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Original article

Toll-like receptor agonists stimulate differential functional activation and cytokine and chemokine gene expression in heterophils isolated from chickens with differential innate responses

Michael H. Kogut [a,*], Christina Swaggerty [a], Haiqi He [a], Igal Pevzner [b], Pete Kaiser [c]

[a] *USDA-ARS, Southern Plains Agricultural Research Center (SPARC), 2881 F and B Road, College Station, TX 77845, USA*
[b] *Cobb-Vantress, Inc., Siloam Springs, AR, USA*
[c] *Institute for Animal Health, Compton, Berkshire, UK*

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Abstract

Heterophils isolated from distinct broilers (lines A and B) differ in function and cytokine gene expression profiles. Nothing is known about Toll-like receptor (TLR) expression nor functional activation and cytokine/chemokine gene expression of line A and B heterophils when stimulated with TLR agonists. We found that line A and B heterophils express the same range of TLRs. All the bacterial TLR agonists, peptidoglycan, the synthetic lipoprotein Pam3CSK4, ultra-pure lipopolysaccharide, and flagellin all induced significantly greater functional activation of heterophils from line A compared to B. Only stimulation with the guanosine analog, loxoribine, (LOX) induced a significantly greater functional response in B over A. Additionally, all heterophils from line A stimulated with the bacterial TLR agonists had dramatic upregulation of pro-inflammatory cytokine and chemokine mRNA expression, whereas heterophils from line B had little or no upregulation of these genes. However, stimulation of all heterophils from line B with the bacterial TLR agonists and LOX induced a significant upregulation of IFN-α, with little transcription of this cytokine gene in line A heterophils. These findings suggest that the difference in heterophil functional efficiency between these parent lines is due to recognition of pathogens and activation of signaling pathways that induce innate cytokine and chemokine responses.

Keywords: Toll-like receptors; Chickens; Heterophils; Cytokines; Chemokines; Innate immunity

1. Introduction

Traditionally, the correlation between genetics and disease resistance in chickens has focused on the acquired immune response [1,2]. Given the ability of the innate immune response to recognize a variety of pathogens and then provide instruction for the initiation of the correct acquired immune responses [3], we hypothesized that the innate immune response could be a more useful marker when genetically selecting chickens for disease resistance. Instead of selecting chickens for resistance to a single pathogen, we are more interested in innate biomarkers that could indicate the potential to mount the most effective immune response against multiple microorganisms.

Recognition of potential pathogenic microbes by the innate immune system is the function of a class of cellular receptors known as the pattern-recognition receptors (PRRs), which include the Toll-like receptors (TLRs). The TLR superfamily represents an evolutionarily conserved signaling system that is a decisive determinant of the innate immune and inflammatory responses. The innate system uses these germ-line encoded receptors to recognize evolutionarily conserved molecular motifs (pathogen-associated molecular patterns [PAMPs]) of infectious microbes [3]. Microbial product-induced activation leads

* Corresponding author. Tel.: +1 979 260 3772; fax: +1 979 260 9332.
E-mail address: kogut@ffsru.tamu.edu (M.H. Kogut).

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to the activation of intracellular signaling pathways that initiate microbicidal killing mechanisms, the production of pro- and/or anti-inflammatory cytokines, and upregulation of co-stimulatory molecules required for antigen presentation to the acquired immune system [4].

Members of the TLR family, first discovered in *Drosophila* [5], are type 1 transmembrane receptors with significant homology in the cytoplasmic domain to the IL-1 receptor type 1. In humans and mice, 11 TLRs have been identified, with each member recognizing and responding to different microbial components. TLR2, in association with TLR1 and/or TLR6, induces signals after exposure to bacterial lipoproteins and peptidoglycans [6], whereas lipopolysaccharide (LPS) from Gram-negative bacteria signals through TLR4 [7]. Double-stranded RNA stimulates TLR3 [8]. Bacterial flagellin induces signaling through TLR5 [9], and TLR9 mediates the cellular responses to the CpG motif of bacterial DNA [10]. Immune response modifiers such as imiquimod and the guanosine analog, loxoribine, as well as the small synthetic antiviral molecule, R848, are agonists for TLR7 and/or TLR8 [11]. Two recent studies have described the natural agonist for TLR7 to be single-stranded RNA viruses [12].

In chickens, seven orthologues of human TLRs have been found (two genes corresponding to mammalian TLR1/6/10, TLR2 type 1, TLR type 2, TLR3, TLR4, TLR5, and TLR7) [13]. Stimulation of either avian macrophages or peripheral blood monocytes with different CpG dinucleotides, signaling mediated by TLR9 in mammals, induced differential cytokine gene expression (IL-1β in macrophages and IFN-γ in monocytes) [14] despite the fact that chicken TLR9 has yet to be identified.

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 the neutrophil, avian heterophils are involved in the phagocytosis and killing of invading microbes. We have found that heterophils constitutively express all seven known chicken TLRs and when stimulated with specific TLR agonists, functionally activate heterophil oxidative burst and degranulation and activate the MAPK signaling cascade leading to the upregulation of pro-inflammatory cytokine gene expression [15].

