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Protein tyrosine kinase and mitogen-activated protein kinase signalling pathways contribute to differences in heterophil-mediated innate immune responsiveness between two lines of broilers

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Protein tyrosine phosphorylation mediates signal transduction of cellular processes with protein tyrosine kinases (PTKs) regulating virtually all signalling events. The mitogen-activated protein kinase (MAPK) super-family consists of three conserved pathways that convert receptor activation into cellular functions: extracellular response kinases (ERK), c-Jun N-terminal kinases (JNK) and p38. Previously conducted studies using two chicken lines (A and B) show line A heterophils are functionally more responsive and produce a differential cytokine/chemokine profile compared with line B, which also translates to increased resistance to bacterial challenges. Therefore, we hypothesize the differences between the lines result from distinctive signalling cascades that mediate heterophil function. Heterophils from lines A and B were isolated from 1-day-old chickens and total phosphorylated PTK and p38, JNK, ERK, and transcription factor (activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB)) protein levels quantified following interaction with *Salmonella* Enteritidis (SE). Control and SE-treated heterophils from line A had greater ($P \leq 0.05$) PTK phosphorylation compared to line B with increased ($P \leq 0.05$) activation of p38. Conversely, line B heterophils activated JNK ($P \leq 0.05$). There were no differences in ERK between control and activated heterophils for either line. Defined signalling inhibitors were used to show specificity. The AP-1 and NF-κB transcription factor families were also examined, and c-Jun and p50, respectively, were the only members different between the lines and both were up-regulated in line A compared with line B. These data indicate that increased responsiveness of line A heterophils is mediated, largely, by an increased ability to activate PTKs, the p38 MAPK pathway and specific transcription factors, all of which directly affect the innate immune response.

Introduction

In the past 15 years, studies show the innate immune response has a fundamental role providing instruction for the ensuing acquired response (Fearon & Locksley, 1996; Bendelac & Fearon, 1997; Medzhitov & Janeway, Jr, 1997a, 1997b; Parish & O'Neill, 1997), which begins with recognition of self from non-self by detecting pathogen-associated molecular patterns (PAMPs) recognized by pattern recognition receptors (PRRs) on host cells (Romagnani, 1992; Fearon & Locksley, 1996; Anderson, 2000; Muzio *et al.*, 2000; Akira, 2001; Akira *et al.*, 2001; Janeway, Jr & Medzhitov, 2002).

Activation of protein tyrosine kinases (PTKs) comprises one of the initial steps for the induction of virtually all signalling cascades which ultimately leads to mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation and subsequent production of inflammatory cytokines (Vitiello *et al.*, 2004; Cloutier *et al.*, 2007; Liu *et al.*, 2007; Kawai & Akira, 2010). The MAPKs are serine/threonine protein kinases that are inducible, highly conserved between species and convert receptor activation into critical cellular functions including, but not limited to, virtually all aspects of innate and adaptive immune responses. There are three major MAPK pathways (Dong *et al.*, 2002; Roux & Blenis, 2004; Liu *et al.*, 2007). The extracellular response kinase (ERK) pathway is involved in normal development including the regulation of cell proliferation and differentiation as well as T cell activation (Th2), cell death, apoptosis, and neutrophil migration and microbial killing (Dong *et al.*, 2002; Johnson & Lapadat, 2002; Alberola-Ila & Hernández-Hoyos, 2003; Wozniok *et al.*, 2007). The p38 pathway is also strongly activated by environmental stressors and pro-inflammatory stimuli, including lipopolysaccharide, and p38 activation increases the expression of messenger RNA (mRNA) that encodes cytokines and their receptors, thus making it a key regulator involved in an immune response (Kyriakis *et al.*, 1994; Roux & Blenis, 2004; Ashwell, 2006). The c-Jun N-terminal kinase (JNK) pathway is preferentially activated by stress signals

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including inflammatory cytokines and lipopolysaccharide, is very complex with regard to its regulation and is controlled, in part, by numerous kinases, enhances neutrophil killing of microbial pathogens, and is known to phosphorylate c-Jun, a member of the activator protein (AP)-1 family of transcription factors (Kyriakis *et al.*, 1994; Dong *et al.*, 2002; Singh & Zhang, 2004; Yeh *et al.*, 2010).

