Profiling pro-inflammatory cytokine and chemokine mRNA expression levels as a novel method for selection of increased innate immune responsiveness

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Abstract

Previous studies using F1 reciprocal crosses and two parental lines of broilers show the sire is instrumental in determining the in vitro leukocyte function and cytokine/chemokine profile. Since the innate immune response is the primary means young chickens have to protect themselves, we hypothesize utilizing a novel genomics approach to select sires based on an elevated pro-inflammatory cytokine and chemokine profile. By identifying sires with increased pro-inflammatory cytokine (interleukin [IL]-1β and IL-6) and chemokine (CXCL12 and CCL12) mRNA expression levels, we expect the progeny will also have elevated profiles. We characterized the pro-inflammatory cytokine and chemokine profile of 119 sires using quantitative real-time RT-PCR (qRT-PCR) and identified two populations with inherently high and low mRNA expression levels of IL-1β, IL-6, CXCL12, and CCL12. Select high and low sires were then used to produce progeny for the second phase of the trial. Blood samples were collected from 214 progeny and the cytokine and chemokine mRNA expression levels determined. Progeny from high sires had significantly (P ≤ 0.02) higher cytokine (IL-1β and IL-6) and chemokine (CXCL12 and CCL12) mRNA expression levels compared to progeny from low sires. We have identified a broiler population of sires with higher and lower than average pro-inflammatory cytokine/chemokine mRNA expression levels and used them to produce progeny with similar profiles.

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Keywords: Chemokine; Chicken; Cytokine; Innate immunity; Selection

1. Introduction

Economic pressure on the poultry industry has directed genetic selection to develop birds that grow rapidly while consuming less feed. Over the past 50 years, selective breeding for increased body weight in broilers has seen a dramatic reduction in the number of days required for a bird to reach processing weight; in the 1950s it took 84 d whereas currently the time has been reduced to 42 d. More specifically, a 1957 broiler weighed 539 g at 42 d compared to 2672 g for a modern broiler (Havenstein et al., 2003). However, selection based on growth characteristics alone can adversely affect immune competence (Bayyari et al., 1997; Beaumont et al., 1999; Han and Smyth, 1972; Janss and Bolder, 2000). The poultry industry suffers major economic losses from both mortality due to infectious diseases and potential contamination of food products due to transfer of microorganisms.
Our laboratories have been evaluating the innate immune system of broilers to assess their capacity to protect against multiple pathogen infections. We have extensively characterized the innate immune response of two parental broiler lines (designated lines A and B). To date, we have shown increased in vitro heterophil function (Swaggerty et al., 2003a,b) corresponds with an increased in vivo resistance to organ invasion by Gram-positive (Swaggerty et al., 2005b) and Gram-negative bacterial infections (Ferro et al., 2004; Swaggerty et al., 2005a). In addition, we have shown increased mRNA expression levels of pro-inflammatory cytokines in heterophils isolated from the more resistant lines compared to the susceptible lines (Swaggerty et al., 2004). Collectively, all data indicate there are clear and measurable differences in heterophil function and innate responsiveness that are under specific genetic control.

Cytokines are essential effector molecules of innate and acquired immunity that initiate and coordinate cellular and humoral responses aimed at eradicating pathogens. Detecting avian cytokines is limited by the lack of specific antibodies and reliable bioassays. However, recent cloning of chicken cytokines and chemokines have enabled development of a more comprehensive array of reagents for investigating innate and acquired immune responses at cellular and molecular levels. Chicken orthologs of the T1 cytokines interferon-γ (IFN-γ) (Digby and Lowenthal, 1995), interleukin (IL)-18 (Schneider et al., 2000), and IL-12 (Balu and Kaiser, 2003; Degen et al., 2004), the pro-inflammatory cytokines IL-1β (Weining et al., 1998) and IL-6 (Schneider et al., 2001), the chemokines CXCLi2 (formerly IL-8) (Bedard et al., 1987; Sugano et al., 1987) and CCLi2, the T2-specific cytokines, IL-4 and IL-13 (Avery et al., 2004), and the anti-inflammatory cytokines transforming growth factor-β4 (TGF-β4) (Jakowlew et al., 1988) and IL-10 (Rothwell et al., 2004) have all been cloned and sequenced. This makes it possible to design probes and primers to quantify cytokine mRNA expression levels using quantitative real-time RT-PCR (qRT-PCR). Although measuring cytokine mRNA expression levels by qRT-PCR does not necessarily equate to the production of bioactive protein, recent publications demonstrate qRT-PCR is the most highly sensitive method available to reliably quantify a broad spectrum of avian cytokines, particularly in the absence of effective bioassays (Beal et al., 2004; Kaiser et al., 2000, 2002, 2003; Smith et al., 2005; Swaggerty et al., 2004; Withanage et al., 2004).