We have extensively characterized the heterophil-mediated innate immune response of two genetically distinct parental lines of broilers (lines A and B). To date, we have shown a distinct difference in in vitro heterophil functional activity between the lines (A > B) [16,17] that corresponds with the in vivo resistance to Gram-positive [18] and Gram-negative [19] bacterial infections. Furthermore, we have found differential basal pro-inflammatory mRNA expression (A > B) in heterophils from the resistant line that were significantly upregulated following stimulation with phagocytic agonists [20]. At the present time, we have no information regarding the expression of TLR or functional activation by TLR agonists of heterophils from these two lines of chickens. Therefore, in the present study, we examined the expression of TLR mRNA on chicken heterophils from lines A and B and, using a panel of TLR agonists, investigated their capacity to induce various effector responses and cytokine and chemokine production by the heterophils from lines A and B.

2. Materials and methods

2.1. Experimental chickens

Broiler chickens used in this study were obtained from a commercial breeder. To maintain confidentiality, the lines were designated A and B. Fertilized eggs were set in incubators (G.Q.F. Manufacturing Company, Savannah, GA; Jamesway Incubator Company, Inc., Ontario, Canada; or Petersime Incubator Co., Gettysburg, PA) and maintained at wet and dry bulb temperatures of 32.2 and 37.8 °C, respectively. After 10 days of incubation, the eggs were candled; non-fertile and non-viable eggs were discarded. The viable eggs were returned to the incubator until day 18, when they were transferred to hatchers (Humidaire Incubator Company, New Madison, OH or Petersime Incubator Co.) and maintained under the same temperature and humidity conditions until hatch. At hatch, straight-run chickens (not separated by sex) were placed in their respective pens (4 feet × 4 feet) containing wood shavings, provided supplemental heat, water, and a balanced, unmedicated 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. TLR ligands

The synthetic lipoprotein Pam3CSK4 (palmitoyl-3-cysteine–serine–lysine-4; PAM), peptidoglycan (*Staphylococcus aureus*; PGN), the synthetic dsRNA analog, poly(I:C), ultra-pure lipopolysaccharide (from *Salmonella minnesota*; p-LPS), flagellin (from *Salmonella typhimurium*; FLG), and the guanine analog loxoribine (LOX) were all purchased from InVivoGen (San Diego, CA) and prepared in sterile physiological water as per manufacturer's instructions. The nuclease-resistant phosphorothioate ODN was purchased from Integrated DNA Technologies (Coralville, IA) and further purified by ethanol precipitation. The ODN was dissolved in sterile phosphate-buffered saline (PBS, pH 7.2) at a concentration of 1 mg/ml. The sequence of the synthetic ODN used in this study was CpG-ODN#17, GTC GTT GTC GTT GTC GTT [28].

2.3. Isolation of peripheral blood heterophils

Avian heterophils were isolated from the peripheral blood of day-old chickens as described previously [21]. Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood was mixed with 1% methylcellulose (25 centipoises; 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% comprised 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.

2.4. PCR analysis of TLR expression in heterophils

Total RNA was isolated from the heterophils using RNeasy Mini Kits (Qiagen, Valencia, CA). The cDNA was synthesized using ThermoScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA) poly (dT_{20}) as primer. RNase-free-DNase I (Promega, Madison, WI, USA) was used to remove genomic DNA contamination in RNA samples at 37 °C for 30 min and heat-denatured at 75 °C for 5 min prior to reverse transcriptase (RT) reaction. PCR amplification was conducted with gene-specific primers (Table 1). The BD Advantage™ 2 Polymerase mix (BD Biosciences Clontech, Palo Alto, CA) was used for the PCR reaction. PCR reactions started with an initial denaturation at 94 °C for 1 min followed by 30 cycles (for β-actin), 40 cycles (for TLRs) of 94 °C for 30 s and 68 °C for 1 min (except for TLR5, 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 1 min were used), with a final extension at 68 °C for 5 min. The PCR products were analyzed after electrophoresis on a 2.0% agarose/ethidium bromide gel.

2.5. TLR stimulation

TLR agonists were used at the following concentrations: PAM (100 μg/ml), PGN (100 μg/ml), flagellin (FGN) (200 μg/ml), poly(I:C) (50 μg/ml), LOX (200 μg/ml), CpG-ODN (5 μg/ml) and p-LPS (10 μg/ml). Optimal TLR agonist concentrations

