 4 °C in RNAlater™, the cells were centrifuged again to remove the supernatant and stored at –20 °C until thawed for RNA isolation. The RNeasy® Kit (Qiagen Inc., Valencia, CA) was used according to the manufacturer's protocol to isolate the total RNA from these samples. The RNA samples were treated with an on-column DNase, RNase-free DNase Set (Qiagen Inc., Valencia, CA) to remove any possible contamination from chicken genomic DNA. Isolated RNA samples were quantified using a Nano Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

2.5. Quantitative real time polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed using a QuantiTech® SYBR® Green RT-PCR Kit (Qiagen Inc., Valencia, CA) and an Eppendorf Mastercycler® ep *realplex*^{4} (Eppendorf North America, Westbury, NY). Primers used for housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), IL-6 and iNOS amplification are listed in Table 1. The RT-PCR mixture consisted of 0.20 μL QuantiTect RT Mix, 10 μL 2× QuantiTect SYBR Green RT-PCR Master Mix, 0.4 μL of 0.5 μM of each specific primer listed in Table 1, 8 μL of 1 ng/μL template RNA, and 1 μL of RNase-free water to make the final reaction volume of 20 μL. The cycling profile for all the reactions was 1 cycle of 50 °C for 30 min, 95 °C for 15 min, and 40 cycles of 94 °C for 15 s, 57 °C for 20 s, and 72 °C for 20 s. Each treatment was run in triplicates. Five-fold dilutions of total cellular RNA from chicken thrombocytes were used to generate standard curves for analysis. Amplification efficiencies calculated for each primer set are listed in Table 1. No-template controls were used in order to confirm target specific amplification, and no-reverse transcriptase controls were used to detect primer dimer formation. Relative quantification of the gene expression was determined through the use of the relative fold change calculation according to Pfaffl (2001). Data for each experiment represent means of six biological replicates (chickens) ± standard error.

2.6. NO assay

The Griess Reagent System (Promega Corporation, Madison, WI) was used to investigate nitric oxide (NO) formation by measuring nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO. A nitrite standard reference curve was prepared for each assay for accurate quantitation of experimental samples according to the manufacturer's protocol. The nitrite concentration of the reference curve ranged from 0 to 100 μM. The assay was performed as suggested in the manufacturer's protocol. Briefly, 50 μL of each experimental sample (supernatant from control and stimulated chicken thrombocytes and MQ.NCSU cells) was added

Table 1
Quantitative real-time polymerase chain reaction primer sets for messenger RNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene), interleukin (IL)-6, and inducible nitric oxide synthase (iNOS).

Target RNA	Accession no.	Primer sequence (5′–3′)	Efficiency (E)
GAPDH	NM_204305	F: ATGCCATCACAGCCACACAGAAGA R: ATGCCATCACAGCCACACAGAAGA	0.98
IL-6	AJ30954	F: ATGTGCAAGAAGTTCACCGTGTGC R: TTCCAGGTAGGTCTGAAAGGCGAA	0.91
iNOS	NM_204961.1	F: ATGGGAACAGAGATTGGAGTGCGA R: TACAACAGCTCGGTCCTTCCACAA	1.01

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