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ERK 1/2 AND p38 MAPK PATHWAYS ARE BOTH INVOVLED IN THE EXPRESSION OF INTERLEUKIN-6, -8, AND CYCLOOXYGENASE-2 IN THROMBOCYTES STIMULATED WITH LIPOPOLYSACCHARIDE

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ABSTRACT

This study was conducted to determine if the p38 and ERK1/2 MAPK pathways are involved in transcription of IL-6, IL-8, and COX-2 in the chicken thrombocyte response to ligation of Toll-like receptor 4 (TLR4) by *Salmonella minnesota* lipopolysaccharide (LPS). Thrombocytes were isolated and subsequently treated with either p38 MAPK or ERK inhibitor, and then with LPS during *in vitro* cell culture. Transcription of IL-6, IL-8, and COX-2 mRNA was determined using real-time PCR. The experiments were repeated using pM, nM and μM concentrations of both inhibitors to test cell sensitivity. Stimulation with LPS induced expression of IL-6, IL-8 and COX-2 mRNA. This expression was reduced by treatment with 25 μM ERK inhibitor as well as by treatment with 70 μM p38 MAPK inhibitor. However, treatment with 25 pM and nM ERK inhibitor, and with 70 pM and nM p38 MAPK inhibitor, yielded no decrease in mRNA expression in relation to treatment with LPS alone. Inhibition with both the ERK and p38 MAPK inhibitors was in accordance with previously established concentrations for mammalian cells. It is now apparent that the p38 and ERK1/2 MAPK pathways are both linked to gene expression of a cytokine, a chemokine and an enzyme involved in the thrombocyte innate response to TLR4 ligation by bacterial LPS.

DEDICATION

I'd like to dedicate this thesis to my mother, Dr. Lorraine Collins. No one I know has set a better example of unending strength and perseverance. I could not have achieved this without your love, support and encouragement.

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LITERATURE REVIEW

Avian Innate Immunity

The innate immune system of aves is similar to that of mammals. Many components such as cytokines, complement, and TLRs have proven to be highly conserved, with the exception of some blood cells (e.g., heterophils, thrombocytes).

Chickens also have many of the same innate immune cells found in mammals. Dendritic cells engulf, process, and present pathogens to naïve T cells using finger-like extensions. Immature dendritic cells circulate in the blood prior to migration into tissues. At this point, they are capable of migrating to secondary lymphoid tissue to stimulate maturing T cells. Monocytes and macrophages are also part of chicken innate immunity. At the time of hatch, chicken macrophages are fully capable of phagocytosis (Qureshi et al, 2000). Monocytes are leukocytes produced in the bone marrow which enter the bloodstream and, after a few days, migrate into the tissues. Once in the tissues, they mature into macrophages. Macrophages phagocytize, process, and present antigens complexed with class II MHC to stimulate sensitized T cells against the presented antigen.

Interestingly, chickens lack the variety of granulocytes found in the mammalian immune system, including functional eosinophils (Vegad & Katiyar, 1995; Kaiser et al, 2005). The primary granulocyte is the heterophil, the avian counterpart to a neutrophil. Like macrophages, heterophils are highly phagocytic. In addition, they secrete proteolytic enzymes and reactive oxygen intermediates (Kogut et al, 2001; Swaggerty et al, 2003). However, the contents of heterophilic granules differ significantly from those of neutrophils (Harmon, 1998). They contain cationic peptides, lysozyme, acid phosphatase, acid hydrolases, Cathepsin, and α-glucosidase (Brune and Spitznagel, 1973; Rausch and Moore, 1975; Daimon and Claxton-Martins, 1977; Fujimori and Yamada, 1978; Maxwell, 1984). Chickens also have cells homologous to

mammalian natural killer (NK) cells. They bear a phenotypic resemblance to mammalian NK cells and display cytotoxic activity against target cell lines (Gobel et al, 1994). These cells are large granular lymphocytes characterized by intracellular expression of CD3 and a lack of receptors definitive of B or T lymphocytes (Gobel et al, 1994). It was previously believed that they were capable of cytolytic activity upon leaving the bone marrow, but in 2004 it was discovered that human NK cells require stimulation, possibly by T-cell secreted IL-2, for cytolytic activity to occur (Ferlazzo et al, 2004). Unlike human NK cells, chicken NK cells do not express MHC class II (Gobel et al, 1994). Chickens also have mast cells, which mediate anaphylaxis as well as the response to parasitic infection. Chicken mast cells are similar to those in mammals, degranulating upon stimulation, to release histamine and other preformed mediators. Secreted factors act to recruit granulocytes, lymphocytes, and monocytes or macrophages to the site of infection (Lin & Befus, 1999). In the avian gut, mast cell histamine release is associated with ion secretion leading to secretory diarrhea, which aids in bacterial clearance (Collins et al, 2007). Chicken mast cells are most prevalent in the ovaries and the lamina propria of the small intestine (Swayne and Weisbrode, 1990). Our laboratory also observed them in the Harderian gland (Scott et al, 2005) lining the subepithelium of the ducts. Rose et al (1980) found that they have the same preformed immune mediators as their mammalian counterparts.

Chicken thrombocytes are the avian counterpart to mammalian platelets. Like platelets, they primarily function in hemostasis, but in contrast to platelets, are nucleated. This means that thrombocytes are capable of responding to external stimuli by altering gene expression. They are morphologically similar to leukocytes, and also have a role in innate immunity and activation of acquired immunity (Traill et al, 1983). Activated thrombocytes express a range of immune mediators including cytokines, chemokines, leukotrienes, and prostaglandins. In addition to birds, nucleated thrombocytes can be found in reptiles and fish. While they primarily function in

coagulation, these cells are also phagocytic, having many lysosomes containing acid phosphatase, basic proteins, and fatty acids (Wigley et al, 1999; Sweeny & Carlson, 1968). They number between 20 and 30 thousand per milliliter of blood, and comprise over 90% of adherent cells in the peripheral blood, (Campbell, 1988; Grecci et al, 1980). Unlike heterophils, these cells are incapable of undergoing oxidative burst (Lam, 1997). Thrombocytes are of interest because, among other reasons, they are the most abundant leukocytes in circulation.

Mammalian platelets are formed by blebbing off large cells, megakaryocytes, located in the bone marrow. Circulating platelets are generally viable for 7 to 10 days (Weiss, 1975). Being non-nucleated, they have no genomic DNA, but do contain mRNA and transcriptional machinery derived from the megakaryocyte (Healy et al, 2006). Interestingly, pre-mRNA, found only in the cell nucleus, has been detected in platelet cytoplasm (Denis et al, 2005). Some primary transcripts found in platelets include TNF-β, IL-7, Stem Cell Factor, cMpl, IgE receptor subunits, and nuclear factor-E2, a transcription factor (Soslau et al, 1997). When stimulated, they express new receptors for adhesive proteins, aggregate, and release cytokines (Gear and Camerini, 2003). Platelets also make direct contact with granulocytes, endothelial cells, monocytes, and lymphocytes in order to activate them (Halvorsen et al, 1993; Klinger, 1997; Gear and Camerini, 2003). Each cell has approximately 35 α granules containing platelet derived growth factor, clotting factors, and platelet factor 4 (Klinger, 1997). They also have dense bodies containing serotonin, epinephrine, histamine, dopamine, ADP, ATP and calcium (Holmsen & Weiss, 1979; Wurzinger, 1990).

Avian thrombocytes express many of the same receptors and secreted factors as platelets. These include CD40L, which binds to the CD40 on B cells to aid in stimulation and survival, on macrophages to stimulate nitric oxide synthesis, and on bursal lymphoma cells to induce apoptosis (Tregaskes et al, 2005). Also common to both platelets and thrombocytes are

glycoproteins IIb and IIIa, which mediate cohesion leading to formation of the hemostatic plug (Kunicki & Newman, 1985). Thrombocytes also express MIP-1β, a chemokine that recruits and activates granulocytes, and platelet derived growth factor, which stimulates angiogenesis (Lam, 2002; Horiuchi et al, 2001). Other factors include β-thromboglobulin, a marker of thrombocyte activation, anti-apoptotic factors, lysosomal compounds, and B-G associated class III MHC antigens (Wachowicz and Krakewski, 1979; DaMatta et al, 1999; Taffarel and Oliveira, 1993; Daimon and Uchida, 1982; Salomonsen et al, 1991).