Table 1
Real-time quantitative RT-PCR probes and primers

RNA target		Probe/primer sequence	Genbank accession numbers[a]
28S	Probe	5′-(FAM[d])-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	F[b]	5′-GGCGAAGCCAGAGGAAACT-3′	
	R[c]	5′-GACGACCGATTGCACGTC-3′	
IL-1β	Probe	5′-(FAM[d])-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′	AJ245728
	F	5′-GCTCTACATGTCGTGTGTGATGAG-3′	
	R	5′-TGTCGATGTCCCGCATGA-3′	
IL-6	Probe	5′-(FAM[d])-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′	
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′	
IL-18	Probe	5′(FAM[d])-CCGCGCCTTCAGCAGGGATG-(TAMRA)-3′	AJ416937
	F	5′-AGGTGAAATCTGGCAGTGGAAT-3′	
	R	5′-ACCTGGACGCTGAATGCAA-3′	
IFN-α	Probe	5′-(FAM[d])-CTCAACCGGATCCACCGCTACACG-(TAMRA)-3′	U07868
	F	5′-GACAGCCAACGCCAAAGC-3′	
	R	5′-GTCGCTGCTGTCCAAGCATT-3′	
CXCLi1	Probe	5′-(FAM[d])-CCACATTCTTGCAGTGAGGTCCGCT-(TAMRA)-3′	AF277660
	F	5′-CCAGTGCATAGAGACTCATTCCAAA-3′	
	R	5′-TGCCCATCTTTCAGAGTAGCTATGAACT-3′	
CXCLi2	Probe	5′-(FAM[d])-CTTTACCAGCGCGTCCTACCTTGCGACA-(TAMRA)-3′	AJ009800
	F	5′-GCCCTCCTCCTGGTTTCAG-3′	
	R	5′-TGGCACCGCCAGCTCATT-3′	
CCLi4	Probe	5′-(FAM[d])-ACACAACACCAGCATGAGGGCACTG-(TAMRA)-3′	AJ243034
	F	5′-CATAGTCTGGCTTGGCGTCTT-3′	
	R	5′-GCGCATTGACTGACTTGCA-3′	

[a] Genomic DNA sequence.
[b] Forward.
[c] Reverse.
[d] 5-Carboxyfluorescein.

for stimulation of heterophils were previously reported [15]. Unless otherwise noted, heterophils were stimulated in 2-ml Eppendorf tubes and incubated at 39 °C and 5% CO_2 for 1 h.

2.6. Quantitative RT-PCR

Cytokine mRNA levels in control heterophils and heterophils following stimulation with TLR agonists were quantitated using a method reported previously [15]. Total RNA was prepared from heterophils using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Purified RNA was eluted in 50 µl of RNase-free water and stored at −70 °C.

Primer and probe sets for the cytokines and 28S were designed using the Primer Express software program (PE Applied Biosystems, Foster City, CA). Details of the probes and primers are given in Table 1. The cytokine probes were designed, from the sequence of the relevant genes, to lie across intron:exon boundaries. Cytokine and 28S rRNA probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein at the 5′ end and with the quencher *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′ end.

Quantitative RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following cycle profile: one cycle of 50 °C for 2 min, 60 °C for 30 min, and 95 °C for 5 min, and 40 cycles of 94 °C for 20 s, 59 °C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-χ-rhodamine, which is not involved in amplification, was used to correct fluorescent fluctuations, resulting from changes in the reaction conditions, for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye passes a significance threshold. In this work, the threshold values of the change in the reporter dye are given in Table 2 for all reactions described.

To generate standard curves for the cytokine and 28S rRNA-specific reactions, total RNA, extracted from control and appropriately stimulated heterophils, was serially diluted (10^{-1} to 10^{-5}) in sterile RNase-free water. Each qRT-PCR experiment contained triplicate no-template controls and test samples, and a $\log_{10}$ dilution series of standard RNA. Each experiment was performed in triplicate, with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCRs for the $\log_{10}$ diluted RNA was used to generate standard curves.

2.7. Oxidative burst

Production of an oxidative burst by TLR agonist-stimulated chicken heterophils was measured by oxidation of DCFH-DA to fluorescent DCF, as described previously [14] with modification. One milliliter of chicken heterophils (8×10^6 cells/ml) was added to 2-ml microcentrifuge tubes and then incubated with individual TLR agonists and DCFH-DA (final concentration 10 µg/ml) for 1 h at 41 °C. The aliquots of cell cultures (150 µl) were then dispensed to black 96-well plates and fluorescence measured using a GENios Plus Fluorescence Microplate Reader (TECAN US Inc., Research Triangle Park, NC) at 485 nm excitation and 530 nm emission wavelengths. The fluorescent units (RFU) were recorded after 1 h.

