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Research paper

Bacterial toll-like receptor agonists induce sequential NF-κB-mediated leukotriene B_4 and prostaglandin E_2 production in chicken heterophils

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ABSTRACT

Studies of the response of the primary avian polymorphonuclear leukocyte, the heterophil, to microbe associated molecular patterns (MAMPs) through toll-like receptors (TLR) has concentrated on the activation of the respiratory burst, release of intracellular granules, and the induction of cytokine and chemokine expression. Virtually no studies have been described on the role of lipid mediators, leukotrienes and prostaglandins, as effectors of the avian inflammatory response. We have previously shown that flagellin (FLG), the bacterial lipoprotein mimic palmitoly-3-cysteine–serine–lysine-4 (PAM), and unmethylated CpG motifs of bacteria DNA (CpG) are all potent activators of the avian innate immune system. In the present studies, we hypothesized that FLG, PAM, and CpG are also capable of eliciting the production of these lipid mediators of inflammation by avian heterophils. Compared to non-stimulated control heterophils, all three TLR agonists were potent inducers (3–5-fold increase) of a rapid production (30 min) of leukotriene B_4 (LTB_4) followed by a later release (60–120 min) of prostaglandin (PGE_2) by the heterophils. LTB_4 and PGE_2 production were derived from lipoxygenase-5 (5-LO) and cyclooxygenase-2 (COX-2) enzymatic activities, respectively, as the selective 5-LO (caffeic acid) and COX-2 (NS-398) inhibitors eliminated LTB_4 and PGE_2 production from the MAMP-stimulated heterophils. These results demonstrate that both the lipoxygenase and cycloxygenase pathways are operational in avian heterophils in response to bacterial MAMPs. Treatment of heterophils with either FLG, PAM, or CpG also induced a significant increase in DNA binding by NF-κB family members' p50, c-Rel, and RelB. Additionally, the production of LTB_4 and PGE_2 were inhibited following treatment of heterophils with the specific pharmacologic inhibitor of NF-κB (Bay 11–7086), thus suggesting that TLR pathway activation of NF-κB controls LTB_4 and PGE_2 production. This the first report of the production of lipid mediators of inflammation by avian heterophils in response to PAMPs. Since FLG, lipoproteins, and bacterial CpG DNA are abundant during bacterial infections, these data support their role in the inflammatory response mediated by avian heterophils.

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1. Introduction

Polymorphonuclear leukocytes (PMNs) are vital cellular components of innate immunity, and function by killing pathogenic microbes following phagocytosis. The primary PMN in poultry is the heterophil, the avian equivalent to the mammalian neutrophil. Like neutrophils, heterophils provide a rapid deployment of the effector arm of the innate immune system of the bird, displaying a variety of pathogen recognition receptors (PRRs), including toll-like receptors (TLRs), which account for the recognition of a multitude of invading microbes (Kogut, 2001; Kogut et al., 2002, 2003a,b, 2005a; Iqbal et al., 2005; Philbin et al., 2005). The TLR family in chickens consists of ten genes (*TLR1LA,*

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1LB, 2A, 2B, 3, 4, 5, 7, 15, and 21) (Fukui et al., 2001; Smith et al., 2004; Iqbal et al., 2005; Kogut et al., 2005a; Philbin et al., 2005; Higgs et al., 2006; Boyd et al., 2007; Keestra et al., 2007; Temperley et al., 2008; Keestra et al., 2008; Brownlie and Allan, 2011). Five of these genes (*TLR2A, 2B, 3, 4, 5, and 7*) are orthologues to mammal TLRs (Temperley et al., 2008). TLR21 is an ortholog of fish and amphibian TLR21, but chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9 (Keestra et al., 2008). TLR1LA, 1LB, and 15 are unique to birds (Higgs et al., 2006). In fact, chicken TLR15 was the first TLR to be described as a sensor for secreted microbe-derived proteases (de Zoete et al., 2011).

We have found that heterophils constitutively express all ten known chicken TLRs (Kogut et al., 2005a, 2006; Nerren et al., 2010). Stimulating heterophils with specific TLR agonists activate a significant oxidative burst and degranulation (Kogut et al., 2005a, 2006) and induce the up-regulation of pro-inflammatory cytokines (IL-1β, IL-6) and inflammatory chemokines (CXCLi2, CCLi4) (He et al., 2003; He and Kogut, 2003; Kogut et al., 2005b, 2006).

Ligation of PRRs activates intracellular signaling cascades that lead to the induction of NF-κB-dependent genes and the synthesis of inflammatory mediators that participate in host defenses against pathogens (Aderem and Ulevitch, 2000; Dalpke and Heeg, 2002; Janeway and Medzhitov, 2002; O'Neill, 2002; Kawai and Akira, 2011). Thus far, all of our studies on the response of heterophils to TLR agonists have centered on the induction of pro-inflammatory cytokines and chemokines. However, eicosanoids, specifically prostaglandins and leukotrienes, are lipid mediators that play a central role in both innate and acquired immunity (Funk, 2001; Peters-Golden et al., 2005; Hattermann et al., 2007; Chen et al., 2001; Valera et al., 2007; Sadikot et al., 2007). Studies have reported an association between TLR signaling and the lipid mediator system in mammals (Funk, 2001; Peters-Golden et al., 2005; Hattermann et al., 2007; Chen et al., 2001; Valera et al., 2007; Sadikot et al., 2007); whereas, only a single study has been reported in avian species (Scott and Owens, 2008).

