Developmental and Comparative Immunology 73 (2017) 124–130

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Lipopolysaccharide induced Interleukin-6 production is mediated through activation of ERK 1/2, p38 MAPK, MEK, and NFκB in chicken thrombocytes

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ARTICLE INFO

Article history:
Received 24 January 2017
Received in revised form
21 March 2017
Accepted 21 March 2017
Available online 23 March 2017

Keywords:
Thrombocyte
p38
ERK
MAPK
NFκB
Proinflammatory cytokine
Interleukin-6
Inhibitor

ABSTRACT

Thrombocytes express Toll-like receptor 4 and apparently use both mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB) pathways for nuclear signaling. However, it is not well known if the same enzyme systems found in mammalian cells are fully functional in chickens. Therefore, kinase inhibitors were used with thrombocytes to block kinases in lipopolysaccharide (LPS) stimulated cells to determine if interleukin (IL)-6 expression and production would be diminished. Results demonstrated that extracellular-signal-regulated kinase (ERK)1/2 and p38 MAPK pathways influence gene expression of IL-6 through treatment with either ERK or p38 MAPK inhibitor. In addition, thrombocyte lysates from cells treated with ERK, p38, mitogen-activated protein kinase kinase (MEK)1/2 and inhibitor of nuclear factor kappa-B kinase (IKK) inhibitor showed different levels of the phosphorylated form of ERK1/2, p38 and NFκB. Furthermore, IL-6 gene expression and production were significantly upregulated in LPS stimulated thrombocytes relative to all inhibitor-treated cells.

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1. Introduction

Avian thrombocytes serve a similar hemostatic function as mammalian platelets and are the most numerous of all chicken leukocytes, playing an integral role in innate immunity (Horiuchi et al., 2004). Thrombocytes express a variety of cytokines and chemokines involved in the inflammatory immune response. As part of the pro-inflammatory innate response, thrombocytes express genes for interleukin (IL)-1β, IL-6, IL-8, IL-12 and cyclooxygenase-2 (COX-2) (Ferdous et al., 2008; Ferdous and Scott, 2015; Scott and Owens, 2008; St Paul et al., 2012). Thrombocytes also express anti-inflammatory cytokines including IL-10 and transforming growth factor (TGF)-β (St Paul et al., 2012), as well as Toll-like receptors (TLRs) 1LA, 1LB, 2-2, 3, 4, 5, 7, 15 and 21 (Ferdous et al., 2008, 2016; Scott and Owens, 2008; St Paul et al., 2012). The TLRs recognize pathogen-associated molecular patterns that are essential components of pathogen survival [e.g. Gram-negative bacterial lipopolysaccharide (LPS), Gram-positive bacterial flagellin, bacterial and viral CpG-containing DNA] and some endogenous proteins released during infection (e.g. Heat Shock Protein-60, -70, and fibrinogen) (Akira and Takeda, 2004).

Thrombocytes bind bacterial LPS through TLR4, which initiates an inflammatory response (Ferdous et al., 2008; Scott and Owens, 2008). Scott and Owens reported that LPS exposure activated the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) signal pathways, and this led to increased gene expression of IL-6 and COX-2, with increased production of prostaglandin (PG) E_2 (Scott and Owens, 2008). The two studies by our laboratory (Ferdous et al., 2008; Scott and Owens, 2008) were the first published reports to show increased pro-inflammatory gene expression in thrombocytes resulting from exposure to LPS. Scott and Owens showed that inhibition of MEK1/2 by PD98059 blocked LPS-induced gene expression, implicating the involvement of this pathway in the expression of pro-inflammatory responses (Scott and Owens, 2008). That study also demonstrated that inhibition of inhibitor of kappa B kinase (IKK)

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http://dx.doi.org/10.1016/j.dci.2017.03.017
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with BMS345541 significantly reduced LPS-induced gene expression, documenting the involvement of the NFκB pathway in the thrombocyte pro-inflammatory response.

The current study focused on the effects of inhibiting specific signaling molecules in two mitogen-activated protein kinase (MAPK) pathways that are implicated in the expression of IL-6 in response to LPS. IL-6 initiates an inflammatory response to pathogens, activates both T and B lymphocytes, and promotes the differentiation of monocytes into macrophages (Burdin et al., 1995; Gessani et al., 1993; Rincon et al., 1997). In this study, SB203580 was used to inhibit p38, which is directly downstream of MEK3/6 in one of the MAPK pathways. An inhibitor was used to suppress the activity of extracellular-signal-regulated kinase (ERK), which is directly downstream of MEK1/2 in another MAPK pathway. These signal molecules were chosen for inhibition because previous studies in our laboratory have demonstrated the involvement of MEK in pro-inflammatory gene expression via ERK activation, but it was undetermined what role the p38 MAPK pathway would have in chicken thrombocyte gene expression. Previously, Scott and Owens were able to inhibit COX-2 gene expression if MEK1/2 was blocked by PD98059 (Scott and Owens, 2008). IL-6 gene expression was unaffected by this inhibitor at the concentration used. Also, there was no prior information regarding direct ERK or p38 MAPK inhibition on innate responses initiated by LPS. It was supposed, but unknown, that blocking ERK instead of MEK1/2 would lead to the same altered gene expression previously observed (Scott and Owens, 2008). This particular pathway, unlike the p38 MAPK pathway, has usually been associated with cell growth and differentiation rather than initiation of pro-inflammatory responses (Chae et al., 2005). The p38 MAPK pathway, on the other hand, has been linked to induction of pro-inflammatory gene expression (Widmann et al., 1999). In addition to inhibitors for ERK and p38 MAPK, we also used the two inhibitors employed by Scott and Owens for analysis of activated and released proteins in the MAPK and NFκB pathways (Scott and Owens, 2008). Experiments were conducted to gather more information on TLR4-linked pathways, gene expression and production of bioactive mediators in chicken thrombocytes.