Table 2
Standard curve data from real-time quantitative RT-PCRs on total RNA extracted from COS-7 cells transfected with pCIneo expressing the relevant cDNA

Source	[a]ΔRn	Log dilutions	[b]C_t	[c]R^2	Slope
28S[d]	0.03	10^{-3}–10^{-7}	7–21	0.9181	2.7097
IL-1β	0.03	10^{-1}–10^{-5}	13–23	0.9628	3.1335
IL-6	0.03	10^{-1}–10^{-5}	11–26	0.9829	4.453
IL-18	0.03	10^{-1}–10^{-5}	11–25	0.9978	3.392
IFN-α	0.03	10^{-1}–10^{-5}	15–21	0.9876	4.141
CXCLi1	0.03	10^{-1}–10^{-6}	11–23	0.9953	3.3165
CXCLi2	0.03	10^{-1}–10^{-5}	12–22	0.9963	3.1137
CCLi4	0.03	10^{-1}–10^{-6}	16–24	0.9958	3.211

[a] ΔRn = change in the reporter dye.
[b] C_t = threshold cycle level: the cycle at which the change in the reporter dye levels detected passes the Δrn.
[c] R^2 = coefficient of regression.
[d] For 28S, RNA from chicken splenocytes was used.

2.8. Degranulation assay

Degranulation was detected by quantifying the amount of β-D glucuronidase activity in the culture medium following stimulation of the heterophils with opsonized SE. Heterophils (8×10^6) were incubated with each TLR agonist for 1 h on a rocker platform at 39 °C in a 5% CO_2 incubator. The reaction was stopped by transferring the tubes containing the cells to an ice bath for 5–10 min. The cells were then centrifuged at 250*g* for 10 min at 4 °C. The supernatants were then removed and used for the assay. A 25 µl aliquot of each supernatant was added to quadruplicate wells in a non-treated, black CoStar flat-bottom ELISA plate and incubated with 50 µl of freshly prepared substrate (10 mM 4-methylumbelliferyl-β-D-glucuronide, 0.1% Triton X-100 in 0.1 M sodium acetate buffer) for 4 h at 41 °C. The reaction was stopped by adding 200 µl of stop solution (0.05 M glycine and 5 mM EDTA; pH 10.4) to each well. Liberated 4-methylumbelliferone was measured fluorimetrically (excitation wavelength of 355 nm and an emission wavelength of 460 nm) with a GENios Plus Fluorescence Microplate Reader (TECAN US Inc., Research Triangle Park, NC). These values were converted to micromoles of 4-methylumbelliferone generated using a standard curve of known concentrations.

2.9. Statistical analysis

The anti-coagulated blood from 50 chickens was pooled, and the heterophils were isolated from each treatment group

as described above. Each oxidative burst and degranulation 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 were compared to those of non-stimulated control heterophils.

3. Results

3.1. TLR expression in heterophils

We used PCR to compare the expression of TLR mRNA in chicken heterophils from lines A and B. The data show that heterophils constitutively expressed TLRs (1, 6, 10), TLR2 type 2, TLR3, TLR4, TLR5, and TLR7 (Fig. 1).

3.2. Functional activation of heterophils by TLR agonists

We examined the functional characteristics of heterophils from lines A and B after exposure to various TLR agonists. Several heterophil functions were measured including oxidative burst, degranulation, and pro-inflammatory cytokine and chemokine mRNA expression.

3.2.1. Oxidative burst

With the exception of poly(I:C), LOX, and CpG, heterophils from line A produced a significantly greater oxidative burst in response to TLR agonists than the heterophils from line B (Fig. 2). Interestingly, only LOX stimulated a significantly greater oxidative burst in line B heterophils than line A. Poly(I:C) had no biological effect on the heterophils from either line.

3.2.2. Degranulation

As observed in the oxidative burst assay, in general, heterophils from line A produced a significantly greater amount of degranulation in response to TLR agonists than did the heterophils from line B (Fig. 2). With the exception of poly(I:C) and LOX, all of the TLR agonists stimulated a significant release of the primary granule, β-D-glucuronidase from the heterophils from line A when compared to heterophils from line B (Fig. 3). Only LOX stimulated a significantly greater degranulation in line B heterophils than in line A. Poly(I:C) had no biological effect on the heterophils from either line.

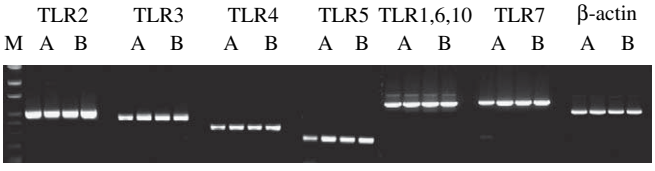

Fig. 1. TLR mRNA expression in chicken heterophils. mRNA was extracted from 10^6 cells, and TLR expression measured by PCR using primers specific for each TLR (Table 1). Duplicate RNA samples from each line were amplified and analyzed by 2% agarose gel electrophoresis, and the products visualized with ethidium bromide. M = markers, A = line A, B = line B.