Transcription factors are essential for the regulation of gene expression and are found in all living organisms. There are two key families of transcription factors in MAPK pathway signalling: the AP-1 family, which includes c-Jun, c-Fos, FosB, Fra-1 and Fra-2, JunD and JunB; and the NF-κB family, which comprises p50, p52, p65 (RelA), c-Rel and RelB (Kawai & Akira, 2007; Hayden & Ghosh, 2008). Activation of transcription factors control the expression of cytokines/chemokines, antimicrobial effectors, and other inflammatory mediators by binding to target DNA sequences and are therefore required for an effective immune response (Fujioka *et al.*, 2004; Hayden *et al.*, 2006; Kawai & Akira, 2007). Upstream PTK and MAPK signalling events lead to the activation of AP-1 and/or NF-κB members which, in turn, controls the expression of inflammatory cytokines and co-stimulatory molecules required for T-cell activation and as a result are essential for regulating and directing virtually all immune responses against invading pathogens (Huang *et al.*, 2004; Vitiello *et al.*, 2004; Cloutier *et al.*, 2007; Kawai & Akira, 2007; de Greeff *et al.*, 2009).

As the first cells to migrate to the site of infection, polymorphonuclear leukocytes (PMN) are critical components of the innate immune response and subsequent inflammatory response (Hachicha *et al.*, 1998; Yamashiro *et al.*, 2001; Kobayashi *et al.*, 2002). Heterophils are the primary PMN in chickens, and are the avian counterpart to mammalian neutrophils and function to modulate the acute innate host response through rapid phagocytosis of invading microbes and foreign particles, which activates signalling mechanisms, production of oxygen intermediates, releasing proteolytic enzymes and cytokines/chemokines (Kaiser *et al.*, 2000; Kogut *et al.*, 2001, 2003, 2007; Mannering & Cheers, 2002; He *et al.*, 2003). Collectively, these studies clearly indicate the significant role of heterophils in the innate immune response and subsequent protection in young birds (Kogut *et al.*, 1994).

Over the past several years our laboratory has evaluated the heterophil-mediated innate immune response of parental broilers (lines A and B). To date, we have shown a phenotype characterized by increased *in vitro* heterophil function (Swaggerty *et al.*, 2003b) corresponds with an increase in *in vivo* resistance to organ invasion by Gram-positive (Swaggerty *et al.*, 2005b), Gram-negative bacterial infections (Ferro *et al.*, 2004; Swaggerty *et al.*, 2005a; Li *et al.*, 2008) and protozoan parasites (Swaggerty *et al.*, 2011, in press). All immunologic evaluations have shown a dichotomy of innate immune responsiveness with heterophils from line A being functionally more responsive compared with heterophils isolated from line B chickens. Based on all of our previous studies and the fact that there are no studies comparing the PTK and MAPK signalling pathways of two genetically distinct lines of broiler chickens, we hypothesized that the differential response was due to differences in intracellular signalling pathways; therefore, the objective of the present study was to quantify phosphorylation of total PTK and activation of ERK, JNK, p38, AP-1 and NF-κB in heterophils isolated from line A and B chickens to determine where the differences occur. Knowledge and understanding of these basic but essential signalling pathways will provide specific information on key regulatory points that are likely to have a direct outcome and point to why one line of birds (line A) is more resistant to *Salmonella* and other food-borne and poultry pathogens compared with line B. The poultry industry will benefit from these data as they will offer additional gene targets for development of a novel selection method based on increased innate immune responsiveness.

Materials and Methods

Experimental chickens. Parent broiler chickens used in this study were obtained from a commercial breeder. To maintain confidentiality, lines were designated A and B. Fertilized eggs were incubated and hatched under standard conditions (wet and dry bulb temperatures of 32°C and 37.5°C, respectively) (Austic & Nesheim, 1990). At hatch, chickens were placed in floor pens (8 feet × 8 feet) containing wood shavings and provided with supplementary heat. They were given 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 metabolizable energy/kg diet, and all other nutrient rations met or exceeded the standards established by the National Research Council (1994).

Bacteria. A poultry isolate of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE, #97-11771) was obtained from the National Veterinary Services Laboratory (Ames, Iowa, USA) and was cultured in tryptic soy broth (Difco Laboratories, Becton Dickinson Co., Sparks, Maryland, USA) overnight at 41°C. Stock SE (1×10^9 colony-forming units/ml) was prepared fresh for each experiment as previously described and kept on ice until used (Swaggerty *et al.*, 2003b).