Myeloid cells contain ample amounts of esterified arachidonic acid (AA) and constitutively express all of the enzymes necessary to hydrolyze and metabolize it via the 5-lipoxygenase (5-LO) pathway and are capable of generating large quantities of products termed leukotrienes (LTs) within seconds to minutes of encountering an activating stimulus (Lewis et al., 1990). LTs are produced by all myeloid cell lineages in response to a variety of stimuli and are involved in a wide array of pathologic inflammatory and acquired immune responses (Lewis et al., 1990; Kanaoka and Boyce, 2004). Much less well appreciated is their role in innate immune responses. Since LTs are molecules that can be generated in response to microbial stimuli and that mediate a variety of antimicrobial functions, LTs are ideally suited for a role in innate immunity. The capacity of microbes to stimulate LT generation can best be understood by considering the molecules through which they interact with leukocytes and the effects of receptor ligation on requisite signal transduction pathways. Leukocytes interact with microorganisms through cell surface receptors for either opsonin molecules or intrinsic pathogen-associated molecular patterns (PAMPs). Ligation of pattern recognition receptors by PAMPs activates intracellular signaling cascades that culminate in the induction of NF-κB-dependent genes and the synthesis of inflammatory mediators that participate in antimicrobial defense. TLR2 mediates LT synthesis (McCurdy et al., 2003), but since LPS/TLR4 signaling does not result in increases in intracellular calcium, it is not sufficient to trigger LT synthesis (Akira and Takeda, 2004). However, short exposure of leukocytes to LPS can prime them for enhanced LT synthesis in response to an activating stimulus (Aderem et al., 1986).

Cyclooxygenase (COX) is the rate-limiting enzyme in the biosynthesis of prostaglandins (PG) and related eicosanoids from arachidonic acid metabolism (Simmons et al., 2004). Two isoforms of COX have been identified and cloned in eukaryotic cells. COX-1 is constitutively expressed in most cells and thought to be involved in regulation of physiological functions while COX-2 is inducible by many pro-inflammatory stimuli (Simmons et al., 2004). Prostanoids, including prostaglandins (PG) and thromboxanes, are produced when arachidonic acid is released from the plasma membrane by phospholipases and metabolized by COX. Although it is well known that the lipid mediators generated by COX-2 enzymatic activity are involved in the regulation of inflammation, their role in the innate immune response to bacterial infections is less well defined (Bos et al., 2004; Simmons et al., 2004).

A key feature of the innate immune response to invading pathogens is the recruitment of polymorphonuclear leukocytes (PMN) to infectious foci. The importance of TLR-mediated immune cell recruitment in host defense has been established (Andonegui et al., 2003; Sheahan et al., 2008) and largely ascribed to the production of chemokines and the expression of adhesion molecules following NF-κB activation by TLR ligands (Andonegui et al., 2003; Sabroe et al., 2005). In contrast, only a few studies specifically addressed the effect of TLR signaling on the generation of PGs and LTB_4 (Surette et al., 1993; Hattermann et al., 2007; Lefebvre et al., 2010), which are also very potent regulators of leukocyte trafficking (Ford-Hutchinson et al., 1980). Consequently, the putative role of LTB_4 and PGE in TLR-mediated inflammation is not fully appreciated especially in non-mammalian species. Activation of TLRs and PMN accumulation at infection sites are critical events of host defense. Given the pivotal role of TLR signaling and PMN trafficking in host defense against microbial pathogens, we hypothesized that activation of TLR on avian heterophils by specific ligands will result in the production of PGE_2 and LTB_4. Therefore, we decided to specifically address the issue of bacterial TLR ligands in inducing the production PGE and LTB_4 by avian heterophils.

2. Material and methods

2.1. Experimental chickens

Non-vaccinated, day-of-hatch straight run broiler chickens used in this study were obtained from a commercial breeder. Upon arrival, paper liners from the chick

transport containers were cultured for the presence of *Salmonella* using described procedures (Andrews et al., 1978). Chickens were placed in their respective pens (4 feet × 4 feet allowing 0.12 m^2 of pen space per bird) containing wood shavings, provided supplemental heat, water, and a balanced, un-medicated corn and soybean meal based chick starter diet ad libitum. The feed was calculated to contain 23% protein and 3200 kcal of metabolizeable energy/kg of diet, and all other nutrient rations met or exceeded standards established by the National Research Council.

2.2. Toll-like receptor ligands

The synthetic lipoprotein Pam3CSK4 (palmitoyl-3-cysteine–serine–lysine-4; PAM) and flagellin (from *Salmonella typhimurium*; FLG) were purchased from InVivoGen (San Diego, CA) and prepared in sterile physiological water as per manufacturer's instructions. The synthetic ODNs were purchased from TriLink BioTechnologies (San Diego, CA, USA). The sequences of synthetic CpG-ODNs used in the present study were: GTCGTTGTCGTTGTCGTT (He et al., 2003). The CpG-ODN was dissolved in sterile phosphate-buffered saline (PBS, pH 7.2) at a concentration of 1 mg/ml.

2.3. Reagents

A rabbit anti-human 5-lipoxygenase polyclonal antibody was purchased from Cayman Chemicals (Ann Arbor, MI). The IκB phosphorylation inhibitor, BAY 11–7086, was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Caffeic acid (3,4-dihydroxycinnamic acid) and NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide) were purchased from EMD Chemicals, Inc. Gibbstown, NJ). All inhibitors were dissolved in DMSO and working concentrations of the inhibitors were prepared in RPMI 1640 tissue culture medium from the stock solutions. The final concentration of DMSO in the experiments was less than 0.5%.

2.4. Isolation of peripheral blood heterophils

Avian heterophils were isolated from the peripheral blood of day-old chickens as described previously (Kogut et al., 1996). Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood was mixed with 1% methylcellulose (25 centiposes; Sigma Chemical Co., St. Louis, MO) at a 1.5:1 ratio and centrifuged at 25 × *g* for 30 min. The serum and buffy coat layers were retained and suspended in Ca^{++}, Mg^{++}-free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co.). This suspension was layered over a discontinuous Ficoll-Hypaque (Sigma Chemical Co.) gradient (specific gravity 1.077 over specific gravity 1.119). The gradient was then centrifuged at 250 × *g* for 60 min. After centrifugation, the 1.077/1.119 interfaces and 1.119 band containing the heterophils were collected and washed twice in RPMI 1640 medium (Sigma Chemical Co.) and resuspended in fresh RPMI 1640. Cell viability was determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Hema-3 stained (Curtin Mathison Scientific, Dallas, TX) cytospin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically >98% pure and >95% viable. On average, the other 2% was comprised of monocytes (at most 0.5%), lymphocytes (at most 0.8%), and thrombocytes (at most 0.7%). The cell concentration was adjusted to 1×10^7 heterophils/ml and stored on ice until used.