As research on thrombocytes has accumulated, it has become obvious that these cells serve a role in more than just coagulation and phagocytosis. It is now clear that they are integral in the avian response to pathogenic invasion. Traill et al (1983) demonstrated that anti-bursal cell serum identifies markers common to B cells and thrombocytes. Higashihara et al (1986) showed that thrombocytes share a polypeptide with MDCC-MSB1 cells. The K1 antibody, which has been used to discriminate thrombocytes from B and T cells via flow cytometry, binds a heterodimeric adhesion molecule expressed on both macrophages and thrombocytes of the chicken (Kaspers et al, 1993; Bohls et al, 2005). Anti-VLA4 recognizes markers on thrombocytes, hematopoietic progenitor cells, and eosinophils (McNagny et al, 1992). Along with the numerous secreted immune modulators, thrombocytes have many surface antigens characteristic of immune cells.

It has been demonstrated that avian thrombocytes express multiple TLRs, a critical and highly conserved component of the innate immune system in vertebrates (T. Scott and M. Owens, unpublished data). Bursal cDNA was first used to demonstrate the presence and functionality of chicken TLR2 (Fukui et al, 2001). More recently, differential induction of nitric oxide via TLR ligation was demonstrated in turkey monocytes (He et al, 2008). Scott and Owens (2008) demonstrated mRNA expression of TLR4 and gene expression via the NFκB and ERK

intracellular signal pathways. This work demonstrated not only that these cells respond to a pathogen associated molecular pattern (PAMP) via a highly conserved MAPK pathway, but also that they express IL-6 and COX-2 in response to bacterial stimulation (Scott and Owens, 2008).

Pathogen recognition often begins with binding of a bacterial or viral component such as lipopolysaccharide or dsRNA by a TLR. Ligation of TLRs results in activation of specific signal pathways, many of which are also highly conserved. Initiation of the intracellular signal leads to alteration of gene expression and results in production of some type of response, such as secretion of pro-inflammatory molecules.

Chickens express many orthologues of human TLRs. Thus far, orthologues of mammalian TLR-1 -2, -3, -4, -5, -7, and one similar to TLR-1, 6, and 10 have been identified. These will be discussed further below.

Avian cells express an array of cytokines that is more narrow in scope compared to mammalian systems (Hughes et al, 2007). Cytokines are secreted glycopeptides that bind specific target receptors, initiating an intracellular signal cascade. Depending on the cytokine and target cell, the signal cascade leads to a response, often a change in gene expression. Cytokines can be subdivided into interleukins (IL), tumor necrosis factors (TNF), transforming growth factors (TGF), interferons (IFN), colony stimulating factors (CSF), macrophage inflammatory protein (MIP) and monocyte chemoattractant protein (MCP). In general, there is low homology between avian and human cytokine sequences (Kaiser et al, 2005).

In the IL subclass of cytokines, chickens express orthologues to IL-1β, -10, -19, -22, -26, four subtypes of IL-17, most T_H1, T_H2, and T cell-proliferative interleukins, and IL-3, 6, 7, 9, and 16 (Kaiser et al, 2005). Most ILs are expressed by leukocytes, although many non-immune cells secrete them as well. They serve a variety of functions, including recruitment of immune cells to sites of infection, and inducing proliferation and activation of cytolytic cells.

IFNs are secreted by immature dendritic cells and virally infected cells in response to infection or tumor growth. They inhibit viral RNA production (Type I) and activate (Type II) natural killer cells and macrophages. In chickens, orthologues to human IFN-α, -β, and –γ have been discovered thus far (Guo et al, 2008). Ligation of many IFN receptors results in activation of the JAK/STAT signal pathway and p53-mediated apoptosis in target cells. IFNs also initiate or upregulate expression of certain genes, such as that for the Myxovirus resistance protein (Mx). This protein has intrinsic GTPase activity and is used for sensing the presence of "nucleocapsid-like structures" during infection. It also traps viral components in areas of the cell where new viral particles cannot be assembled (Li et al, 2007). Ligation of IFN receptors also leads to production of Very Large Inducible GTPase 1 (VLIG-1) (Guo et al, 2008). This is a large enzyme that regulates transcription of hundreds of genes involved in pathogen defense (Klamp et al, 2003).

TNFs bind target receptors to initiate a signal cascade leading to cell death via the caspase pathway. Chickens express ten known TNF-like cytokines (Kaiser et al, 2005). They bind target receptors on endothelial cells to increase vascular permeability, thus allowing an influx of immune cells and other inflammatory molecules. Most TNF receptors are type II transmembrane proteins, meaning they make a single pass through the lipid bilayer, and the N-termini lie on the cytoplasmic face of the plasma membrane.

Chemokines, a subclass of cytokines, are 67-127 amino acid-long glycoproteins that function to draw immune cells into an area of damage or site of infection. Most are secreted, but a few exist which are membrane-bound (Kaiser et al, 2005). The chicken repertoire of chemokines differs significantly from that of mammals (Guo et al, 2008). Chemokines are categorized into subfamilies based on the first two N-terminal cysteine residues. In CXCL chemokines, these residues are separated by one amino acid. The cysteine residues are adjacent in the CCL

subfamily. XCL chemokines have only two cysteine residues, with one near the N terminus and the other downstream. The few known CX_3CL chemokines have three amino acids separating the two N-terminal cysteines.

Chemokines are a critical component of the innate arm of the immune system. They can have endocrine, paracrine, or autocrine function, and mediate inflammation as well as hematopoiesis. Thus far, orthologue genes to human CCL-1 through -5, -8, 11, -13, -15, -16, -18, and -23 have been discovered. In addition, orthologues to CXCL-1 through -8, CXCL-12 through -14, CX_3CL1, and XCL-1 have been discovered (Kaiser et al, 2005). CCL chemokines are grouped into the MIP and MCP families (Kaiser et al, 2005). Functionally, chemokines are divided into those which enhance cytokine (type I) responses and those that enhance antibody (type II) responses. Some chicken cytokines with known functions include CXCR4, which is involved in regulation of progenitor cell trafficking, XCL-1, which is a T cell chemoattractant, CXCL13, a B cell attractant, and CAF, a chemotactic and angiogenic factor (Tamamura et al, 2006; Moser et al, 2004; Kaiser et al, 2005). Ah294 is a possible orthologue of mammalian CCL5, which is a chemoattractant for monocytes, memory T_H cells, and eosinophils (Hughes et al, 2007). Phylogenetic analysis groups the chicken ah221 gene with the mammalian MCP family of cytokines (Hughes et al, 2001).

Toll-like Receptors

TLRs are highly conserved members of the IL-1 receptor family that can be found on cell surface and internal compartment membranes across many animal phyla (Boyd et al, 2007). They were named for their structural similarity to a protein coded by the *Drosophila Toll* gene which is required for the establishment of the dorsal-ventral axis in developing larvae (Medzhitov et al, 1997). Later, these receptors were identified as a critical component of the innate immune

system. Members of IL-1R family share an approximately 200 amino acid cytoplasmic region called a TIR (Toll/IL1R) domain. Extracellular regions contain leucine-rich repeat domains (Akira, 2003). All TLRs have leucine-rich repeats and one or two cysteine-rich regions in the ligand-binding region of the external domain (Kindt et al, 2007). A short transmembrane domain spans the plasma membrane. The cytoplasmic, or TIR (Toll/IL-1R) domain of these receptors is structurally similar to the cytoplasmic domain of the IL-1 receptor, and is very much homologous among the different TLRs.

At present, eleven TLRs have been identified in humans and mice. Eight human orthologues have been identified in chickens (Iqbal et al, 2005). Most TLRs function as either hetero- or homodimers, and all bind to a specific bacterial or viral component. Collectively, these components are called pathogen associated molecular patterns (PAMPs), and are conserved among the types of pathogens in which they are found.

Mammalian TLRs that reside on internal membranes include TLR-3, -7, -8, and -9. These internal receptors bind PAMPs found inside infected cells, including double- and single-stranded viral RNA, and CpG dinucleotides of bacterial DNA. TLR-1 and -2 dimerize on the plasma membrane to bind components of bacterial parasites (Kindt et al, 2007). TLR-2 and -6 dimerize to bind components of gram positive bacteria and fungi (Kindt et al, 2007). TLR-4 monomers come together to bind lipopolysaccharide (LPS), a major component of gram negative bacterial cell walls. This homodimer also binds the F protein of respiratory syncytial virus. Finally, TLR-5 forms a homodimer to bind flagellin (Kindt et al, 2007). The functions of human TLR-10 and -11 are at present unknown.