Heterophil isolation. Heterophils were isolated from peripheral blood of 150 chickens per line 1 day post-hatch. Following blood collection, heterophils were isolated as previously described (Swaggerty *et al.*, 2003b). Briefly, blood from chickens was collected in Vacutainer® tubes containing disodium ethylenediamine tetraacetic acid (EDTA) (BD Vacutainer, Franklin Lakes, New Jersey, USA) and mixed thoroughly. The blood and EDTA for each line was pooled and diluted 1:1 with RPMI 1640 media containing 1% methylcellulose and centrifuged at $40 \times g$ for 15 min at 4°C. The supernatant was transferred to a new conical tube and diluted with Ca^{2+}-free and Mg^{2+}-free Hank's balanced salt solution (1:1), layered onto discontinuous Histopaque® gradients (specific gravity 1.077 over 1.119) and centrifuged at $190 \times g$ for 1 h at 4°C. The Histopaque® layers were collected, washed with RPMI 1640 (1:1) and pelleted at $485 \times g$ for 15 min at 4°C. The cells were then re-suspended in fresh RPMI 1640, counted on a haemocytometer, and diluted to 1×10^7/ml in RPMI. Heterophil preparations were consistently 95% pure and >95% viable. All tissue culture reagents and chemicals were obtained from Sigma Chemical Company (St Louis, Missouri, USA) unless noted otherwise.

Protein tyrosine kinase assay. Total phosphorylated PTK was quantified using a commercially available kit (catalogue number PTK101; Sigma). Heterophils (4×10^6/ml) were treated with RPMI (control) or SE for 1 h at 39°C on a rocker and the reaction stopped by adding 100 µl of 20% perchloric acid. Additional samples treated with the inhibitor genistein, a broad-spectrum PTK inhibitor, were also included (200 µM) to show specificity (Sigma Chemical Co.). Heterophils were pelleted ($485 \times g$ for 15 min), the supernatant discarded, and the cells washed with phosphate-buffered saline. Following the wash, the supernatant was decanted and ice-cold lysis buffer (2 ml) added; the cells were

re-suspended, and kept on ice for 30 min. Lysed cells were removed by centrifugation (10,000 × *g* for 15 min at 4°C) and the supernatant collected and diluted 1:1 in tyrosine kinase buffer and the manufacturer's instructions followed. Following addition of the stop solution, the plate was read at 492 nm within 5 min (GENios Plus Fluorescence Microplate Reader; TECAN US Inc, Research Triangle Park, North Carolina, USA).

MAPK family (p38, ERK, and JNK) immunoassays. Total p38, ERK1/2, and JNK1/2 protein levels were quantified using commercially available immunoassay kits (BioSource International, Inc., Camarillo, California, USA). Briefly, heterophils (1×10^7) were treated with RPMI (control) or SE for 1 h at 39°C on a rocker. Specific p38 (SB203580), ERK (PD98059), and JNK (SP600125) inhibitors were used (100 μM) to pre-treat cells for 1 h at 39°C prior to stimulation in order to validate specificity (Calbiochem, San Diego, California, USA). Following stimulation, heterophils were centrifuged (6220 × *g* for 5 min at 4°C), and the supernatant discarded. Cells were washed twice with ice-cold phosphate-buffered saline (16,430 × *g* for 10 min at 4°C) and then lysed with the appropriate freshly-prepared extraction buffer (1 ml) for 30 min on ice (vortexing the sample every 10 min). Supernatants were collected and stored at –70°C until the immunoassays were performed according to the manufacturer's instructions. As described in the protocol, the ERK samples were boiled for 5 min prior to dilution. Briefly, 100 μl freshly prepared standards and samples (diluted 1:10) were added to the appropriate wells and incubated overnight at 4°C in the dark. The initial 2-h incubation was extended overnight to optimize sensitivity for chicken heterophils (Genovese *et al.*, 2007). The following morning, the plate was washed four times with wash buffer using a plate washer. The detection antibody (100 μl) was added to each well except the chromogen blank and incubated for 1 h at room temperature in the dark. The plate was washed four times with the wash buffer, the horseradish peroxidase antibody (100 μl) added, again leaving the chromogen blank, and incubated for 30 min at room temperature in the dark. The plate was washed four times; chromogen solution (100 μl) was added to all wells and incubated for 30 min at room temperature in the dark. Stop Solution (100 μl) was added and the plate read at 450 nm within 5 min (GENios Plus Fluorescence Microplate Reader). Results were calculated using the four-parameter algorithm standard curve.

ELISA to measure activation of AP-1 and NF-κB transcription factors. Transcription factors from the AP-1 and NF-κB families were also evaluated. The AP-1 family members including c-Jun, c-Fos, FosB, Fra-1, JunD, and JunB were measured using a commercially-available enzyme-linked immunosorbent assay (ELISA) (TransAM AP-1 family transcription factor assay kit; Active Motif, Carlsbad, California, USA); NF-κB family members including p65, p50, p52, c-Rel, and RelB were measured with the TransAM NF-κB family transcription factor assay kit (Active Motif). Sample preparation was the same for both assays. Briefly, heterophils (1×10^7) were treated with RPMI (control) or SE for 1 h at 39°C on a rocker, and the cells were collected by centrifugation (2430 × *g* for 5 min at 4°C), and lysed with the appropriate freshly-prepared lysis buffer. Lysis was carried out on ice for 30 min and samples were vortexed every 10 min. Lysates were centrifuged at 9720 × *g* for 5 min at 4°C and the supernatants collected and stored at −70°C until the assay was performed. The ELISAs were carried out following the manufacturer's protocol. Following addition of the stop solution, absorbance readings were taken at 450 nm within 5 min (GENios Plus Fluorescence Microplate Reader).