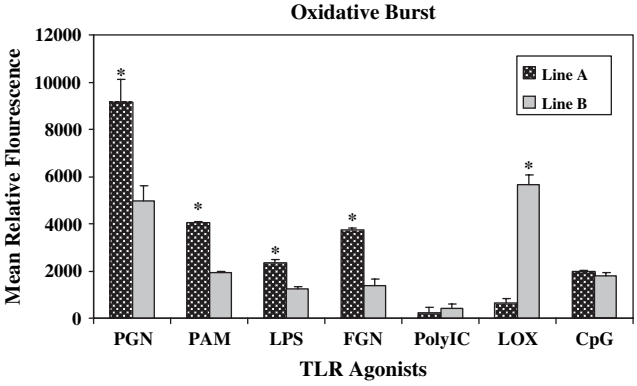

Fig. 2. Respiratory burst of heterophils stimulated by various TLR agonists. Reactions contained 8×10^5 heterophils/well, 10 μg DCFH-DA/ml, and varying concentrations (μg/ml) of the TLR agonists. Plates were incubated for 1 h at 28 °C, and the relative fluorescent units were measured. Data represent the mean ± S.E.M. of three independent assays for each TLR agonist. Functional differences were determined by ANOVA. Significant differences were further separated by Duncan's multiple range test. Asterisk above the bars indicates the significant difference ($p < 0.05$) of relative fluorescent units between the TLR agonists-stimulated and non-stimulated age-matched heterophils from each line.

3.2.3. Expression of pro-inflammatory cytokine and chemokine mRNA

For the qRT-PCR experiments, replicate experiments on different days were highly repeatable, with a coefficient of variation for four replicate RT-PCR assays of $\log_{10}$ serially

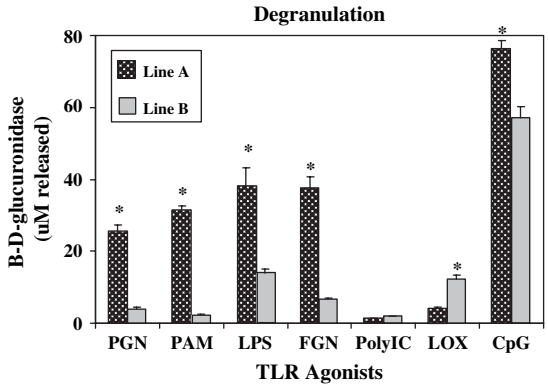

Fig. 3. Degranulation by heterophils from line A and line B stimulated by various TLR agonists. Heterophils (8×10^6/ml) from each line were incubated with various concentrations (μg/ml) of the TLR agonists for 1 h at 39 °C. Data represent the mean ± S.E.M. of three independent assays for each TLR agonist. Functional differences were determined by ANOVA. Significant differences were further separated by Duncan's multiple range test. Asterisk above the bars indicates the significant difference ($p < 0.05$) in β-D-glucuronidase release between the TLR agonists' stimulated and non-stimulated age-matched heterophils from each line.

diluted RNA for the different reactions as shown in Table 2. There was a linear relationship between the amount of input RNA and the C_t values for the various reactions (Table 2). Regression analysis of the C_t values generated by the $\log_{10}$ dilution series gave R^2 values for all reactions in excess of 0.98 (Table 2). The increase in cycles per $\log_{10}$ decrease in input RNA for each specific reaction, as calculated from the slope of the respective regression line, is given in Table 2.

To account for the variation in sampling and RNA preparations, the C_t values for cytokine-specific product for each sample were standardized using the C_t value for 28S rRNA product for the same sample from the reaction run simultaneously. To normalize RNA levels between samples within an experiment, the mean C_t value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA C_t values about the experimental mean were calculated. Using slopes of the respective cytokine and 28S rRNA $\log_{10}$ dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific C_t values. Standardization does not dramatically alter the distribution of the results as a whole (data not shown).

The adjusted cytokine-specific C_t values for the non-stimulated heterophils from each line for each of the cytokines and chemokines are shown in Table 3. Heterophils from line A had significantly higher basal levels of IL-1β, IL-6, CXCLi2, CXCLi1, and CCLi4 compared to heterophils isolated from line B chickens. No statistical differences were observed in the basal expression of IFN-α in heterophils from lines A and B.

All bacterial TLR agonists significantly upregulated mRNA expression of the cytokines IL-1β and IL-6 and the chemokines CXCLi2, CXCLi1, and CCLi4 in heterophils from line A (Figs. 4 and 5). There was virtually no fold change in pro-inflammatory cytokine (<1 fold; Fig. 4) and chemokine (<3 fold; Fig. 5) mRNA expression in heterophils from line B chickens stimulated with TLR agonists. Conversely, there was a significant upregulation in IFN-α mRNA expression in heterophils from line B stimulated with all TLR agonists (Fig. 4); whereas, there was <1 fold change in IFN-α gene expression in heterophils from line A chickens stimulated with the same TLR agonists.