In the avian immune system, human orthologues of TLR-1 through TLR -7 have been identified (Iqbal et al, 2005). To date, no avian orthologues of TLR-8, -9, or -10 have been discovered (Yilmaz et al, 2005). TLR-2 and -4 are the best characterized thus far. Two isoforms

of chicken TLR-2 have been reported, and interrupted remnants of the gene for one of these isoforms was discovered in the human genome, suggesting that gene duplication occurred prior to the divergence of mammals and birds 300 million years ago (Boyd et al, 2007). While the two isoforms of chicken TLR2 share 88.5% nucleic acid identity, only isoform 2 has demonstrated a strong response to classic mammalian TLR2 agonists (Fukui et al, 2001). Both TLR-2 and -4 share around 50% amino acid sequence identity with the human counterparts, and evidence suggests that these TLRs were distinct before the divergence of mammals and birds (Yilmaz et al, 2005).

As in mammals, TLR4 ligation is responsible for eliciting the avian immune response to LPS. It was previously believed that TLR2 isoform 2 bound LPS to initiate this response, but a 2008 study revealed that this response is generated exclusively by TLR4 in conjunction with MD (myeloid differentiation protein)-2 (Keestra & van Putten, 2008). In the mammalian system, LBP binds LPS and transfers it to CD-14, which then delivers the molecule to MD-2 (Pallson-McDermott & O'Neill, 2004). The conformational change induced in MD-2 by LPS ligation triggers transfer of the molecule to TLR4 (Kim et al, 2007; Ohto et al, 2007). In this study, a protein similar to CD14 was discovered, but no orthologue to LBP was apparent (Keestra & van Putten, 2008). It seems, however, that CD14, MD-2, and LPS interaction during initiation of the LPS response is similar in chickens and mammals (Keestra & van Putten, 2008). While all other known TLRs aside from TLR3 activate only MyD88-dependent signal pathways, TLR4 ligation recruits multiple adapter molecules, namely TIRAP (in the MyD88 dependent pathway), TRAM (TRIF-related adapter molecule), and TRIF (TIR-domain-containing adapter-inducing IFN-β) (Keestra & van Putten, 2008). Dual activation of TRIF and TRAM is unique to the TLR4-activated pathway (Yamamoto et al, 2003). The authors of this publication attribute the previous judgment on TLR2-LPS ligation to contaminating lipoproteins (Hirschfeld et al, 2000). In some

genetic lines of chickens, TLR4 has been mapped to a gene region associated with susceptibility to *Salmonella* infection (Leveque et al, 2003).

TLRs are highly conserved receptors, the ligation of which activates highly conserved intracellular signal cascades. Activation of these cascades leads to a variety of responses, depending on the ligand and cell type. TLRs are a critical component of the innate immune system in all animal phyla examined to date. TLR4, in particular, allows immune cells such as avian thrombocytes and mammalian macrophages to initiate a local response to invading gram negative bacteria through activation of both MyD88-dependent and -independent MAPK pathways.

Intracellular Signal Cascades in the Innate Immune Response

Ligation of all TLRs induces activation of intracellular signaling cascades. In particular, the p38 mitogen-activated protein kinase (MAPK), extracellular-signaling regulated kinase (ERK), and Nuclear factor (NF)-κB pathways are activated. Induction of these pathways via TLR ligation activates cell-mediated immunity, apoptosis, antimicrobial peptide production, or pro-inflammatory molecule production. TLR ligation recruits MyD88 to the TIR domain of the receptor. The IRAK1/IRAK4 complex then docks on MyD88, and IRAK4 phosphorylates IRAK1, creating a binding site for TRAF6. The newly formed IRAK1/TRAF6 complex dissociates from IRAK4 to activate TAK1. From this point, TAK1 may activate IKK in the NFkB or MEK in the p38 MAPK pathway. TAK1 also leads to activation of TPL2, which then activates MKK1/2 in the ERK1/2 pathway (Banerjee & Gerondakis, 2007).

ERK1/2 can be found in varying levels in all tissues (Chen et al, 2001). Activation of the ERK pathway via TLR4 ligation begins with recruitment of Ras to the cytoplasmic tail of TLR4. Ras binds temporarily to a docking site on the tail and recruits Raf1. Raf1 phosphorylates and activates MEK1/2, a serine/threonine kinase, which in turn phosphorylates and activates ERK1/2.

ERK1/2 is then free to enter the nucleus and activate Elk, a transcription factor. Ligation of peptidoglycan by TLR2 and CpG by TLR9 also induce activation of the ERK pathway via Ras. However it is important to note that ERK activation via TLR4 ligation is Ras-independent (Chen et al., 2004; Xu et al., 2003; David et al., 2005). The ERK pathway has traditionally been linked to cell survival and proliferation, but recent research shows that it is also integral to the innate immune response (Roux & Blenis, 2004; Galdiero et al, 2005).

Inhibition of MEK1/2 in the ERK pathway in U937 human monocytic leukemia cells as well as in chicken thrombocytes has led to decreased mRNA expression of TNF-α, IL-1β, and COX-2 (Galdiero et al, 2005; Scott & Owens, 2008). Interestingly, LPS-induced expression of IL-6 and -8 are unchanged in U937 cells after treatment with PD98059 (Galdiero et al, 2005). However, LPS-induced expression of IL-6 and TNF were suppressed in alveolar macrophages treated with MEK1 inhibitor PD98059 (Carter et al, 1999).

TLR-4 ligation also leads to activation of the p38 MAPK cascade. This pathway, unlike the ERK pathway, has traditionally been linked to inflammation (Chae et al, 2005). Ligation leads to activation of TAK, which phosphorylates and activates MEK3/6. MEK3/6 then phosphorylates and activates p38. This molecule then enters the nucleus and activates MSK1/2 which leads to transcription of pro-inflammatory molecules, including IL-6, IL-1, TNF-α and IL-8 (Galdiero et al, 2005; Scott and Owens, 2008).

Inhibition of p38 suppresses LPS-induced production of TNF-α, IL-6, and IL-10 in humans (Kaminska, 2005). As with the ERK pathway, inhibition of the p38 MAPK pathway using SB203580 significantly lowered the levels of IL-6 and TNF produced in human alveolar macrophages stimulated with LPS (Carter et al, 1999). In contrast, Galdiero et al (2005) found that LPS-induced expression of IL-6 and -8 was unchanged when U937 human monocytic cells

were treated with SB203580. In murine macrophages, this pathway is apparently critical for expression of IL-6 and -12 (Zhu et al, 2005).

Despite varying results for expression of specific cytokines, it is clear that the p38 MAPK and ERK pathways are critically involved in inflammation. Furthermore, these pathways appear to be highly conserved, as their involvement in regulation of the inflammatory response has been demonstrated in mice, humans, and chickens, among others (Zhu et al, 2005; Galdiero et al, 2005; Scott and Owens, 2008; Aggeli et al, 2001).

The present study is an expansion upon previous research in our laboratory, which demonstrated the involvement of the ERK and NFκB pathways in the thrombocyte inflammatory response to LPS stimulation. This work has been expanded to include the p38 MAPK pathway, which, in many other organisms, is integral in the inflammatory response. In addition, this study has focused on the involvement of MAPKs rather than MAPKKs in the expression of inflammatory molecules. Expression of a cytokine, a chemokine, and an enzyme in response to LPS stimulation was examined. It was found that both the ERK and p38 MAPK pathways are involved in the thrombocyte response to LPS, and that both pathways regulate expression of interleukin-6, interleukin-8 and cyclooxygenase-2.

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 bind bacterial lipopolysaccharide (LPS) through TLR4, which initiates an inflammatory response (Ferdous et al., 2008; Scott and Owens, 2008). Part of the general innate response is the production of pro-inflammatory molecules including IL-6, IL-8 and cyclooxygenase-2 (COX-2). IL-6, a cytokine, initiates an inflammatory response to pathogens, activates both T and B lymphocytes, and promotes the differentiation of monocytes into macrophages (Gessani et al., 1993; Burdin et al., 1995; Rincon et al., 1997). IL-8, a CXC chemokine, is released locally at sites of infection or tissue damage. Upon release, this chemokine acts as a chemoattractant, recruiting heterophils which are activated to further enhance inflammatory responses to pathogens. COX-2 is an enzyme that catalyzes the initial conversion of arachadonic acid to various prostanoids, primarily PGE_2, PGD_2, and thromboxane A_2. Prostanoids are then released by cells to act as inflammatory mediators affecting surrounding tissues (Herlong and Scott, 2006).