Statistical analyses. Anti-coagulated blood from 150 chickens per group was pooled and heterophils isolated. Each blood collection and heterophil isolation was conducted on four separate days (heterophils pooled from a total of 600 chickens per line). All comparisons and statistical analyses were performed on controls versus treated values for each line; no comparisons were made between lines. The mean and standard error of the mean were calculated from pooled data. Statistical analyses (Student's *t* test) were performed using Microsoft® Excel 2007 ($P < 0.05$).

Results

Protein tyrosine kinase. Heterophils were isolated from 1-day-old chickens and the number of international units (IU) of total phosphorylated PTK was quantified (Figure 1). Basal levels of PTK were significantly ($P < 0.05$) greater in line A heterophils compared with line B heterophils (0.46 ± 0.01 and 0.36 ± 0.01 IU, respectively). Heterophils from both lines responded following stimulation, but line A heterophils had significantly ($P < 0.05$) greater levels of phosphorylation following stimulation with SE (3.03 ± 0.23 IU) compared with line B heterophils (1.25 ± 0.06 IU). Treatment with genistein prior to stimulation with SE returned levels to those observed for both line A (0.44 IU) and B (0.36 IU) controls (Figure 1).

MAPK family. We also wanted to determine whether there were measurable differences between the components of the MAPK super-family signalling pathways. Total p38, ERK, and JNK proteins (pg/ml) were quantified in control and SE-stimulated heterophils from line A and B chickens. Basal levels of p38 were higher ($P < 0.05$) in line A heterophils (1145 ± 211) compared with line B heterophils (603 ± 65). Following

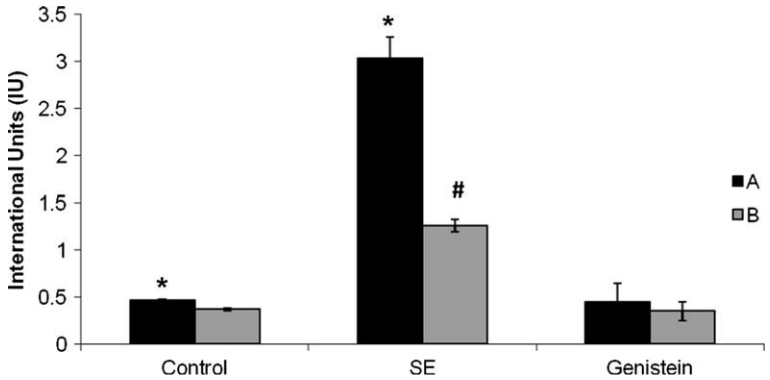

Figure 1. *Total phosphorylated PTK activity. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and the total international units of phosphorylated PTK quantified using a commercially available ELISA. Line A control and SE-stimulated values were significantly ($P \leq 0.05$) higher than those observed for line B (indicated by *). Treatment of heterophils with genistein, a broad-spectrum PTK inhibitor, prior to SE exposure kept the PTK levels comparable with controls. Significant differences between treatments indicated by # ($P \leq 0.05$). Results shown represent the mean of four separate experiments, and error bars are the mean ± standard error.*

stimulation with SE, p38 activity increased in both lines; however, the levels were significantly ($P < 0.05$) higher in line A heterophils (1813 $\pm$ 140) compared with line B heterophils (1375 $\pm$ 114) (Figure 2). Pre-treatment of heterophils with SB203580, a p38 inhibitor, prior to stimulation maintained the p38 levels comparable with those observed in the control preparations.

Compared with PTK and p38 activity, a different pattern was observed for JNK. Basal levels of JNK (pg/ml) were higher ($P < 0.05$) in line B heterophils (126 $\pm$ 18) compared with line A heterophils (39 $\pm$ 12). Following stimulation with SE, JNK concentrations increased in both lines; however, the levels were significantly ($P < 0.05$) higher in heterophils from line B (510 $\pm$ 42) compared with line A heterophils (404 $\pm$ 29) (Figure 3), thus indicating that line B heterophils are regulated by a different signalling mechanism to that regulating line A heterophils. Treatment of heterophils with SP600125, a JNK inhibitor, prior to stimulation maintained levels similar to controls.