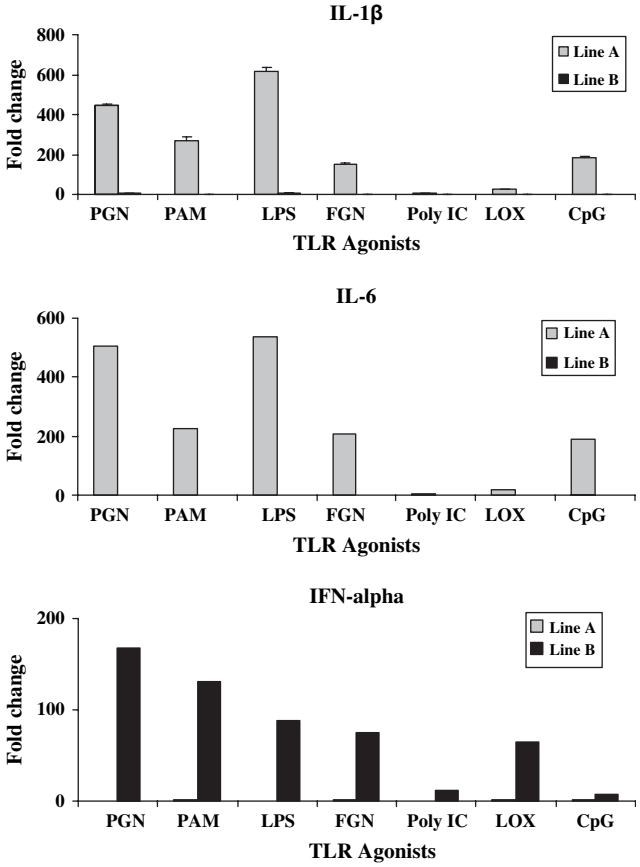

Fig. 4. Quantitation of innate cytokine mRNA expression (IL-1β, IL-6, IFN-α) induced in heterophils from line A and line B following stimulation by different concentrations (μg/ml) of various TLR agonists. Data are expressed as the fold change in cytokine mRNA levels when treated samples were compared to non-stimulated control heterophils from age-matched chickens from each line. Error bars show S.E.M. from triplicate samples from three separate qRT-PCR experiments.

4. Discussion

Our laboratories have been interested in ascertaining measures of innate immunity applicable to the development of new, more generally disease-resistant lines of poultry. The heterophil is the first cell to migrate to the site of infections, where they undertake antimicrobial and pro-inflammatory functions to control infections [21]. Therefore, heterophil functional efficiency is of interest when evaluating the potential worth of innate immunity as a tool for selection of poultry. We have previously reported differences in the in vitro heterophil functional efficiency between two parental lines of broilers (A > B) and between the F1 reciprocal crosses, suggesting that heterophil functional efficiency may be genetically associated and heritable [16,17]. The heterophil functional advantages of line A also appear to correlate with biological differences including increased resistance against bacterial infections [19,20] and the ability to mount an effective pro-inflammatory cytokine response when stimulated with phagocytic agonists [20].

Table 3
Corrected 40-C_t basal levels of cytokine and chemokine mRNA expression in heterophils isolated from day-old chickens

Line	IL-1β	IL-6	IFN-α	CXCLi2	CXCLi1	CCLi4
A	22.85 ± 1.65*	23.79 ± 1.85*	21.58 ± 1.29	20.68 ± 1.12*	23.21 ± 1.57*	27.22 ± 1.83*
B	17.66 ± 1.04	13.94 ± 0.89	23.52 ± 0.66	15.49 ± 0.77	16.21 ± 1.37	14.48 ± 0.88

Asterisks indicate significant differences ($p < 0.05$) between line A and line B.

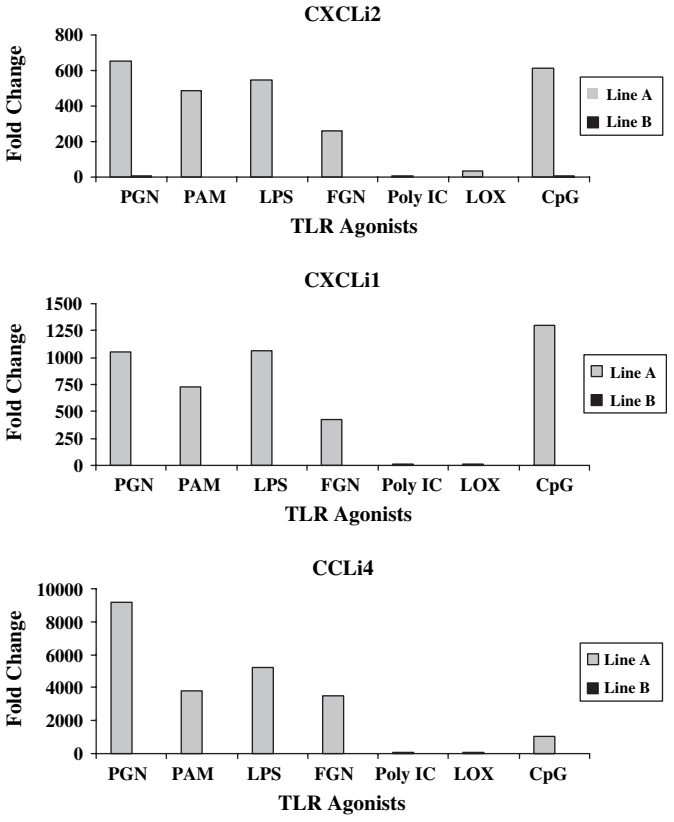

Fig. 5. Quantification of chemokine mRNA (CXCLi2, CXCLi1, CCLi4) induced in heterophils from line A and line B following stimulation by different concentrations (μg/ml) of various TLR agonists. Data as expressed as the fold change in cytokine mRNA levels when treated samples were compared to non-stimulated control heterophils from age-matched chickens from each line. Error bars show S.E.M. from triplicate samples from three separate qRT-PCR experiments.