Unlike thrombocytes, mammalian platelets lack nuclei and thus also the capacity to respond to pathogenic invasion through gene expression. Platelets do, however, store CC and CXC chemokines, cationic peptides, interleukins, and adhesive compartmentalized α-granules (Klinger and Jelkmann, 2002). Upon stimulation by contact with foreign pathogens, platelets may degranulate, releasing antimicrobial peptides and inflammatory mediators (Boehlen and Clemetson, 2001). It has also been shown that platelets are capable of aggregating, engulfing bacteria and viruses into vacuoles, and then fusing vacuoles with α-granule containing compartments (Klinger, 1997; Elzey et al, 2005).

Ferdous et al. (2008) found that incubating chick thrombocytes with LPS led to a significant increase in the expression of pro-inflammatory cytokines, specifically IL-1β, IL-6 and IL-12. Scott and Owens (2008) reported that LPS exposure activated the NF-κB and MEK signal pathways through TLR4 ligation, and this led to significantly increased gene expression of IL-6 and COX-2, with increased production of PGE_2. These two studies by our laboratory were the first published reports to show increased pro-inflammatory gene expression in thrombocytes resulting from exposure to LPS. Once gene expression was demonstrated through LPS activation, Scott and Owens (2008) showed that inhibition of MEK1 by PD98059 blocked this LPS-induced gene expression, implicating the involvement of this pathway in the expression of pro-inflammatory responses. That study also demonstrated that inhibition of IKK with inhibitor BMS345541 significantly reduced the LPS-induced gene expression, documenting the involvement of the NF-κB pathway in the thrombocyte pro-inflammatory response.

The current study focused on the effects of inhibiting specific signaling molecules in two MAPK pathways that are implicated in the expression of three pro-inflammatory gene products- a cytokine, a chemokine and an enzyme- in response to TLR4 binding LPS. 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 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 for inflammatory responses. Previously, Scott and Owens (2008) were able to inhibit COX-2 gene expression if MEK1/2 was blocked by PD98059. IL-6 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 TLR4

binding with LPS. It was supposed, but unknown, that blocking of 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). Since thrombocytes are terminal-stage cells incapable of further proliferation, would the role of the MEK1/2 to ERK transduction be different for a cell type completely committed to innate immunity? When MEK1/2 was inhibited in Scott and Owens' study (2008), there was a departure of effect between IL-6 and COX-2 gene expression. Would inhibition at the ERK level reveal a similar departure or would gene expression for IL-6, IL-8 and COX-2 all be decreased when inhibition occurs at a level closer to the nucleus? Also, would the assumed p38 MAPK's role in expression of pro-inflammatory genes found in other innate cells (i.e. neutrophils, heterophils, and macrophages) be a working mechanism in chicken thrombocytes? With these questions in mind, a series of experiments were conducted with the idea that more information could be gathered on TLR4 linked pathways and gene expression. The data presented were derived from both microarray analysis and real-time PCR in order to learn more about the pro-inflammatory potential of chicken thrombocytes.

MATERIALS AND METHODS

Thrombocyte Recovery

Blood was obtained from 9 - 12 week old Single Comb White Leghorn (SCWL) female chickens. 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 x 10^7 cells in a final volume of 1 milliliter.

ERK and p38 MAPK Inhibitors and Thrombocyte Culture

Five milligrams ERK inhibitor (Calbiochem) was resuspended in 1.0 mL dimethyl sulfoxide (DMSO), producing a 15 mM solution. Inhibitor suspension was added to each ERK inhibitor-treated tube, resulting in a final inhibitor concentration of 25 μM. An equal volume of DMSO was added to tubes not treated with inhibitor. One milligram SB203580, p38MAPK inhibitor (Calbiochem), was resuspended in 1.0 mL DMSO, producing a 2.6 mM solution. Inhibitor suspension was added to SB203580-treated tubes and an equal volume of DMSO was added to tubes not treated with inhibitor. This resulted in a final inhibitor concentration of 70 μM.

All tubes were then incubated at 41°C on a rocking platform for 30 min in a humidified water-jacketed incubator. After 30 min, the tubes were removed and 2 μL (10 μg/mL final concentration) of lipopolysaccharide from *Salmonella minnesota* was added to LPS treated tubes. Two microliters of DEPC treated water was added to those not treated with LPS. All tubes were incubated at 41°C on a rocking platform for 60 min in a humidified water-jacketed incubator.

The tubes were removed and centrifuged at 5000 x *g* for 120 s at room temperature. The resulting supernatant was removed and each cell pellet was resuspended in 100 μL RNA*later*™ (Qiagen). All tubes were held frozen at -20°C.

Preliminary Expriments

Based upon effective inhibitory concentrations from mammalian cell treatment, final concentrations of 25 μM ERK inhibitor and of 70 μM p38 inhibitor were chosen. To determine if these concentrations were appropriate for chicken thrombocytes, a preliminary experiment was performed. Different treatments were applied to four tubes of cells for each inhibitor. The first tube of cells served as a negative control and was not treated with LPS or inhibitor. The second tube was treated with inhibitor alone, the third with LPS, and the fourth with both inhibitor and LPS. Results are found in Appendix 1.

RNA samples from control cells, LPS-stimulated cells, and cells treated with μM concentrations of each inhibitor with and without LPS stimulation were subjected to microarray analysis (C. Keeler, University of Delaware). Approximately 4,000 genes potentially involved in TLR4-linked signal pathways were examined. This was done to verify our initial real-time PCR results and to further determine the scope of genes induced via TLR4 pathways. These results are presented in Appendix 2.

First Experiment

In order to determine if there was a difference in response to the inhibitors between two different genetic lines of chickens, the preliminary experiment described above was repeated with the same concentrations of inhibitors. However, both inhibitors were used in respective cultures in order to compare against the same DMSO control. Blood was taken from six birds of each genetic line for each test.

Second Experiment

Dilutions of both the ERK and p38 inhibitors were made in the pM, nM and μM range to determine thrombocyte sensitivity to treatment. The treatments in the first experiment were

repeated, with the addition of 25 pM and nM ERK inhibitor with and without LPS, and 70 pM and nM p38 inhibitor with and without LPS.

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 DNase digest. 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). Reactions were completed using 0.5 μM forward and reverse primers for GAPDH, IL-6, IL-8 and COX-2 (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. Two reactions per treatment per bird were performed. In addition, Treatment 1 (no inhibitor and no LPS) for each bird was 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 (denaturation), 57°C for 20 s (annealing), and 72°C for 20 s (extension).

mRNA	Primer sequence (5'-3')		Accession No.
Reference			
GAPDH	F	ATGCCATCACAGCCACACAGAAGA	**NM_204305**
	R	GCACACGGAAAGCCATTCCAGTAA	
Targets			
IL-6	F	ATGTGCAAGAAGTTCACCGTGTGC	**AJ309540**
	R	TTCCAGGTAGGTCTGAAAGGCGAA	
IL-8	F	TTTCAGCTGCTCTGTCGCAAGGTA	**AJ009800**
	R	CTTGGCGTCAGCTTCACATCTTGA	
COX-2	F	TCCACCAACAGTGAAGGACACTCA	**M64990**
	R	ATCCCACTCTGGATGCTCCTGTTT	

Table 1. Forward (F) and reverse (R) primer sets for chicken thrombocyte real-time reverse transcriptase polymerase chain reactions of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Interleukin-6 (IL-6), Interleukin-8 (IL-8) and cyclooxygenase-2 (COX-2).

Statistical Analysis

In order to determine treatment differences, least-squares analysis of variance was used to compare fold-change in mRNA expression of COX-2, IL-6 and IL-8. When appropriate, post-ANOVA comparisons of treatment means were accomplished by pairwise t-tests. In cases where the inhibitor controls (inhibitor and no LPS) were not different from the overall untreated control (no inhibitor and no LPS), the data was presented in bar graphs with the overall untreated control only representative of all controls.

RESULTS

Both p38 MAPK and ERK Inhibitors Interfered With mRNA Expression in LPS- Stimulated Thrombocytes.

In the experiment comparing expression of IL-6, IL-8 and COX-2 in two different genetic lines of chickens, no differences in expression of these molecules in response to LPS were found, so the data were pooled for genetic lines. Between the control cells (-/-/-) and those treated with LPS alone (+/-/-), expressions of IL-6, IL-8 and COX-2 mRNA were significantly different (Fig. 1A, B and C). There were also significant reductions in IL-6, IL-8 and COX-2 mRNA expressions for LPS stimulated cells treated with p38 MAPK (+/+/-) and ERK inhibitors (+/-/+) compared to those treated with LPS (+/-/-).