The levels of ERK were measured in control and SE-stimulated heterophils preparations from lines A and B (Figure 4). There were no differences observed between lines or between control and stimulated samples and treatment of heterophils with PD98059, an ERK inhibitor, prior to stimulation also had no effect.

Transcription factors. Activation of transcription factors from the AP-1 family were measured, including c-Jun, c-Fos, FosB, Fra-1, JunD, and JunB. Each protein was measured in control and SE-stimulated heterophils isolated from line A and line B chickens. The only difference between lines A and B was observed with c-Jun (Figure 5). Control levels were comparable between the two lines whereas levels were significantly ($P < 0.05$) higher in line A heterophils stimulated with SE while the stimulated values observed in line B heterophils were not different from the control values. All other AP-1 members were comparable between lines A and B, and therefore probably do not contribute to the differential responsiveness observed between these lines of birds.

Activation of transcription factors from the NF-κB family, including p50, p52, p65 (RelA), c-Rel, and RelB, were also measured in control and SE-stimulated heterophils isolated from lines A and B (Figure 6). Activation of p50 was higher ($P < 0.05$) in SE-treated heterophils compared with controls from line A, whereas activation between control and SE-treated heterophils were comparable for line B. Both lines showed a significant down-regulation for p65 and Rel-B activation but since the responses were similar these are not likely to be factors that contribute to the differential responses between these lines of chickens. No other differences were observed.

Discussion

In the present study we determined that there were differences between signalling pathways in heterophils isolated from two distinct and well-characterized lines of broiler chickens. We examined the heterophil-mediated innate immune responsiveness of two lines of chickens and have shown that line A chickens are more resistant to bacterial challenge (Ferro *et al.*, 2004; Swaggerty *et al.*, 2005b; Li *et al.*, 2008) and *in vitro* studies show that line A heterophils have increased bactericidal/killing ability (Swaggerty *et al.*, 2003b) and have elevated pro-inflammatory cytokine/chemokine mRNA expression levels compared with line B (Swaggerty *et al.*, 2004; Kogut *et al.*, 2006). All of these studies were focused on downstream events and/or end products, which led us to ask whether the differences were initiated at the receptor level and/or upstream signalling events. Evaluation of key receptors (TLR4, TLR5, TLR15) and adaptor proteins (MyD88, TIR-domain-containing adaptor-inducing interferon, Toll-interleukin-1 receptor domain-containing adaptor protein) on heterophils isolated from lines A and B showed the only difference was in TLR15 mRNA expression, where line A heterophils had significantly higher expression levels compared with line B heterophils (Nerren *et al.*, 2009). These results strongly suggest that upstream signalling events, not merely receptors, are major mediators to the observed phenotypic differences between line A and line B chickens.

The lack of availability of avian-specific reagents has limited the intricate studies required to fully dissect critical signalling networks. However, there is 96 to 99% sequence identity between chicken and mammalian MAPK members (http://blast.ncbi.nlm.gov), thus allowing for the use of commercially available bioassays and

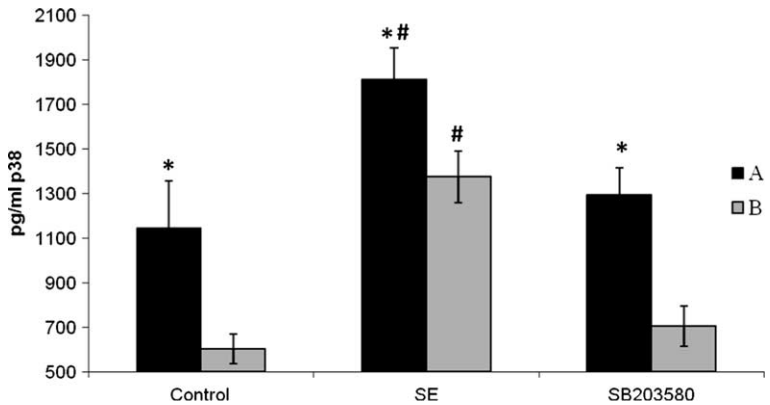

Figure 2. *Total p38 protein levels. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and p38 protein levels (pg/ml) quantified using a commercially available ELISA. Line A control and SE-stimulated values were significantly ($P \leq 0.05$) higher than those observed for line B (indicated by *). Treatment of heterophils with SB203580, a specific p38 inhibitor, prior to SE exposure kept the levels similar to controls. Significant differences between treatments indicated by # ($P \leq 0.05$). Results shown represent the mean of four separate experiments, and error bars are the mean $\pm$ standard error.*