Since heterophils are the prototypic avian innate immune cells and TLRs are the prototypic innate immune receptors, we investigated heterophil function and the pro-inflammatory cytokine and chemokine gene expression profile in heterophils from lines A and B following in vitro stimulation with TLR agonists. As seen previously when stimulated with phagocytic agonists [20], heterophils from the genetically distinct chickens have a differential functional response following stimulation with TLR agonists. Heterophils from line A responded to stimulation with all TLR agonists that were bacterial components (PGN, PAM, LPS, FGN, CpG) with, in general, a significant increase in bacterial killing mechanisms (oxidative burst and degranulation). Stimulation of TLRs normally associated with recognizing viral PAMPs (TLR3 and TLR7) had no effect on inducing oxidative burst and degranulation. These results are similar to those we have reported previously for chicken heterophils [15].

Intriguing differences in the functional responses were observed with heterophils from line B. Although bacterial TLR agonists induced an increased oxidative burst and degranulation response in heterophils from line B chickens, the responses were significantly lower than those found in heterophils from line A. These differential heterophil functional activities between the lines of broiler chickens (A > B) are virtually identical to those we have observed following stimulation of the heterophils from each line with phagocytic agonists [16,17]. It is possible that the modest functional responsiveness of heterophils from line B to a diverse array of agonists is due to a general reduction in the number of cell surface receptors that either recognize targets coated with opsonic molecules (complement and Fc receptors) or directly recognize (TLR) the agonists expressed on these cells. Although all of these receptors are expressed on line B heterophils [16,17] (Fig. 1), we are limited by the lack of avian-specific tools available at this time to test this hypothesis. We are currently developing monoclonal antibodies and qRT-PCR probes to be able to quantitate expression of these receptors. Alternatively, reduced functional responsiveness of heterophils from line B to a diverse array of agonists when compared to heterophils from line A could result from a lack of downstream activation of the signaling cascades that control receptor-mediated oxidative burst and degranulation. For example, the mitogen-activated protein kinase (MAPK)-signaling pathways play a predominant and essential role in immune responses [22]. We have demonstrated the importance of various members of the MAPK superfamily in mediating functional activation by TLRs (p38 and extracellular signal-regulated kinase 1/2 [ERK 1/2], [15]) on chicken heterophils. Preliminary experiments have measured significantly less protein tyrosine kinase (PTK) activity in heterophils from line B (≥50% less PTK activity) when compared to heterophils from line A following stimulation with various agonists (Swaggerty and Kogut, unpublished data). Further studies are ongoing to determine if, in fact, this reduction in total PTK has some genetic basis. Furthermore, we are also assessing the functional variability in these genetically divergent chickens by examining the frequency of genetic polymorphisms in genes associated with the MAPK superfamily and other PTKs.

Another intriguing difference in the functional activity of heterophils from line B was that the TLR7 agonist, the guanosine analog LOX, induced significant increases in both oxidative burst and degranulation, whereas LOX had no stimulatory effect on the functional activities of the heterophils from line A. LOX activates both human and murine immune systems, but is particularly efficient at activating antiviral activities through the induction of type I interferons (IFN) [8]. Why heterophils from line B chickens can be functionally activated via TLR7 whereas heterophils from line A chickens cannot is unknown. One can speculate that the TLR7 system may be a redundant back-up system of innate host defenses in these birds. Based on the results from these and our previous experiments, we know that heterophils from line B are not as functionally active as heterophils from line A. However, they are able to produce reactive oxygen radicals during the respiratory burst (Fig. 1) [16,17]. These reactive oxidants target the macromolecules of microbial pathogens that result in oxidative damage to proteins, lipids, and nucleic acids. Oxidative damage to guanine and cytosine nucleobases would result in oxidized nucleosides structurally similar to LOX and other guanosine analogs; thus further inducing innate

function via TLR7. In fact, oxidized nucleosides have been found at sites of infection and inflammation [23].