2.5. TLR stimulation

TLR agonists were used at the following concentrations: PAM (100 μg/ml), FGN (200 μg/ml), and CpG-ODN (5 μg/ml). Optimal TLR agonist concentrations for stimulation of heterophils were previously reported (Kogut et al., 2005a). Unless otherwise noted, heterophils were stimulated in closed 2-ml eppendorf tubes and incubated at 41 °C and 5% CO_2 for 1 h.

2.6. Inhibitor treatments

Heterophils were pre-incubated for 30 min at room temperature on a rocking platform with either the lipoxygenase inhibitor (caffeic acid; 5 μM), the COX-2 inhibitor (NS-368; 2 or 5 μM), or the IκB phosphorylation inhibitor (Bay 11–7086, 50 μM). Following this pre-incubation, the heterophils were then stimulated with TLR agonists (in the presence of the inhibitors) as described above.

2.7. Immunoblotting of 5-lipoxygenase (5-LO)

Heterophils were lysed with SDS-PAGE sample buffer, and boiled for 3 min. The cell lysates were normalized to 2×10^6 cells per well and fractionated on a 4–20% SDS-PAGE (NuSep Inc., GA). Proteins were then transferred to an Immun-Blot® PVDF membrane (Bio-Rad Laboratory, Hercules, CA) and probed with 5-lipoxygenase polyclonal antibody (Cayman Chemical, Ann Arbor, MI). Opti-4CN (Bio-Rad Laboratory) was used for detection.

2.8. LTB_4 and PGE_2 production assays

The production of LTB_4 and PGE_2 was measured with a commercially available enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI). Briefly, heterophils were cultured for 1 h with either TLR agonist (using optimal agonist concentrations that induced heterophil functions, see Kogut et al., 2005a) and EIAs were performed on cell supernatants from TLR stimulated heterophils according to the manufacturer's instructions.

2.9. COX-2 activity assay

Quantitation of COX-2 activity in TLR-stimulated heterophils was determined by using EIAkit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The COX-2 assay kit measures the peroxidase activity of cyclooxygenase. The peroxidase activity is

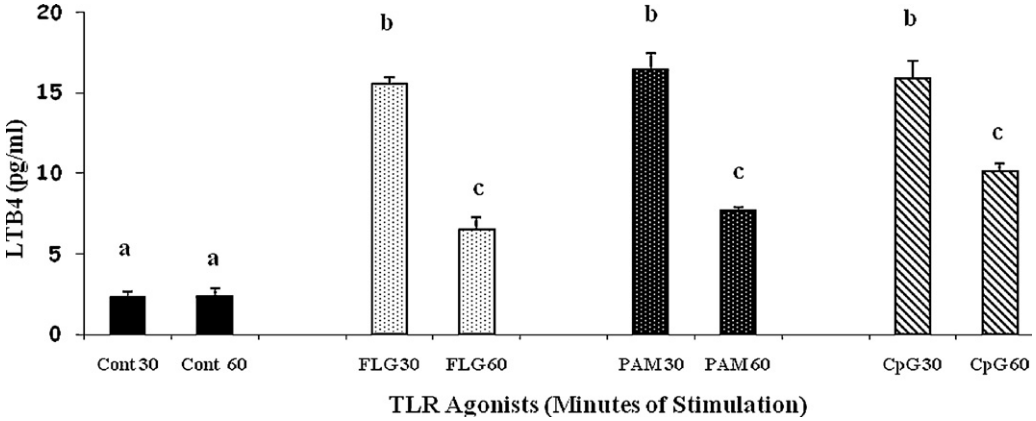

Fig. 1. TLR agonist stimulation of LTB_4 production by heterophils. Isolated peripheral blood heterophils were stimulated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for 60 min as described in Section 2. LTB_4 EIAs were performed on cell supernatants from TLR stimulated heterophils (see Section 2). Data are expressed as mean ± SE of triplicate experiments. Columns with different superscript letters indicate significant differences when compared to non-stimulated control heterophils ($p < 0.05$).

measured colorimetrically monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

2.10. NF-κB activation analysis

The ELISA-based Trans-Am transcription factor kit (Active Motif, Carlsbad, CA) was used to detect and quantify NF-κB activation. The kit uses a patented technology to attach an oligonucleotide containing a TPA-responsive element (5′-TGAGTCA-3′) or an NF-κB binding consensus sequence (5′-GGGACTTTCC-3′) to a 96-well plate according to the transcription factors analyzed. The active forms of the subunits for NF-κB (p65, p52, p50, c-Rel, RelB) in whole cell extracts can be detected using specific antibodies for epitopes that are accessible only when the nuclear factors are activated and bound to their target DNA. Preparation of cell extract was done according to the manufacturer's instructions. The specificity of the assays was checked by measuring the ability of soluble wild-type or mutated NF-κB oligonucleotides to inhibit binding.

2.11. Statistical analysis

The anti-coagulated blood from 50 chickens was pooled and the heterophils were isolated from each treatment group as described above. Each LTB_4 and PGE_2 assay was conducted four times over a two-month period with pooled heterophils (heterophils pooled from 50 chickens for each preparation; i.e., 200 chickens in total were used as cell donors). At least three replicates were conducted for each assay with the heterophils from each pool of chickens. The data from these four repeated experiments were pooled for presentation and statistical analysis.

The mean and standard error of the mean were calculated for each of the treatment groups. Differences between the non-stimulated heterophils and the agonist stimulated heterophils were determined by analysis of variance. Significant differences were further separated using Duncan's multiple range test. The data obtained using heterophils stimulated with each TLR agonist was compared to non-stimulated control heterophils.