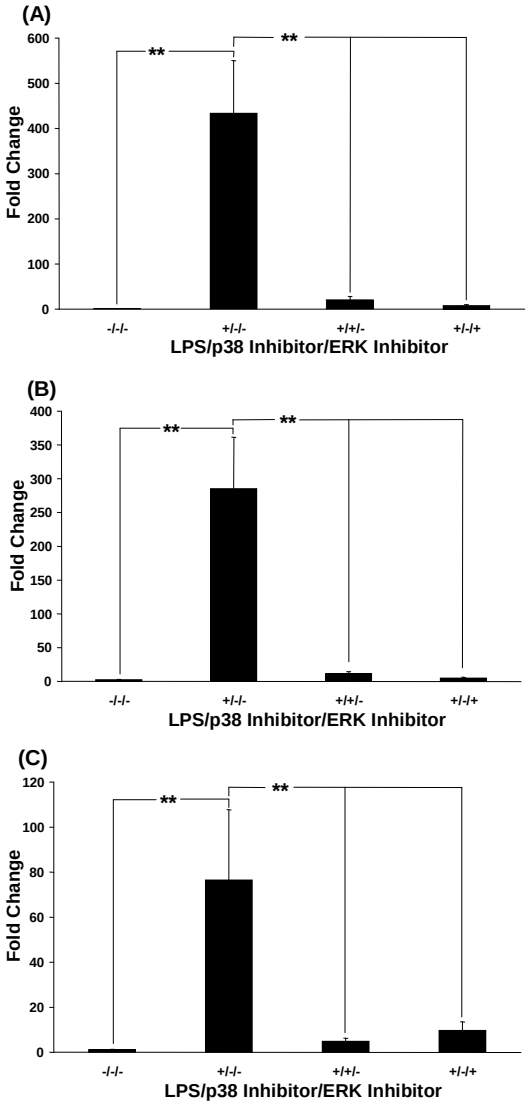

Figure 1. Interleukin-6 (IL-6) (A), Interleukin-8 (IL-8) (B), and cyclooxygenase-2 (COX-2) mRNA expression (C) in chicken thrombocytes following 30 min pre-treatment with or without p38 MAPK inhibitor SB203580 (70 μM) or ERK inhibitor (25 μM), and then 1h incubation of thrombocytes (1 x 10^7) at 41°C in the presence or absence of *S. minnesota* lipopolysaccharide (LPS: 10 μg/mL). The mRNA expression of all three molecules varied relative to LPS and inhibitor treatment combinations. Data are presented as means ± SEM (n=12). **$P \leq 0.01$.

Varying Concentrations of Both p38 MAPK and ERK Inhibitors Interfered with mRNA Expression in LPS-Stimulated Thrombocytes.

The effect of treating thrombocytes with varying concentrations of the two inhibitors resulted in varying expression patterns for the three genes of interest. For p38 MAPK inhibitor, IL-6 and IL-8 expression following LPS stimulation (+/-) was significantly elevated over that of unstimulated cells (-/-) (Fig. 2A and B), while the COX-2 response was elevated but not significantly (Fig. 2C). Interestingly, all three pro-inflammatory indicators had high induction of mRNA following LPS stimulation and inhibitor pre-treatment at the pM concentration. Gene expressions for all three were reduced at the nM concentration of p38 MAPK inhibitor, and the μM pre-treatment was effective in blunting LPS stimulated gene expression for all three.

In the experiment using dilutions of ERK inhibitor, treatment with LPS alone (+/-) significantly induced the expression of IL-6, IL-8 and COX-2 over the control thrombocytes (-/-). The μM concentration of ERK inhibitor resulted in reduced expression of all three genes while inhibitor treatment at pM and nM concentrations did not effectively interrupt gene expression in LPS stimulated thrombocytes (Fig. 3A, B, and C).

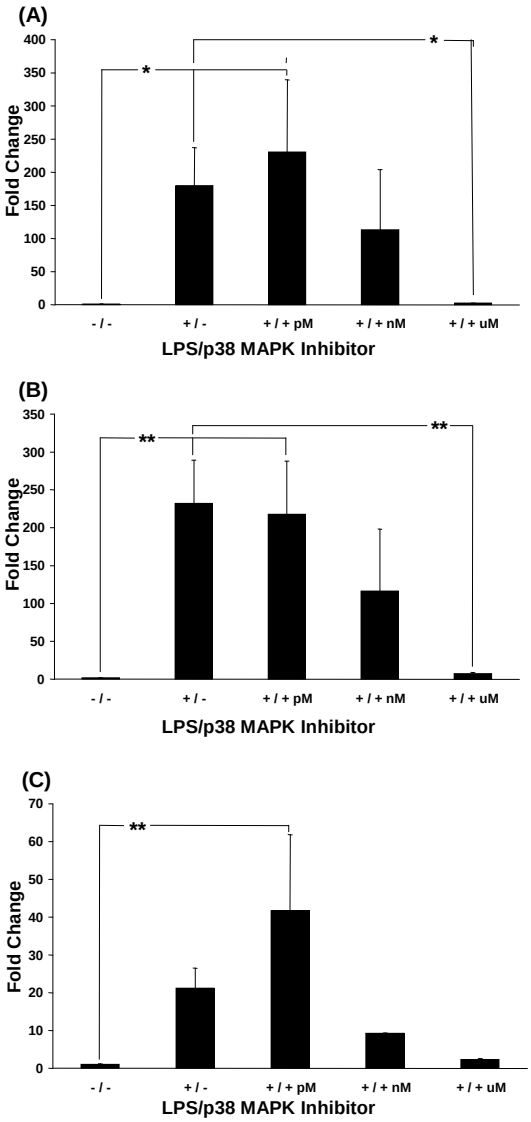

Figure 2. Interleukin-6 (IL-6) (A), Interleukin-8 (IL-8) (B), and cyclooxygenase-2 (COX-2) mRNA expression (C) in chicken thrombocytes following 30 min pre-treatment with or without p38 MAPK inhibitor SB203580 (70 μM, nM or pM), and then 1h incubation of thrombocytes (1 x 10^7) at 41°C in the presence or absence of *S. minnesota* lipopolysaccharide (LPS: 10 μg/mL). The mRNA expression of all three molecules varied relative to LPS and p38 MAPK inhibitor treatment combinations. Data are presented as means ± SEM (n=6). *$P \leq 0.05$, **$P \leq 0.01$.

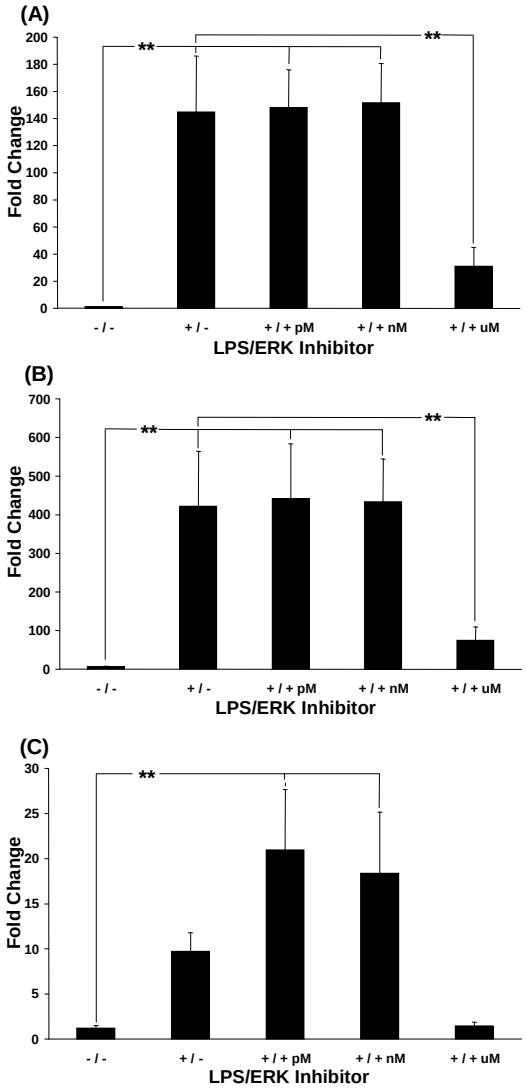

Figure 3. Interleukin-6 (IL-6) (A), Interleukin-8 (IL-8) (B), and cyclooxygenase-2 (COX-2) mRNA expression (C) in chicken thrombocytes following 30 min pre-treatment with or without ERK (25 μM, nM or pM), and then 1 h incubation of thrombocytes (1 x 10^7) at 41°C in the presence or absence of *S. minnesota* lipopolysaccharide (LPS: 10 μg/mL). The mRNA expression of all three molecules varied relative to LPS and p38 MAPK inhibitor treatment combinations. Data are presented as means ± SEM (n=6). *$P \leq 0.05$, **$P \leq 0.01$.