Pro-inflammatory cytokines and chemokines have a key role in initiating an innate immune response and assist in generating a local inflammatory response (Ferro et al., 2004; Hughes et al., 2007). An increase in IL-1β, IL-6, and IL-8 (CXCLi2) mRNA expression is associated with increased resistance to extraintestinal Salmonella enteritidis (SE) infections in chickens (Ferro et al., 2004). In addition to an effective pro-inflammatory cytokine response, a strong chemotactic response is influential in determining resistance to SE. In chickens, an IL-8-like chemokine is involved in heterophil recruitment to the site of infection following challenge with SE (Kogut, 2002). Based on our previous genetic analysis with lines A and B, we hypothesized that by selecting sires within a broiler population with increased pro-inflammatory cytokine and chemokine responses, the progeny of these sires will have a more efficient innate immune response. Using a functional genetic approach (qRT-PCR), the objectives of the present study were (1) identify sires with inherently “high” and “low” pro-inflammatory cytokine and chemokine mRNA expression profiles, and (2) evaluate pedigreed progeny from selected “high” and “low” sires for cytokine and chemokine profiles. The goal of the selection trial is to increase the pro-inflammatory cytokine and chemokine responses of a population of broilers while maintaining the other desired growth characteristics that make this broiler population valuable. The long-term goal is to produce a broiler population with increased livability in the field accompanied by increased resistance against diverse pathogens.

2. Materials and methods

2.1. Blood collection and cell isolation

The chickens used in the present study are a commercial pure line reared on a farm following proprietary industry standards and guidelines. Blood collection was conducted according to federal regulations and overseen by an attending veterinarian. Sire blood samples were collected from 21-week-old broilers while the progeny were bled at 6-weeks-of-age. In both instances blood (3–5 ml) was collected from the big vein in the wing and transferred to vacutainer tubes containing disodium ethylenediaminetetraacetic acid (EDTA) (BD vacutainer, Franklin Lakes, NJ) and mixed thoroughly. The samples were shipped overnight to our laboratory in an insulated cooler with cold-packs. Upon receipt of the samples, the blood and EDTA were transferred to a conical tube and 8–10 ml 1% methylcellulose prepared in RPMI was added, mixed thoroughly, and centrifuged at 35 × g for...
20 min at 4 °C. The supernatant containing the peripheral blood leukocytes (PBL), including heterophilis and monocytes, was transferred to a new conical tube and 10 ml clear RPMI added, and the cells pelleted by centrifugation (485 x g for 15 min at 4 °C). The cells were re-suspended and washed in fresh RPMI (1 ml) and pelleted (485 x g for 15 min at 4 °C), the supernatant removed, and the cells re-suspended in lysis buffer (Qiagen RNeasy mini RNA extraction kit, Qiagen Inc., Valencia, CA), and frozen.

2.2. Isolation of total RNA

The lysed cells were transferred to QIAshredder homogenizer columns (Qiagen) and centrifuged for 2 min at ≥8000 x g. Total RNA was extracted from the homogenized lysates according to the manufacturer’s instructions, eluted with 50 μl RNase-free water, and stored at −80 °C until qRT-PCR analyses were performed.

2.3. Quantitative real-time RT-PCR

Cytokine mRNA expression was quantitated using a well-described method. Primers and probes for cytokines, chemokines, and 28S RNA-specific amplification have been described (Kaiser et al., 2000; Kogut et al., 2003) but for clarity are provided (Table 1). The qRT-PCR was performed using the TaqMan fast universal PCR master mix and one-step RT-PCR master mix reagents (Applied Biosystems, Cheshire, UK). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following cycle profile: one cycle of 48 °C for 30 min, 95 °C for 20 s, and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Quantification was based on the increased fluorescence detected by the 7500 Fast Sequence Detection System due to hydrolysis of the target-specific probes by the 5’ nuclease activity of the rTth DNA polymerase during PCR amplification. To correct for differences in RNA levels between samples within the experiment, the correction factor for each sample was calculated by dividing the mean Ct value for 28S rRNA-specific product for each sample, by the overall mean Ct value for the 28S rRNA-specific product from all samples. Fold change in mRNA expression levels between high and low corrected 40 – Ct values were calculated as follows:

\[ \text{Fold change} = \frac{2^{(\text{high sire corrected mean for each cytokine or chemokine} - \text{low sire corrected mean for each cytokine or chemokine})}}{2^{\text{high sire corrected mean for each cytokine or chemokine}}} \]