3. Results

3.1. TLR agonist stimulation of LTB_4 production

All three TLR agonists were potent inducers (3–5-fold increase) of a rapid production (30 min) of LTB_4 (Fig. 1). However, there was a significant ($p \leq 0.05$) reduction in LTB_4 production induced by the TLR agonists from 30 to 60 min after stimulation.

3.2. Expression of 5-LO

LTB_4 is metabolized from cellular arachidonic acid via the 5-LO pathway. Therefore, we assayed for the expression of inducible 5-LO in heterophils stimulated with the various TLR agonists. Inducible 5-LO was rapidly expressed (5 min) in heterophils stimulated with all three TLR agonists when compared to the non-stimulated control heterophil (Fig. 2). 5-LO expression was reduced to control cell levels by 20 min after stimulation with the TLR agonists. Accordingly, this corresponds to the rapid production of LTB_4 within 30 min of stimulation by TLRs.

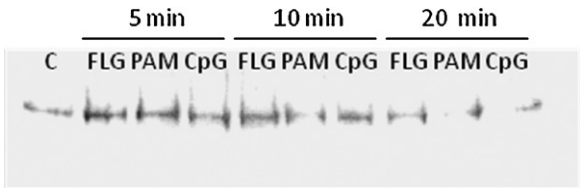

Fig. 2. TLR agonist stimulation of inducible 5-LO. Freshly prepared heterophils were incubated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for times indicated. Heterophils were then lysed with SDS-PAGE sample buffer, and boiled for 3 min. The cell lysates were normalized to 2×10^6 cell per well and fractionated on a 4–20% SDS-PAGE. Proteins were then transferred to a Immun-Blot® PVDF membrane and probed with 5-lipoxygenase polyclonal antibody. Identical results were found in two independent experiments.

3.3. Effect of caffeic acid on LTB4 production

To further confirm that TLR agonists induce production of LTB_4 through the 5-LO pathway, we pre-incubated heterophils with caffeic acid before stimulation with the TLR agonists and measured LTB_4 production. The production of LTB_4 was reduced to control levels by pre-treatment of the heterophils with caffeic acid (Fig. 3) confirming 5-LO pathway induction by TLR activation in avian heterophils.

3.4. TLR agonist stimulation of PGE_2 production

All three TLR agonists were potent inducers (2–3-fold increase) of PGE_2 in avian heterophils after stimulation when compared to the non-stimulated control heterophils (Fig. 4). However, the induction of PGE_2 was time-dependent with significant production over control cells found after 60 min of stimulation and production increasing through 120 min. By 180 min PGE_2 production decreased to control cell levels (data not shown).

3.5. Expression of COX-2

Prostaglandins are produced when arachidonic acid is released from the plasma membrane by phospholipases and metabolized by COX. Therefore, using a COX-2 ELISA assay kit, we measured the production of inducible COX-2 in heterophils following incubation with FLG, PAM, or CpG (Fig. 5). All three TLR agonists were potent inducers of inducible COX-2 within 30 min of stimulation with maximum production found after 60 min when compared to the non-stimulated control heterophils (Fig. 5). COX-2 production significantly diminished between 60 and 90 min to control levels (data not shown). The increased COX-2 production corresponded to the production of PGE_2 within 60–120 min of stimulation by TLRs.

3.6. Effect of COX-2 inhibitor on PGE_2 production

To further confirm that TLR agonists induce production of PGE_2 through the inducible COX-2 pathway, we pre-incubated heterophils with the COX-2 specific inhibitor, NS-368, before stimulation with the TLR agonists and measured PGE_2 production. NS-368 had a significant concentration dependent inhibitory effect on PGE_2 production by the TLR agonists-stimulated heterophils, thereby confirming the COX-2-dependent pathway of PGE_2 production (Fig. 6).

3.7. NF-κB activation analysis

All three TLR agonists induced a significant increase in DNA binding by the NF-κB family members, c-Rel, RelB, and p50 (Fig. 7A–C). As seen previously, (Kogut et al., 2008) we detected a significant increase in NF-κB activation within 15 min of stimulation of heterophils with the agonists when compared to non-stimulated control heterophils. Again, we must point out that we cannot rule out DNA binding by the NF-κB family members, p65 and p52. As we found previously, Western blot analysis of the chicken heterophils and mouse macrophages using the anti-p65 and p52 antibodies from the TransAm kit showed no cross-reactivity with chicken p65 or p52, respectively (data not shown). According to the manufacturer, the antibody against p65 was mapped to the N-terminal region of the gene where there is less than 30% level of identity between the chicken p65 and murine p65, whereas, the antibody against p58 was mapped to an area of the gene where there is less than 25% level of identity between the chicken p52 and murine p52. As expected, TLR agonist activation of the transcription factor NF-κB (Fig. 7A–C) was totally blocked by pretreatment of the cells with selective inhibitor of NF-κB, Bay 11–7086.

3.8. NF-κB mediates LTB_4 and PGE_2 production

To evaluate whether NF-κB activation plays a role in TLR agonist stimulation of the lipid mediators of inflammation, pre-incubated heterophils with the selective inhibitor of NF-κB, Bay 11–7086, before stimulation with the TLR agonists and measured LTB_4 and PGE_2 production. Inhibition of NF-κB activation significantly diminished ($p < 0.05$) the production of both LTB_4 and PGE_2 by TLR-stimulated heterophils (Fig. 8A and B).

4. Discussion

Few data are available about the activation of the arachidonic acid (AA) cascade in avian cells, neither in this context nor in response to the activation of other innate immune receptors including nucleotide-binding oligomerization domain (NOD)-like receptors, dectin-1, and mannose receptors. Addressing this issue is relevant since eicosanoids play an important role in connecting innate and adaptive immunity in mammals (Luster and Tager, 2004; Peters-Golden et al., 2005). The present results further accentuate the functional relevance of TLR receptors in avian heterophils, specifically their effect on arachidonic acid metabolism. TLR agonists used in this study elicited the production of both LTB_4 and PGE_2; thus demonstrating the existence of two programs for arachidonic acid metabolism in heterophils: (a) the constitutive metabolic route conveying arachidonic acid into the 5-lipoxygenase pathway, and (b) the inducible route linked to COX-2 induction expression.