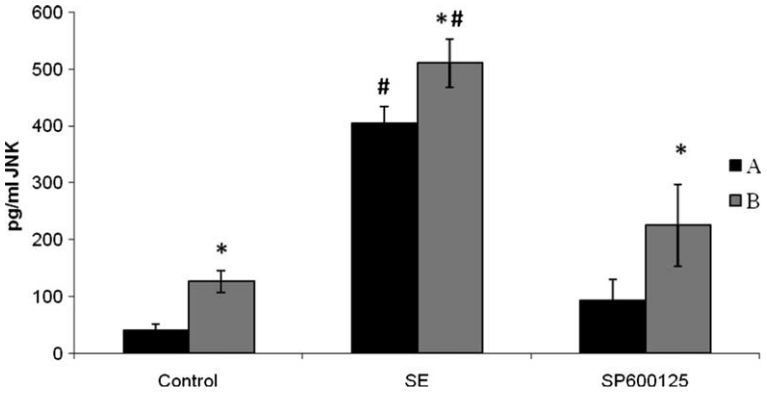

Figure 3. *Total JNK protein levels. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and JNK protein levels (pg/ml) quantified using a commercially available ELISA. Line B control and SE-stimulated values were significantly ($P \leq 0.05$) higher than those observed for line A (indicated by *). Treatment of heterophils with SP600125, a specific JNK inhibitor, prior to SE exposure kept the levels similar to controls. Significant differences between treatments indicated by # ($P \leq 0.05$). Results shown represent the mean of four separate experiments, and error bars are the mean ± standard error.*

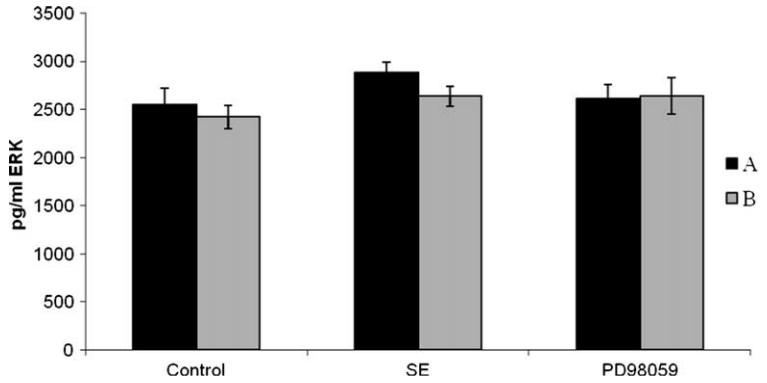

Figure 4. *Total ERK protein levels. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and ERK protein levels (pg/ml) quantitated using a commercially-available ELISA. There were no differences between control and SE-stimulated levels for either line. Treatment of heterophils with PD98059, a specific ERK inhibitor, prior to SE exposure had no effect. Results shown represent the mean of four separate experiments, and error bars are the mean ± standard error.*

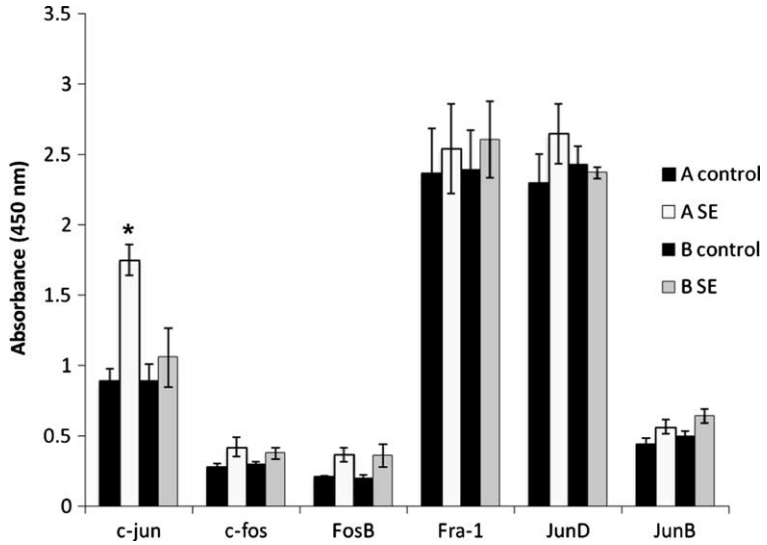

Figure 5. *Activation of the AP-1 transcription factor family. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and c-Jun, c-Fos, FosB, Fra-1, JunD, and JunB activation determined using a commercially-available ELISA. Activation of c-Jun was significantly (*$P \leq 0.05$) higher in line A SE-treated heterophils whereas line B was unchanged from its control levels. All others had similar activation patterns. Absorbance was read at 450 nm. Results shown represent the mean of four separate experiments, and error bars are the mean ± standard error.*