DISCUSSION

In chicken thrombocytes, stimulation with LPS leads to a number of inflammatory responses through TLR4-linked cytoplasmic pathways. The current experiments demonstrated that expression of IL-6, IL-8, and COX-2 genes are mediated by two of the three major MAPK signaling pathways. Specifically, both ERK and p38 MAPK pathways regulate gene expression for these pro-inflammatory molecules.

Previous work in our laboratory (Scott and Owens, 2008) has shown that LPS stimulates the NF-κB pathway of thrombocytes, resulting in increased expression of IL-6 and COX-2 mRNA and PGE_2 production. It was also demonstrated that MEK1/2 inhibition negatively affected expression of COX-2, but not IL-6 mRNA. In contrast, downstream ERK inhibition in the current experiments negatively affected expression of both IL-6 and COX-2, in addition to IL-8. Furthermore, the p38 MAPK pathway, previously shown to be involved in pro-inflammatory molecule expression in other innate cells (Khatri and Sharma, 2006; Chen et al., 1999) was also involved in the chicken thrombocyte innate response.

As reported by Scott and Owens (2008), IL-6 expression increased dramatically in response to LPS. In the present experiment, too, there was a significant response, and this expression was suppressed by treatment with μM concentrations of both p38 and ERK inhibitors. Furthermore, 70 nM of p38 inhibitor suppressed the IL-6 response. In a study using LPS-stimulated chicken heterophils, treatment with PD98059 reduced ERK concentrations (Kogut et al., 2007). In contrast to Kogut et al. (2007), treatment of thrombocytes with 10 μM PD98059 in our laboratory did not result in significantly lowered IL-6 expression (Scott and Owens, 2008) as expected. It is possible that the 10 μM concentration of PD98059 did not affect ERK activity enough to suppress the IL-6 response or that these are probable differences in pathway sensitivity of heterophils compared to thrombocytes for this particular inhibitor.

Treatments with pM and nM concentrations of ERK and p38 inhibitors were ineffective at suppressing the IL-6 response to LPS. The IC_{50} of p38 MAPK inhibitor for human monocytes is 50-100 nM (Gallagher et al., 1995), a concentration range that was marginally effective with chicken thrombocytes. Seventy μM p38 inhibitor was effective at suppressing LPS-induced expression of IL-6. The listed IC_{50} for ERK inhibitor is ≤ 25 μM for HeLa, A549, and SUM-159 tumor cells (Kumar et al., 1999; Hancock et al., 2005). This concentration was effective at suppressing the IL-6 response of chicken thrombocytes.

In heterophils, stimulation with LPS results in increased mRNA expression of IL-6 along with IL-8 and IL-1β (Kogut et al., 2005). Heterophil receptor-mediated phagocytosis also leads to increased expression of IL-6 and IL-8 (Kogut et al., 2003). Multiple studies have demonstrated IL-6 production by heterophils of different genetic lines, both constitutively (Swaggerty et al, 2004) and in response to stimulation with LPS (Kogut et al, 2006). In mammals, this pro-inflammatory cytokine stimulates neutrophil degranulation and B-cell differentiation, and induces acute phase proteins (Akira et al, 1990; Kaiser et al, 2000; Swaggerty et al, 2006). IL-6 also stimulates thrombopoiesis. Administration of IL-6 to C57BL/10 mice resulted in increased levels of thrombopoietin (TPO) mRNA in the liver, along with increased TPO plasma levels (Kaser et al, 2001). The increase in TPO mRNA was also accompanied by a significant increase in the number of circulating platelets (Kaser et al, 2001). When TPO was neutralized in vivo via injection of anti-TPO polyclonal antibody, this platelet elevation was not seen, indicating that IL-6 regulates platelet levels via TPO induction (Kaser et al, 2001).

Though in this experiment expression of IL-8 mRNA increased significantly in response to treatment with LPS, treatment with pM and nM concentrations of both p38 and ERK inhibitors were ineffective at suppressing the LPS-induced IL-8 response. However, treatment with 70 μM p38 inhibitor and with 25 μM ERK inhibitor were both effective at significantly lowering IL-8

mRNA expression. While thrombocytes are unique, terminal-stage cells, they apparently share many of the immunological signal pathways existing in other leukocytes. In general, CXC chemokines such as IL-8 act as chemoattractants for polymorphonuclear cells, while CC and C chemokines function in macrophage and lymphocyte attraction (Baggiolini et al., 1994; Luster, 1998; Zlotnik and Yoshie, 2000; Zhang et al., 2003). In neonatal chicks, infection with *Salmonella enteritidis* results in increased expression of CXCLi2, a chemokine synonymous to IL-8 that recruits heterophils (Kogut, 2002).

LPS also stimulated thrombocyte expression of COX-2 mRNA, and this response was suppressed by μM concentrations of both inhibitors. Scott and Owens (2008) previously demonstrated an increase in COX-2 mRNA expression by thrombocytes in response to LPS. In macrophages, exposure to Infectious Bursal Disease Virus (IBDV) results in greater expression of COX-2 and IL-8 mRNA (Khatri and Sharma, 2006) and inhibition of p38 and NF-κB in these cells suppressed the COX-2 mRNA response. IBDV infection has also been shown to stimulate production of IL-1β, IL-6, cMGF, and iNOS in chicken macrophages (Kim et al., 1998; Khatri et al., 2005). In J774 macrophages, inhibition of p38 attenuates LPS-induced COX-2 activity, and consequently inhibits the release of PGE_2 (Chen et al., 1999).

While microarray analysis is a valuable hypothesis-generating tool, the amount and reliability of pertinent information gained in this study was limited. Microarray data indicated that the p38 MAPK but not the ERK pathway regulates interleukin-8 expression. This is in contrast to RT-PCR results, which suggested that both pathways were involved in expression of this molecule upon TLR4 ligation. The data for IL-6 expression was in accordance with RT-PCR data, indicating that both pathways regulate expression of this molecule. No information on cyclooxygenase-2 expression was generated from this experiment. This analysis did, however,

support the theory that the NFκB pathway in chicken thrombocytes, as in most other systems examined, predominates MAPK pathways in the inflammatory response to bacterial stimulation.

The current experiments examined the effect of inhibiting two different MAPK pathways on production of a cytokine, a chemokine, and an enzyme involved in the innate response to bacterial LPS. Both the ERK and p38 pathways begin with ligation of TLR4 by LPS. The avian ERK pathway is not yet fully understood, but is known to be Ras-dependent (Kogut et al., 2007). Ligation of TLR4 activates an undetermined signal molecule, which in turn promotes conversion of Ras-GDP to Ras-GTP. Ras-GTP activates Raf1, which in turn activates MEK1/2. MEK1/2 phosphorylates and activates ERK1/2, which then phosphorylates and activates Elk-1. Elk-1 then enters the nucleus and acts as a transcription factor for pro-inflammatory molecules, like those examined in this study. In the p38 MAPK pathway, ligation of TLR4 results in activation of TAK, which phosphorylates MEK3/6. MEK3/6 in turn activates p38 (MAP Kinase 14). p38 activates a form of Elk which enters the nucleus and activates transcription of IL-6, IL-8 and COX-2.

Treatment with 20 to 40 μM PD98059 inhibited phosphorylation of ERK1/2 in limbal epithelial cultures and suppressed production of IL-8, IL-1β, and TNF-α in response to exposure to hyperosmolar medium (Li et al., 2006). In immortalized T/C28a2 human chondrocytes, activation of the ERK, p38 and JNK pathways resulted in enhanced expression of COX-2 and production of PGE_2 (Nieminen et al., 2005). The COX-2 and PGE_2 responses were suppressed by treatment with PD98059. Also, treatment with SB203580 inhibited COX-2 expression and subsequent PGE_2 production at μM concentrations (Nieminen et al., 2005).

Several models show the MAPK pathway involving p38 as the dominant pathway in pro-inflammatory responses, with the ERK pathway involvement primarily devoted to cell growth and differentiation events (Widmann et al., 1999; Lai et al., 2001). The results of the current

study would contradict those models, as stimulation of thrombocytes with LPS resulted in increased expression of IL-6, IL-8 and COX-2 mRNA which were all inhibited by treatment with the ERK inhibitor (Fig. 6). The involvement of the ERK MAPK pathway in pro-inflammatory responses is likely due to the fact that thrombocytes are terminal-stage cells that do not grow or differentiate. They are short-lived and actively function only in immune responses. The model for signaling in the thrombocyte (Fig. 6) is now expanded from that proposed by Scott and Owens (2008). Our model includes two MAPK and the NF-κB pathways for expression of genes encoded for pro-inflammatory molecules. The cytoplasmic signaling pathways in thrombocytes would appear to be primarily devoted to immunologic responses in support of inflammation since these cells express Toll-like receptors (Scott and Owens, 2008) and are poised to activate upon exposure to various pathogens entering the bloodstream.