In the present investigations, we evaluated the potential link(s) between avian TLR activation and the lipid mediator network in galliforms. We investigated the effects of TLR2, TLR5, and TLR21 activation on lipid mediator production by the primary chicken PMN, the heterophil, exposed to PAM, FLG, and CpG, respectively. These studies demonstrate that TLR2, -5, and -21 signaling strongly promotes inflammatory lipid mediator biosynthesis and provide further insights on innate immune response of fowl to bacterial infections. All TLR agonists used in the present study were strong consistent inducers of lipid mediator production, combined with their ability to functionally activate heterophils and induce pro-inflammatory cytokines and inflammatory chemokines, demonstrating the relevance of heterophil accumulation at sites of infection as critical to host defenses (Kogut et al., 2005a,b, 2006, 2008). The results clearly demonstrate that lipid

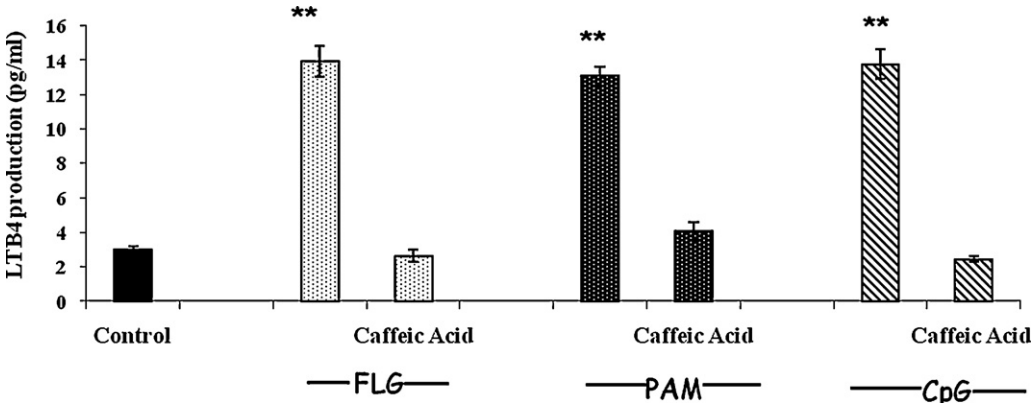

Fig. 3. Effect of lipooxygenase inhibitor (caffeic acid) on TLR agonist stimulation of LTB_4. Heterophils were pre-incubated with for 30 min at room temperature with (caffeic acid (5 μM) and then stimulated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for 60 min as described in Section 2. LTB_4 EIAs were performed on cell supernatants from TLR stimulated heterophils (see Section 2). Data are expressed as mean ± SE of triplicate experiments. Columns with ** superscripts indicate significant differences when compared to non-stimulated control heterophils ($p < 0.05$).

mediators are components of the TLR signaling pathway in avian heterophils. Moreover, these studies reveal a clear connection between TLR stimulation, NFκ-B activation, and lipid mediator biosynthesis.

LTB_4 production by TLR activated chicken heterophils was rapid and transient, being maximal after 30 min, reduced by nearly 70% by 60 min, and no longer observed at 90 min and above. Alternatively, PGE_2 production by TLR activated chicken heterophils was delayed with significant production not found until after 60 min, maximal production after 120 min, and production virtually reduced to control levels by 180 min. We find this timing pattern for the production of these lipid mediators intriguing. While both LTB_4 and PGE_2 are lipid mediators of inflammation with complex activities, PGE_2, coupled to adenylate cyclase, can efficiently suppress functional responses in phagocytes and lymphocytes, thus acting as an endogenous anti-inflammatory agent and immunosuppressor (Christman et al., 1993; Nataraj et al., 2001). Given this dual character of PGE_2, it is distinctly possible that its enhanced, but delayed production by avian heterophils exposed to TLR ligands can either be promoting the inflammatory response or, more likely, quite the opposite, be supporting its resolution. Therefore, the time course of release of various mediators found in the present studies is likely critical to the evolution and resolution of inflammation in chickens. Our data might suggest that the early release of LTB_4 following TLR activation support the development of the immediate innate immune response, while the enhanced capacity for PGE_2 synthesis at a later time may be part of the down-regulatory mechanisms leading ultimately to resolution of inflammation.

This is the first report of the production of lipid mediators of inflammation by avian heterophils in response to

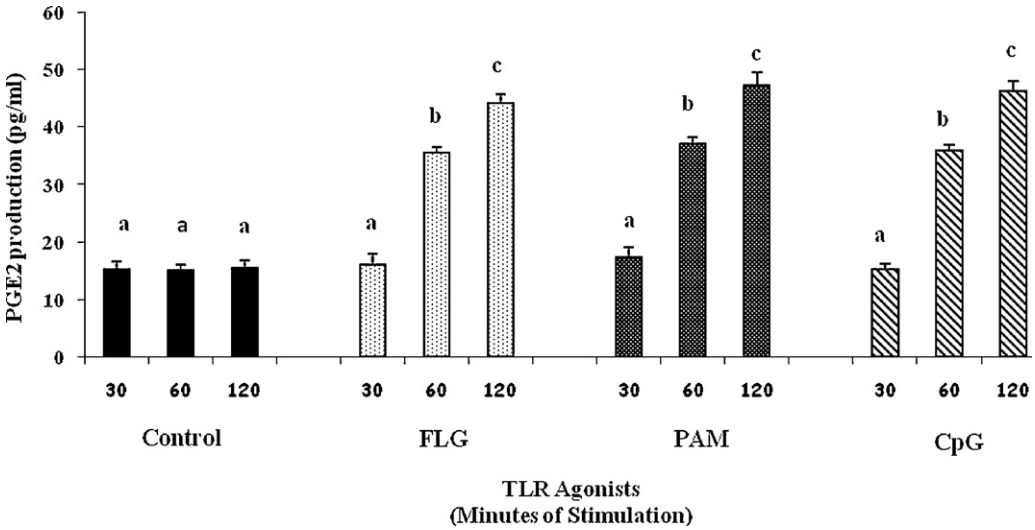

Fig. 4. TLR agonist stimulation of PGE_2 production by heterophils. Isolated peripheral blood heterophils were stimulated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for 30–180 min as described in Section 2. PGE_2 EIAs were performed on cell supernatants from TLR stimulated heterophils (see Section 2). Data are expressed as mean ± SE of triplicate experiments. Columns with different superscript letters indicate significant differences when compared to non-stimulated control heterophils ($p < 0.05$).