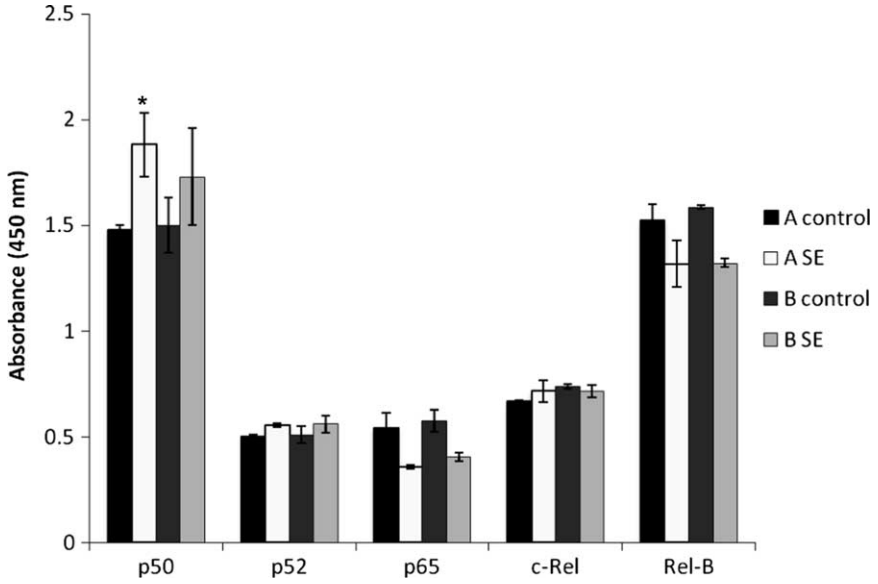

Figure 6. *Activation of the NF-κB transcription factor family. Heterophils were isolated from 1-day-old chickens, stimulated with RPMI (controls) or SE for 1 h at 39°C and p50, p52, p65, c-Rel, and RelB activation quantitated using a commercially-available ELISA. Activation of p50 was significantly (*$P \leq 0.05$) higher in line A SE-treated heterophils whereas line B was unchanged from its control levels. All others had similar activation patterns. Absorbance was read at 450 nm. Results shown represent the mean of four separate experiments, and error bars are the mean ± standard error.*

reagents, which will allow us to advance the field of avian biology. The current study was undertaken to begin to understand whether the differences observed between line A and line B with regard to phenotype were a result of deficiencies or deletions in key signalling pathway components including members of the MAPK superfamily or the NF-κB and AP-1 transcription factor families. The results showed there were no obvious deletions in any specific signalling pathway(s) between heterophils from lines A and B, and that the differences are more likely attributable to slight differences in the early MAPK signalling events. A study by Genovese *et al.* (2007) compared PTK, ERK, and p38 activity in heterophils isolated from wild-type and commercial large-bodied turkeys and showed significantly higher levels of activity for all three signalling components in the wild-type birds over the commercial variety, which corresponds to increased heterophil function (Genovese *et al.*, 2006). As demonstrated in the current study, the observed increase in heterophil function is likely to be a result of increased activation of PTK signalling and elevated p38 levels, suggesting a connection between these signalling pathways and PMN functional efficiency in both chickens and turkeys. The ERK MAPK family was not a differentiating factor between the two lines of chickens tested in the current study, yet in turkeys there is a differential response (Genovese *et al.*, 2006) suggesting that ERK contributes, in part, to the functional efficiency of heterophils from wild-type turkeys but not in the broiler lines we tested. Also, a study using heterophils isolated from 1-day-old Leghorn chickens (egg layers) shows that the bacterial components lipopolysaccharide and flagellin increase ERK activity (Kogut *et al.*, 2007). It is possible that the differences are due to the type of chicken used as the heterophil donor (egg layers versus meat-type chickens in the current study) or the agonist (bacterial component versus live bacteria in this study). Additional studies to dissect the differential responses to distinct agonists may provide beneficial insight into the differences between layer and broiler chickens and provide valuable data for these two diverse phenotypes.