The data from the present study mimic the results from previous studies [20] where heterophils from genetically distinct chickens have a differential pro-inflammatory cytokine profile in response to stimuli, in the present case TLR agonists. Specifically, heterophils from line A have upregulated mRNA expression levels of IL-1β and IL-6 following stimulation with bacterial TLR agonists compared to heterophils from line B chickens. We have previously shown that an increase in both IL-1β and IL-6 mRNA expression by heterophils is associated with increased resistance to extraintestinal *Salmonella enteritidis* infections in line A chickens [20]. In line A chickens, invasion of the intestine by paratyphoid salmonellae stimulates a strong inflammatory response characterized by an increased expression and release of the pro-inflammatory cytokines IL-1 and a massive infiltration of heterophils to the site of infection [19,20]. Based on the results from the present studies, the infiltrating heterophils could also be an additional source of IL-6 following activation of TLRs by bacterial PAMPs. The increased expression of IL-1β and IL-6 may activate a population of heterophils that are better able to respond to and eliminate bacterial pathogens. However, it should be pointed out that it is doubtful that the IL-6 produced by the heterophils is acting as a heterophil activator. We have shown that recombinant chicken IL-6 can neither directly activate heterophils nor prime heterophil functional activity [24]. The function of the IL-6 produced locally by heterophils at the site of infection is unknown but may play a role in the general induction of an acute inflammatory response and production of acute phase proteins. Further research is required to answer this question. However, IL-1β activates mammalian neutrophils [25] and thus may function as a heterophil activator or priming agent.

This paper clearly shows a totally different cytokine expression pattern in heterophils from line B chickens, compared to line A chickens, when stimulated with TLR agonists. The predominant cytokine mRNA that was upregulated by stimulation with all TLR agonists was the type I IFN, IFN-α (Fig. 4). Type I IFNs were the first cytokines discovered due to their antiviral activity [26]. Recent studies have shown that bacteria and bacterial PAMPS can induce type I IFN gene expression by interacting with TLRs [27]. However, unlike their beneficial contributions as antiviral factors, type I IFNs induced by the Gram-positive bacterium, *Listeria monocytogenes*, inhibit host immunity [28]. This predominant expression of IFN-α and anti-inflammatory cytokines [20] by heterophils from line B chickens could account for the characteristic reduced heterophil functional activity and increased susceptibility to bacterial infections described previously [16–20].

The induction of type I IFN by both bacterial and viral infections is mediated by an IFN regulatory factor (IRF) 3-dependent pathway [28]. Based on the data presented here, we speculate that the divergence in the induction of IFN-α gene expression in heterophils from the two lines of chickens results from a differential activation of IRF3. Current studies are ongoing to test this hypothesis.

Chemokines are small, structurally related chemoattractant molecules that regulate the movement of various leukocytes [29]. They can be divided into subfamilies based on the position of the two amino terminal cysteine residues; i.e. adjacent [CC] or separated by a single amino acid [CXC]. In mammals, CXC chemokines mainly attract PMNs, while CC chemokines primarily induce an influx of mononuclear phagocytes [29]. In mammals, both CCL and CXCL chemokines can be broadly subdivided further by function into those that have homeostatic roles and those that are involved in inflammation. The homeostatic chemokines have a single ligand–single receptor relationship. On the other hand, an inflammatory chemokine receptor can have multiple ligands. Further, inflammatory chemokines are present in three multigene families—the MIP-like and MCP-like CCL chemokines, and a family of CXCL inflammatory chemokines (including human IL-8). Several chicken chemokines and chemokine receptors have been identified and cloned (reviewed in Ref. [30]). Recently, analysis of the chicken genome sequence showed that the chicken possesses all three multigene families, but seems to have evolved a different repertoire of members of these families compared to those in mammals [30]. For this reason, much of the nomenclature for chicken inflammatory chemokines present in the literature is at best premature and at worst misleading and incorrect. A new nomenclature has been proposed [30]. The inflammatory chemokine genes are designated either CCLi or CXCLi, the 'i' standing for presumed inflammatory function and numbered in the order in which they occur in the genome. Ten CCLi and only three CXCLi chemokine genes were identified. Of these, CXCLi1 corresponds to the previously described K60, CXCLi2 to the previously described CAF/IL-8, and CCLi4 to the previously described MIP-1β.

In the present study, we observed a rapid induction of expression of the CXC chemokines CXCLi2 and CXCLi1, and the CC chemokine CCLi4 in heterophils from line A compared to heterophils from line B. This is the first report demonstrating that rapid changes in chemokine gene expression occur following TLR agonist-mediated activation of avian heterophils. Characteristically, heterophils play a vital role in the early protection against *Salmonella* infections in the chicken by rapidly migrating to the site of bacterial invasion [19,20]. The present experiments imply that heterophils in line A chickens are capable of directing their own recruitment to the site of inflammation. A secondary influx of heterophils could then be primed locally by pro-inflammatory cytokines to continue the inflammatory response and to orient host acquired immunity towards a Th1 response against *Salmonella* by the production of Th1 cytokines [20]. Interestingly, heterophils from line B chickens had no change in mRNA expression for the three chemokines measured in this study. Why these heterophils were found to have no change in their chemokine profile is unknown at this time. It could be that heterophils from these birds react to TLR agonists with a totally different chemokine profile than those we measured here or it is also possible that the chemokine response from these birds is significantly slower, and we are not able to measure a response in the time frame used here. Both of these possibilities are currently being investigated.

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