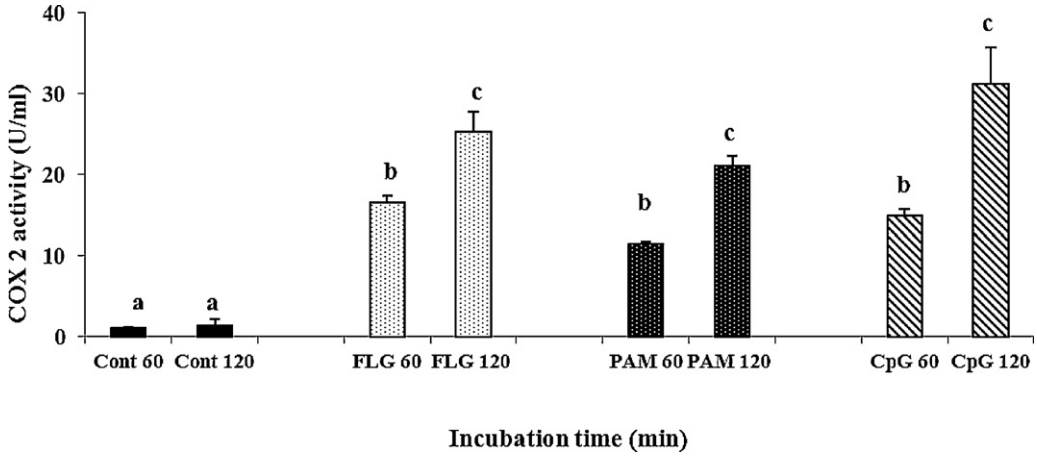

Fig. 5. TLR agonist stimulation of inducible COX-2. Isolated peripheral blood heterophils were stimulated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for 60–120 min. Quantitation of COX activity in TLR-stimulated heterophils was determined by using an EIA kit as described in Section 2. Data are expressed as mean ± SE of triplicate experiments. Columns with superscript b or c indicate significant differences at $p < 0.05$ or $p < 0.01$, respectively, when compared to non-stimulated control heterophils.

MAMPS. Since bacterial flagellin, lipoproteins, and DNA are abundant during bacterial infections, these data support a major role in the inflammatory response mediated by avian heterophils. The ability of chicken macrophages to produce significant quantities of leukotrienes has not been demonstrated (Whelan et al., 1997; Dietert and Golemboski, 1998), but stimulated chicken thrombocytes can synthesize leukotrienes (Hall et al., 2007). Chicken thrombocytes were found to have a LPS-induced (TLR4) increase in mRNA expression of COX-2 with release of PGE_2 into culture media (Scott and Owens, 2008). Likewise, COX-2 expression has been reported in chicken macrophages after viral infection (Khatir and Sharma, 2006) and chick embryo fibroblasts after mitogen stimulation (Xie et al., 1991).

This report confirms and extends the findings of Scott and Owens (2008) with chicken thrombocytes, where the NF-κB signaling influences PGE_2 and LTB_4 production. These results from the present studies are similar to those observed with human neutrophils, demonstrating the significant role of NF-κB activation in inducing the production of eicosanoids following stimulation with physiologically relevant stimuli such as TLR agonists (Burelout et al., 2007; Gaudreault and Gosselin, 2009; Alvarez et al., 2010). The present study revealed that TLR-induced LTB_4 and PGE_2 production was dependent upon early activation of NF-κB, composed of the p50, c-Rel, and/or RelB subunits since treating the heterophils with the IκB phosphorylation inhibitor, BAY 11–7086, prevented the activation of NF-κB with a resulting decreased production of LTB4 and PGE_2. We detected a significant increase in NF-κB activation within 15 min of stimulation of heterophils with TLR agonists with a significant increase in LTB_4 in 30 min and PGE_2 by heterophils 60 min after stimulation with the agonists. These sequential processes imply that NF-κB mediate the production of the lipid inflammatory mediators.

Although little direct information is available on the role of these lipid mediators *in vivo* in chickens during inflammation, there is enough information in the mammalian

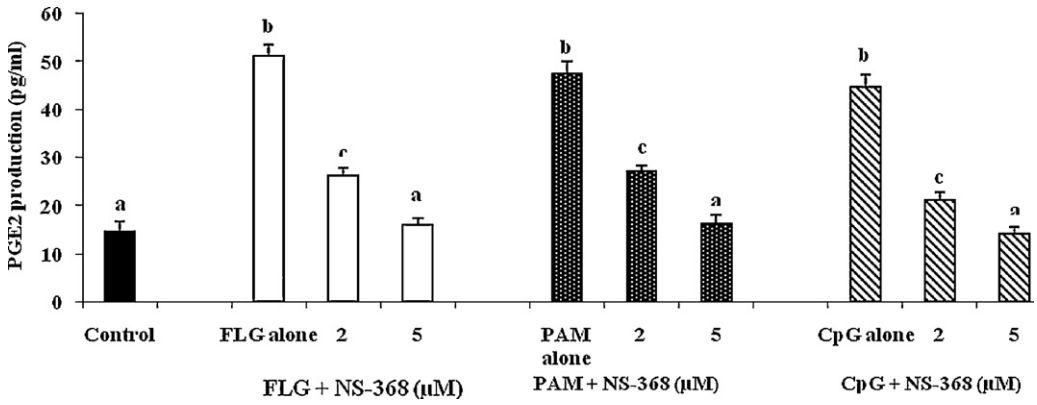

Fig. 6. Effect of cyclooxygenase inhibitor (NS-368) on TLR agonist stimulation of PGE_2. Heterophils were pre-incubated with for 30 min at room temperature with the COX-2 inhibitor, NS-368 (2 or 5 μM) and then stimulated with either FLG (200 μg/ml), PAM (100 μg/ml), or CpG (5 μg/ml) for 60 min as described in Section 2. PGE_2 EIAs were performed on cell supernatants from TLR stimulated heterophils (see Section 2). Data are expressed as mean ± SE of triplicate experiments. Columns with superscript b or c indicate significant differences at $p < 0.01$ or $p < 0.05$, respectively, when compared to non-stimulated control heterophils.