The corrected cytokine mean is calculated:

\[ \text{Average of each replicate} \times \text{cytokine slope} \]

\[ \frac{28S \text{ slope} \times 28S \text{ correction factor}}{2} \]

Table 1

<table>
<thead>
<tr>
<th>RNA target</th>
<th>Probe/ primer sequence</th>
<th>Exon boundary</th>
<th>Accession numbera</th>
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<tr>
<td>28S</td>
<td>5’-(F AM)b)-AGGACCGCT ACGGACCTCC ACC A-(TAMRA)-3’</td>
<td>X59733</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-GGGCAAGGC AGAGGAACCT-3’</td>
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<td></td>
<td>5’-GACCGACGTGGTC ACCGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-(F AM)-CC AC ACTGC AGCTGGAGGAAGCC-(T AMRA)-3’</td>
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<td>Y15006,AJ245728</td>
</tr>
<tr>
<td>F</td>
<td>5’-GCTCT AC ATGTCGTGTGATGAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-TTGCTAGTGGTCCGC ATGA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-(F AM)-AGGAGAATATGCCGCTGAGCAGCTCTCC A-(T AMRA)-3’</td>
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<td>AJ250838</td>
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<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-GTTAGCTGGTAAGCGAAGCAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL2</td>
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<td>AJ009800</td>
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<tr>
<td>R</td>
<td>5’-TGGC ACCGC AGCTC ATT-3’</td>
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<tr>
<td>CCL2</td>
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<tr>
<td>F</td>
<td>5’-GCAGACACT ACT CAGAGACC AAC AG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5’-AGGGCCCTTCTCGTGAF-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Genomic DNA sequence.
* 5-Carboxyfluorescein.
* Forward.
* Reverse.
2.4. Statistical analyses

There were no statistical analyses performed on the original 119 sires. The data obtained on the initial sires showed there was a range of responses and allowed us to identify sires with a naturally high and low baseline value. Three high sires were identified and selected and produced 120 progeny while five low sires were selected and produced 94 progeny. The mean and standard error of the mean for each cytokine and chemokine were calculated for the progeny populations from the high and low sires and statistical analyses performed (Student’s t-test at \( P \leq 0.05 \)).

3. Results

3.1. Identification and characterization of sires

The cytokine and chemokine mRNA expression profile was quantitated for 119 sires. Based on our previous findings (Ferro et al., 2004; Swaggerty et al., 2004, 2006), two pro-inflammatory cytokines (IL-1\( \beta \) and IL-6) and two pro-inflammatory chemokines (CXCLi2 [formerly IL-8] and CCLi2 [formerly macrophage inhibitory protein (MIP)-1\( \beta \)]) were selected as indicators for an efficient innate immune response. The response for each cytokine and chemokine produced a typical bell-shaped curve with approximately 15 sires having very low or very high mRNA expression levels (Fig. 1). We next sought to determine if a particular sire was in the top/bottom category of multiple cytokines and chemokines. In fact, we identified 12 sires that were in the top responders for at least three out of four of the cytokines and chemokines. Similar numbers for the lowest responding sires were also observed (11 had low mRNA expression levels in at least 3 out of 4 categories). We wanted to utilize sires in the top/bottom of each parameter in the selection process to determine if a strong/weak pro-inflammatory cytokine and chemokine response could be transferred to progeny and therefore potentially increase/decrease the efficiency of the innate immune response.

3.2. Characterization of progeny

Of the 12 sires identified as having naturally high mRNA expression levels of pro-inflammatory cytokines and chemokines, three were selected for the second phase of the selection trial, and of the 11 low sires, 5 were selected for the second phase. The cytokine and chemokine profiles of the selected high and low sires used to produce the progeny are summarized in Table 2. The purpose of these experiments was to determine if a strong and/or weak pro-inflammatory cytokine and chemokine response was transferred to progeny. In theory, this would enable us to select for an increased efficiency of the innate immune response. A total of 120 and 94 progeny from high and low sires, respectively, were analyzed and their pro-inflammatory cytokine and chemokine profile determined by qRT-PCR. All data shown as corrected 40 – \( C_t \) values (summarized in Table 3).