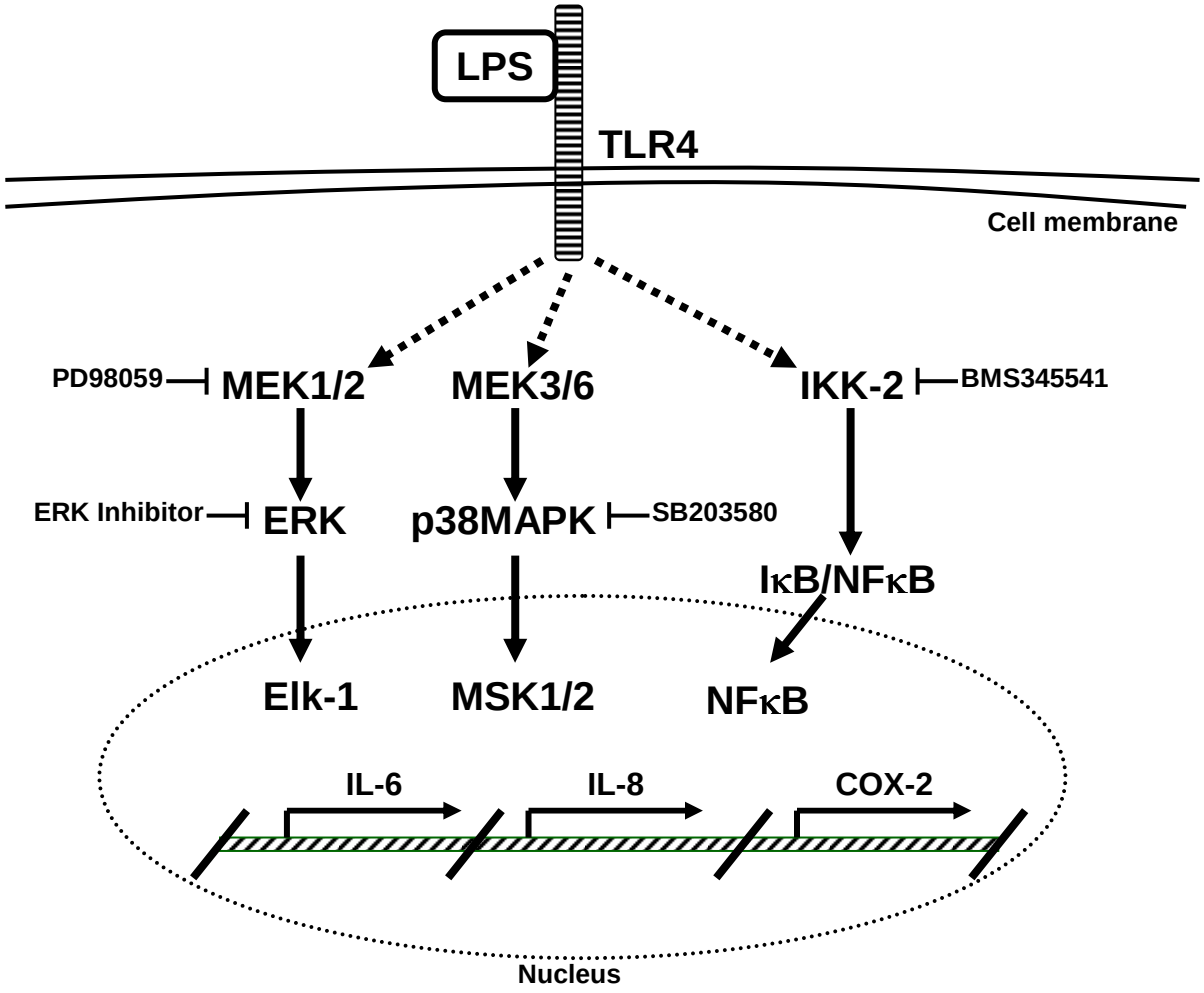

Figure 4. Diagram of LPS stimulation of thrombocyte TLR4-linked MAPK pathways leading to gene expression of IL-6, IL-8 and COX-2. Both the ERK and p38 pathways lead to stimulation of gene expression for these pro-inflammatory molecules based upon inhibition of ERK and p38 by respective inhibitors. Additionally, results of Scott and Owens (2008) revealed the role of the NF-κB pathway in support of the pro-inflammatory response of the thrombocyte. All three pathways would be considered major stimulation cascades for gene expression following Toll-like receptor ligation. Solid arrows represent direct steps in pathways. Dashed arrows represent multiple steps in pathways.

APPENDICES

APPENDIX 1: PRELIMINARY EXPERIMENTATION

Both p38 MAPK and ERK inhibitors interfered with mRNA expression of LPS stimulated thrombocytes.

In this preliminary experiment, using 70 μM p38 inhibitor on SCWL chicken thrombocytes, there were significant differences in IL-6, IL-8 and COX-2 mRNA expressions for LPS (+/-) treated cells compared to negative controls (-/-) and thrombocytes exposed to p38 MAPK inhibitor (+/+) (Fig. A1A, B, and C). Using 25 μM ERK inhibitor on SCWL chicken thrombocytes, there were significant differences in IL-6, IL-8 and COX-2 mRNA expression levels between control cells (-/-) and those treated with LPS (+/-) (Fig. A2A, B and C). The expression levels of cells treated with ERK inhibitor and LPS (+/+) were well below the expression levels of LPS (+/-) treated cells.

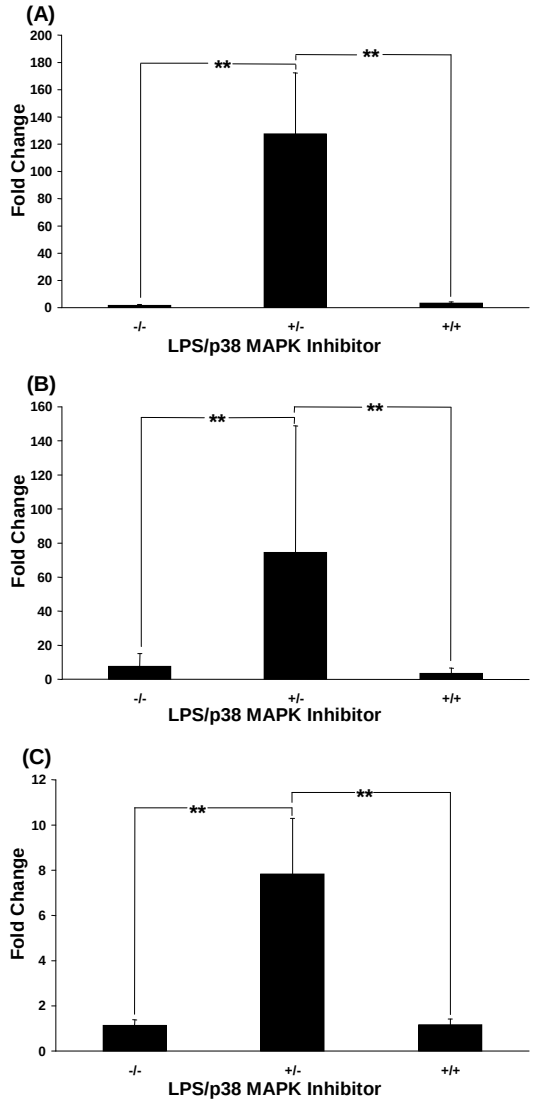

Figure A1. Interleukin-6 (IL-6) (A), interleukin-8 (IL-8) (B), and cyclooxygenase-2 (COX-2) mRNA expression (C) in chicken thrombocytes following 30 min pre-treatment with or without p38 MAPK inhibitor SB203580 (70 μM), and then 1h incubation of thrombocytes (1 x 10^7) at 41°C in the presence or absence of *S. minnesota* lipopolysaccharide (LPS: 10 μg/mL). The mRNA expression of all three varied relative to LPS and p38 MAPK inhibitor treatment combinations. Data are presented as means ± SEM (n=6). $^{**}P \leq 0.01$.