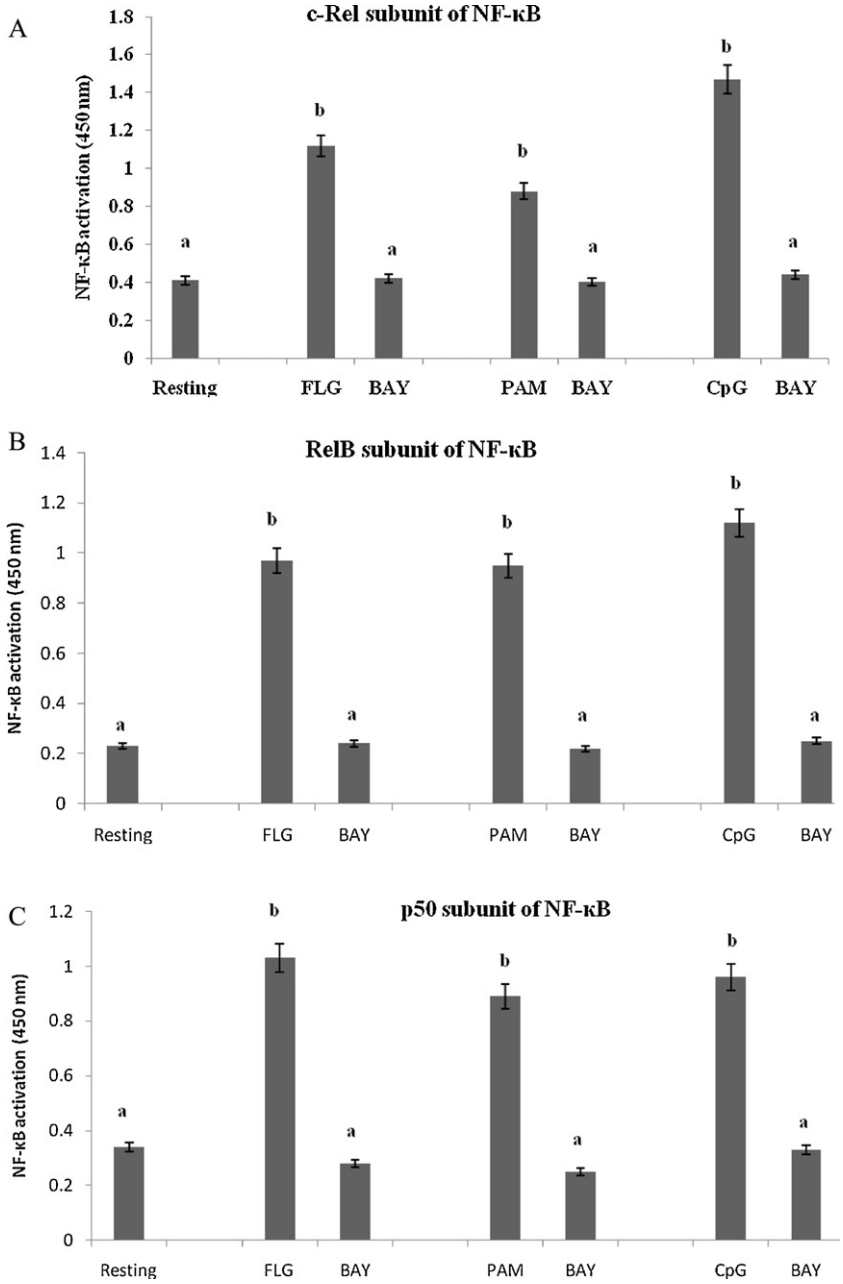

Fig. 7. Effect of TLR agonist stimulation of c-Rel, RelB, and p50 subunits to an NF-κB consensus sequence. Heterophils were stimulated with either FLG, PAM, or CpG in the presence or absence of the NF-κB inhibitor (Bay 11–7086, 50 μM). Cell lysates (10 μg/ml) were tested for binding of the activated c-Rel (A), RelB (B), and p50 (C) subunits to an NF-κB consensus sequence using the Trans-Am NF-κB ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding and are shown as mean ± SE of triplicate experiments. Columns with different superscript letters indicate significant differences when compared to non-stimulated control heterophils ($p < 0.05$).

literature to allow reasonable speculation. Cyclooxygenase (COX)-derived PGE_2 has been shown to inhibit, while 5-lipoxygenase (5-LO)-derived LTB_4 has been shown to enhance a variety of antimicrobial functions, including the phagocytosis by alveolar macrophages in mammals (Aronoff et al., 2004; Peres et al., 2007). Ligation of TLRs also enhances the expression of COX-2, the enzyme responsible for the inducible generation of prostanoids including PGE_2 (Breyer et al., 2001). PGE_2 is produced in abundance at sites of inflammation and infection and itself modulates many aspects of macrophage function (Howe et al., 2002). The immunomodulatory effects of PGE_2 largely result from its ability to increase intracellular cAMP through the stimulatory G protein (Gs)-coupled E prostanoid (EP) receptors EP2 and EP4 (Aronoff et al., 2005). Increases in intracellular cAMP generally suppress innate immune