2. Materials and methods

2.1. Thrombocyte recovery

Blood was obtained from 9 to 12-week old Single Comb White Leghorn (SCWL) female chickens. 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 (AAALAC) criteria. Syringes fitted with needles were used to collect 3 mL of whole blood from the wing vein of each chicken into 0.1 mL volume of 10% ethylenediaminetetraacetic acid (EDTA) solution. The collected blood samples were stored on ice until brought back to the laboratory. Thrombocytes were isolated by gradient centrifugation according to Scott and Owens (2008). Viable cell counts were performed using the Trypan blue method and dilutions made so that each microcentrifuge culture tube contained 1×10^7 cells.

2.2. ERK, p38 MAPK, MEK and IKK inhibitors and thrombocyte culture

The inhibitors used were for ERK (CAS1049738-54-6), p38 MAPK (SB203580, CAS15421-47-6), MEK1 (PD98059, CAS167869-21-8) and IKK (BMS-345541, CAS445430-58-0) from Calbiochem resuspended in dimethyl sulfoxide (DMSO) as required for dilution and resuspension. Concentrations of inhibitors used are described below under different experiments. A maximum volume of 27 μL DMSO was added to all reaction tubes in addition to HBSS. Following addition of inhibitors to suspended thrombocytes, all tubes were incubated at 41 °C on a rocking platform for 30 min in a incubator. After 30 min, the tubes were removed and 2 μL (10 μg/mL final concentration) lipopolysaccharide from *Salmonella minnesota* (ultra pure LPS, InvivoGen) was added to LPS treated tubes. All tubes were incubated at 41 °C on a rocking platform for 60 min in a incubator. The tubes were removed and centrifuged at 5000×*g* for 120 s at room temperature. The resulting supernatant was removed for bioassays. A set of cell pellets was resuspended in 100 μL RNA*later*™ (Qiagen) for real-time polymerase chain reaction. The other cell pellets were lysed for Western-blot analysis. In order to prepare cell lysates, lysis buffer (Cell Signaling Technology®) prepared according to manufacturer's protocol was used to resuspend the cell pellets on ice for 5 min. The lysed cells were pelleted with a 10 min spin at 5000 × *g* at 4 °C. The supernatant was removed and transferred to a separate microcentrifuge tube. All tubes were held frozen at −20 °C. Supernatants were also transferred to labeled tubes and held at −20 °C for later assay assessment of IL-6 from the treated thrombocytes.

2.2.1. Experiment 1

Based upon effective inhibitory concentrations from preliminary investigation in our laboratory, final concentrations of 25 μM ERK inhibitor and 70 μM p38 inhibitor were chosen. For this first experiment 25 μM ERK inhibitor with and without LPS, and 70 μM p38 inhibitor with and without LPS were used. Thrombocytes that were not treated with LPS or inhibitor served as negative controls. Thrombocytes treated only with LPS were considered positive controls.

2.2.2. Experiment 2

Based on the first experiment here and our previous experimental results from Scott and Owens (2008), the final concentrations of inhibitors used per reaction were ERK (25 μM), p38 MAPK (70 μM), MEK1/2 (10 μM) and IKK (5 μM). Again, all other experimental conditions for treating thrombocytes in culture were the same.

2.3. RNA isolation and real-time reverse transcriptase polymerase chain reaction

RNA from each tube of cells was extracted according to a previously established protocol (Scott and Owens, 2008), using a Qiagen RNeasy® kit with the addition of on-column DNase digestion. The final RNA concentration in each treatment tube was determined using a spectrophotometer (Eppendorf BioPhotometer).

Real-time RT-PCR was performed using the Quantitech SYBR Green RT-PCR kit (Qiagen) and an Eppendorf Mastercycler ep *realplex* (Eppendorf North America). Reactions were completed using 0.5 μM forward and reverse primers for GAPDH and IL-6 (Table 1). Dilutions were made for all treatments so that each individual reaction contained 10 ng RNA in a volume of 8 μL. Twelve microliters of master mix containing buffer, reverse transcriptase, water, and forward and reverse primers were added to each experimental reaction. In addition, no inhibitor and no LPS for each sample were repeated without reverse transcriptase as a negative control. A “No Template” negative control with DEPC-treated water in place of RNA was used to ensure purity of master mix components. The cycling profile was 50 °C for 30 min (reverse transcriptase), 95 °C for 15 min (DNA polymerase), 40 cycles of 94 °C for 15 s

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