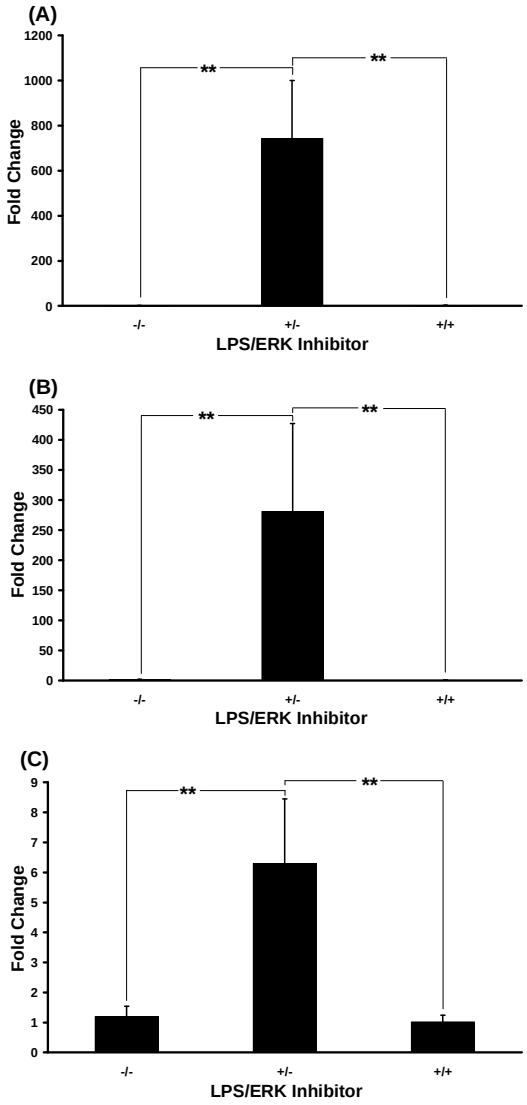

Figure A2. Interleukin-6 (IL-6) (A), interleukin-8 (IL-8) (B), and cyclooxygenase-2 (COX-2) mRNA expression (C) in chicken thrombocytes following 30 min pre-treatment with or without ERK inhibitor (25 μM), and then 1h incubation of thrombocytes (1 x 10^7) at 41°C in the presence or absence of *S. minnesota* lipopolysaccharide (LPS: 10 μg/mL). The mRNA expression of all three varied relative to LPS and ERK inhibitor treatment combinations. Data are presented as means ± SEM (n=6). $^{**}P \leq 0.01$.

APPENDIX 2: MICROARRAY DATA

Both p38 MAPK and ERK Inhibitors Interfere with mRNA Expression of Numerous Cytokines, Chemokines, and TLR-linked Signal Molecules in LPS-Stimulated Thrombocytes

Data from experiments in our laboratory indicated intact ERK and p38 MAPK signal pathways in chicken thrombocytes. With this knowledge, RNA samples were subjected to microarray analysis of approximately 4,000 genes. Examples of affected gene expression can be found in Appendix 2. While this analysis was intended to expand our knowledge of the scope of molecules affected by TLR4-linked pathways, the depth of information gained proved to be limited.

Gene Name	Genbank Accession #	Con vs. LPS	Con vs. p38 Inh.	LPS vs. LPS + p38 Inhib.	p38 Inh. vs. Inh. + LPS
MD-2	XM_418301	-	+1.869	+1.869	
TLR2 (isoform 1)	CK607640	-1.838		-1.736	-1.704
TLR3	NM_001011691	+1.737	+1.893	+1.893	
TLR4	NM_001030693	+2.669		-	
TLR5	BI066471	+4.266		-1.748	
TLR7	BM440145	+2.336		-	+1.679
TLR15	CK611396	+7.316			
TAB1	NM_001006240	-2.288	-2.506	-2.506	+2.497
NFκB (p50)	CK608565	+2.638		-3.497	
MKK3	NM_001012787	-1.595			
p38	BQ038538	-2.212			
cFos	CK607079	+2.385			
TRAF6	CK607050			-2.387	-2.227
TAK1	BM426610			-1.605	
IkB	NM_001001472			+2.085	+1.514

Table A1. List of Toll-like receptor pathway-linked genes differentially expressed in untreated control chicken thrombocytes versus thrombocytes exposed to LPS, and p38 inhibitor. Expression was determined by microarray, and genes in the list exhibited at least a +/-1.5 fold change in expression.

Gene Name	Genbank Accession #	Con vs. LPS	Con vs. ERK Inh.	LPS vs. LPS + ERK Inhib.	ERK Inh. vs. Inh. + LPS
TLR2 (isoform 1)	CK607640	-1.838			
TLR3	NM_001011691	+1.737	+2.038		
TLR4	NM_001030693	+2.669			
TLR5	BI066471	+4.266	+2.164		
TLR7	BM440145	+2.336	-4.237		-2.398
TLR21	CK609262		-9.091	-5.917	
TAB1	NM_001006240	-2.288	-1.637	+1.638	
NFκB (p50)	CK608565	+2.638	+3.147		
MKK3	NM_001012787	-1.595			
p38	BQ038538	-2.212			+2.275
cFos	CK607079	+2.385		-1.898	
MyD88	CK613540		+1.548		

Table A2. List of Toll-like receptor pathway-linked genes differentially expressed in untreated control chicken thrombocytes verses thrombocytes exposed to LPS, and Erk inhibitor. Expression was determined by microarray, and genes in the list exhibited at least a +/-1.5 fold change in expression.

Gene Name	Genbank Accession #	Con vs. LPS	Con vs. p38 Inh.	LPS vs. LPS + p38 Inhib.	p38 Inh. vs. Inh. + LPS
chCCL20	CK613680	**+12.79**	**-2.439**	**-1.538**	+1.198
IL-1β	CK607391	**+5.756**	**+1.600**	**-2.770**	-1.333
iNOS	CK611178	**+5.744**	+1.002	**-2.392**	**-3.021**
MIP-1β	CK611404	**+5.548**	**-6.329**	+1.346	+1.035
IL-18	CK613996	**+4.312**	**+6.088**	-1.443	+1.040
chCXCLi1(IL8)	CK610286	**+4.071**	**-2.899**	+1.126	+1.246
chCCLi1	CK609462	**+2.612**	**+8.221**	+1.182	**+4.410**
IL-6	CK613692	**+1.964**	-1.376	**-2.717**	
IL-4	AJ621249	**+1.773**	**-7.143**		
IL-16	CK614630	**+1.750**	+1.421	+1.037	**-1.639**
IL-12β	AJ564201	**-2.008**	-1.105	**+3.865**	
PDGFβ mRNA	CK609072	+1.268	**+1.507**	-1.062	+1.278
chCCLi3	CK613216	-1.321	**-2.387**	**+2.498**	-1.122
IL12α	AY262751		**-2.481**		
chCCLi7	CK610423	+1.418	**+2.305**	-1.008	**-2.037**

Table A3. List of cytokine and chemokine genes differentially expressed in untreated control chicken thrombocytes verses thrombocytes exposed to LPS, and p38 inhibitor. Expression was determined by microarray, and genes in the list exhibited at least a +/-1.5 fold change in expression.

Gene Name	Genbank Accession #	Con vs. LPS	Con vs. ERK Inh.	LPS vs. LPS + ERK Inhib.	ERK Inh. vs. Inh. + LPS
chCCL20	CK613680	**+12.79**	+1.352	**-6.944**	+1.361
IL-1β	CK607391	**+5.756**	+1.447	-1.456	+1.057
iNOS	CK611178	**+5.744**	**+2.369**	+1.041	+1.024
MIP-1β	CK611404	**+5.548**	+1.296	**-3.021**	+1.416
IL-18	CK613996	**+4.312**	**+4.59**	+1.062	-1.002
chCXCLi1(IL8)	CK610286	**+4.071**	+1.369	-1.431	**+4.789**
chCCLi1	CK609462	**+2.612**	**+3.831**	+1.399	-1.048
IL-6	CK613692	**+1.964**	-1.357	**-2.309**	+1.152
IL-4	AJ621249	**+1.773**	+1.105		
IL-16	CK614630	**+1.750**	**+2.988**	**+1.786**	+1.046
IL-12β	AJ564201	**-2.008**	-1.422	**+2.516**	**+1.784**
PDGFβ mRNA	CK609464	+1.268	**+1.555**	+1.25	+1.018
chCCLi3	CK613216	-1.321	-1.337	-1.029	+1.258
IL12α	AY262751		**-6.289**		
chCCLi7	CK610423	+1.418	**+1.627**	**+1.981**	**+2.196**
chCCL5	CK609464			**-8.403**	

Table A4. List of cytokine and chemokine genes differentially expressed in untreated control chicken thrombocytes verses thrombocytes exposed to LPS, and Erk inhibitor. Expression was determined by microarray, and genes in the list exhibited at least a +/-1.5 fold change in expression.

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