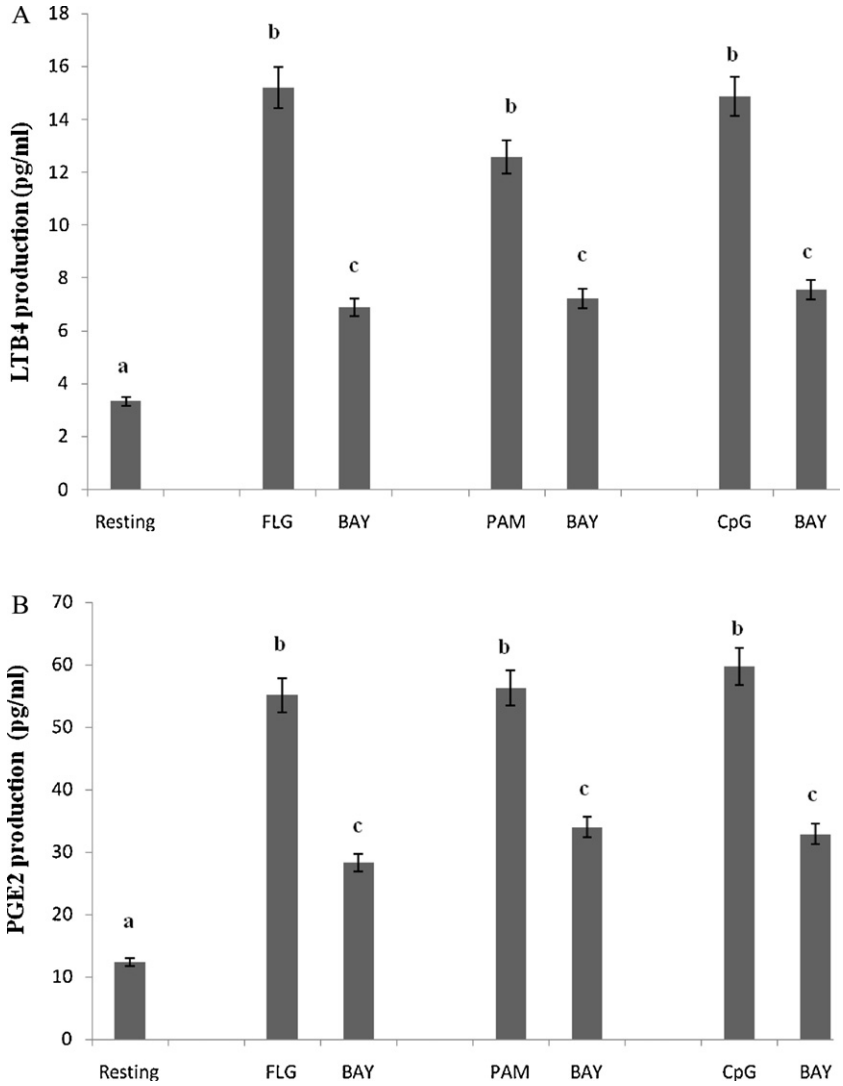

Fig. 8. Effect of NF-κB inhibitor on LTB_4 and PGE_2 production. Heterophils were stimulated with either FLG, PAM, or CpG in the presence or absence of the NF-κB inhibitor (Bay 11–7086, 50 μM). LTB_4 and PGE_2 EIAs were performed on cell supernatants from TLR stimulated heterophils (see Section 2). LTB_4 (A) and PGE_2 (B) data are expressed as mean ± SE of triplicate experiments. Columns with different superscript letters indicate significant differences when compared to non-stimulated control heterophils ($p < 0.05$).

functions of macrophages, including the generation of inflammatory mediators such as TNF-α, the phagocytosis and killing of microbes (Serezani et al., 2005, 2007), and inhibiting the assembly, activation of, and translocation of p47phox to the phagosome membrane (Serezani et al., 2007). PGE_2 contributes to an extensive scope of biological responses, including the modulation of inflammation and immune responses (Harris et al., 2007) that can suppress cytokine and chemokine production by activated macrophages (van der Pouw Kraan et al., 1995; Takayama et al., 2002), influence Th1/Th2 differentiation of T cells (Nagamachi et al., 2007) and regulate cytokine production of dendritic cells (Khayrullina et al., 2008). In contrast, overproduction of prostaglandins has been shown to have damaging consequences to the host (Funk, 2001). Synthesis of PGE_2 typically accompanies the induction of other NFκB-dependent gene products including iNOS and cytokines. The increases in cAMP that follow, therefore, serve to shape and to modulate the emerging innate response to TLR ligation. The effector functions involved in innate immune responses that are influenced by leukotrienes include direct effects on leukocyte accumulation as well as their capacity for microbial phagocytosis (Wirth and Kierszenbaum, 1985) and killing (Demitsu et al., 1989; Bailie et al., 1996) by a myriad of microbicidal mechanisms many of which are activated or amplified by leukotrienes (Serhan et al., 1982; Schmidt et al., 1989; Larfars et al., 1999; Dewald and Baggiolini, 1985), and indirect effects mediated by elaboration of other inflammatory molecules such as cytokines and chemokines, which themselves activate leukocyte recruitment and antimicrobial mechanisms, MCP-1 by monocytes (Huang et al., 2004), IL-8 by neutrophils (Kuhns et al., 2001), and MIP-1β by mast cells (Mellor et al., 2002).

In summary, we have demonstrated that TLR agonists are potent inducers of LTB_4 and PGE_2 in avian heterophils. The production of these lipid mediators of inflammation is time-dependent with LTB_4 produced by heterophils within 30 min of agonists stimulation, whereas, PGE_2 is produced 60–120 min after stimulation. Lastly, the production of LTB_4 and PGE_2 were inhibited by a pharmacological inhibitor of NF-κB implying that the TLR pathway activation this transcription factor controls production of the mediators. The results of this study combined with our earlier studies with TLR ligation provide new perspectives on likely molecular pathways that are prone to pharmacological manipulation during bacterial and viral infections in poultry.

Acknowledgments

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