Phosphorylation of p38 and JNK influences downstream cytokine/chemokine production (Kim & Rikihisa, 2002; Cloutier *et al.*, 2003, 2007). Induction of pro-inflammatory cytokines and chemokines by neutrophils is dependent on the activation of p38 (Cloutier *et al.*, 2007), while JNK has a lesser role in inflammatory cytokine/chemokine production (Cloutier *et al.*, 2003). The differential phosphorylation of MAPK family members could account for the differential cytokine/chemokine responses we have shown previously (Swaggerty *et al.*, 2004, 2006; Kogut *et al.*, 2006). Our findings are in agreement with this pattern where increased levels of p38 are associated with a greater pro-inflammatory cytokine/chemokine profile whereas JNK activity is associated with a lesser pro-inflammatory response. In this instance, the avian PMN response is comparable with the mammalian PMN and therefore it is not surprising that we observed a differential signalling response with line A (increased pro-inflammatory profile) functioning more through p38 activation and line B (greater anti-inflammatory profile) working via the JNK pathway. There are also reports where avian PMN respond differently compared with the mammalian PMN. Human neutrophil killing is enhanced following an increase in JNK activation (Yeh *et al.*, 2010); however, this is not what was observed in the present study where heterophils from line B chickens had elevated levels of JNK, yet their ability to kill SE is reduced compared with line A (Swaggerty *et al.*, 2003a). The Yeh *et al.* study utilized opsonized bacteria while the SE in the current study was non-opsonized; however, this is not likely to be the reason for the differences as opsonized SE produces similar activation of JNK to that which was presented herein (Swaggerty, unpublished data).

The NF-κB family of transcription factors (composed of p50, p52, p65 [RelA], c-Rel, and RelB) generally resides in an inactive state in the cytoplasm and is complexed with inhibitory IκB proteins that upon phosphorylation release IκB to initiate nuclear translocation and either homodimerize or heterodimerize with the NF-κB complex, which then mediates gene expression. The AP-1 proteins (comprised of c-Jun, c-Fos, FosB, Fra-1 and Fra-2, JunD, and JunB) also undergo either homodimerization or heterodimerization upon activation. Interleukin-6 and CXCLi2 mRNA expression levels in avian heterophils are mediated, in part, by AP-1 (specifically c-Jun and JunD) and NF-κB (p50, c-Rel, and RelB) activation (Kogut *et al.*, 2008). In the current study, only c-Jun and p50 were significantly different between lines A and B, indicating a differential mechanism is in place that probably directs the distinct cytokine and chemokine profiles that we demonstrated earlier. As stated previously with regard to studies comparing heterophils from Leghorns versus broilers and purified bacterial components as opposed to live bacteria, it is possible that the differences in transcription factors may also be explained by differences between the two biological systems that were tested. Likewise, there are reports in the mammalian literature describing signalling pathway differences depending on the agonist used (Yum *et al.*, 2001; Strassheim *et al.*, 2004). As we have seen with heterophils, regulation of interleukin-6 and other inflammatory cytokines in mammalian cells is also mediated by NF-κB (Kim & Rikihisa, 2002; Cloutier *et al.*, 2007; Requena *et al.*, 2009; Cao *et al.*, 2010; Khalaf *et al.*, 2010), and interleukin-8 production is regulated more so by AP-1 in T cells (Khalaf *et al.*, 2010), and NF-κB in monocytes (Vitiello *et al.*, 2004). Additionally, deficiencies in AP-1 proteins are associated with increased susceptibility to *S. typhimurium* (Maruyama *et al.*, 2007). As we have discussed, differences in MAPK signalling pathways influence downstream activation of NF-κB and AP-1. Thus, we cannot rule out the possibility that NF-κB proteins are directly affecting AP-1 activity as has been seen in mammalian cells (Fujioka *et al.*, 2004). The authors also recognize that transcription factors may have already been switched on and off during the 1 h stimulation or it is possible that that they are activated at a later time point. Additional studies to fully understand the role and timing of AP-1 and NF-κB activation should be pursued in the future. Another possibility is that the sequence identity for both transcription factor families is too low (http://blast.ncbi.nlm.gov). Sequence identity for NF-κB members ranged from 59 to 74%. Members of AP-1 varied from 47 to 90%; however, no matches were found for FosB or JunB, so the lack of observed changes in these two genes is quite possibly due to lack of recognition by the human antibody. An earlier study by our laboratory shows increased expression of several members from each transcription factor family (Kogut *et al.*, 2008); therefore the differences observed in the present study are probably due to genetic differences between lines A and B. The current study showed AP-1 and NF-κB transcription factors, specifically c-Jun and p50, respectively, were activated differentially following stimulation with SE between lines A and B, both of which share relatively high sequence identity with the human genes (90% and 74%, respectively).

Collectively, these data indicate that the increased responsiveness of line A chickens and/or their heterophils is influenced, in part, by an increased ability to initiate critical signal transduction pathways mediated in general by PTKs as well as specific members of the MAPK super-family, which therefore directly affects the initiation and subsequent production of an effective innate immune response. As a result of these findings, selection of chickens for increased activation of specific signalling pathways may produce a line of birds that is more resistant and more responsive against a broad range of pathogens based on a more efficient innate immune response. Chickens that are inherently more resistant to poultry and food-borne pathogens are likely to have increased liveability in the field and may have decreased levels of food-borne pathogens, thus reducing the potential for transmission from the avian host into the food supply.

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