![Fig. 1. Range of pro-inflammatory cytokine and chemokine mRNA expression levels (corrected 40 – \( C_t \)) observed in the initial sire screening (\( n = 119 \) sires). (A) IL-1\( \beta \). (B) IL-6. (C) CXCLi2. (D) CCLi2.](image-url)
As with the initial sires, the same pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (CXCL2 and CCL2) were quantitated for all progeny samples. There were significant ($P < 0.02$) differences between IL-1β and IL-6 mRNA expression levels (Table 3). The mean IL-1β mRNA expression levels in progeny from high and low sires were 9.94 $\pm$ 0.16 and 9.36 $\pm$ 0.19, respectively. The mRNA expression levels for IL-6 were 17.7 $\pm$ 0.26 and 16.69 $\pm$ 0.29, respectively.

Similar significance ($P < 0.004$) was also observed for the chemokine responses (Table 3). The mean CXCL2 mRNA expression levels for progeny from high and low sires were 10.24 $\pm$ 0.31 and 8.74 $\pm$ 0.40, respectively. The CCL2 mRNA expression levels were 17.12 $\pm$ 0.26 and 15.83 $\pm$ 0.30, respectively.

The fold change in mRNA expression levels between the high and low progeny was also determined (Fig. 2). The fold change between high and low progeny for IL-1β and IL-6 were 1.5 and 2, respectively. The differences were greater in the chemokines where CXCL2 mRNA expression was 2.8-fold greater in the high progeny compared to the low progeny. Additionally, CCL2 mRNA expression in the high progeny was 2.4-fold greater than the levels from the low progeny.

4. Discussion

Traditionally, genetic selection of poultry based on immunity and disease resistance has focused on either inherent resistance to a specific infectious agent such as Marek’s disease virus, avian leukosis virus, or a specific vaccine (Zekarias et al., 2002). This approach has resulted in inbred stocks of chickens resistant to specific pathogens with either poor growth characteristics or a poor response to other pathogens or vaccines. Thus far, to our knowledge, there is no commercially available line of poultry that has been genetically selected and bred for polyvalent resistance to multiple infectious agents or increased responsiveness to diverse vaccines.

Table 2
Cytokine and chemokine mRNA expression levels (40 – $C_t$) of high and low sires used to produce progeny

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>High sires (n = 3)</th>
<th>Low sires (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>IL-1β</td>
<td>15.91</td>
<td>18.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>14.28</td>
<td>16.34</td>
</tr>
<tr>
<td>CXCL2</td>
<td>14.16</td>
<td>15.58</td>
</tr>
<tr>
<td>CCL2</td>
<td>15.71</td>
<td>17.41</td>
</tr>
</tbody>
</table>

a Standard error of the mean.

Table 3
Cytokine and chemokine mRNA expression levels (40 – $C_t$) of progeny from defined high and low sires

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>Progeny from high sires (n = 120)</th>
<th>Progeny from low sires (n = 94)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Mean ± S.E.M.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.32</td>
<td>12.86</td>
<td>9.94 ± 0.16</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.29</td>
<td>22.41</td>
<td>17.7 ± 0.26</td>
</tr>
<tr>
<td>CXCL2</td>
<td>0.0</td>
<td>17.66</td>
<td>10.24 ± 0.31</td>
</tr>
<tr>
<td>CCL2</td>
<td>2.29</td>
<td>21.55</td>
<td>17.12 ± 0.26</td>
</tr>
</tbody>
</table>

a Standard error of the mean.

* P value calculated by performing Student’s $t$-test and comparing the average of high and low sires for each cytokine and chemokine.

Fig. 2. Fold change in mRNA expression levels of high progeny compared to low progeny ($n$ = 120 high progeny; $n$ = 94 low progeny). Fold change calculated as follows: $2^{(\text{high sire corrected mean for each cytokine or chemokine} - \text{low sire corrected mean for each cytokine or chemokine})}$. The corrected cytokine mean is calculated: (Average of each replica-te × cytokine slope)/28S slope × 28S correction factor.
Advancing our earlier studies where innate immune responsiveness was evaluated, the purpose of the present study was to show that the pro-inflammatory cytokine and chemokine profile was passed onto progeny through the sire.

The host immune response to pathogens in the earliest stages of infection is a critical determinant of disease resistance and susceptibility. For the past 6 years we have extensively characterized the innate immune response of parental broilers and have shown that, as in vitro heterophil function increases (Swaggerty et al., 2003b), there is a corresponding increase in resistance against organ invasion by SE (Ferro et al., 2004; Swaggerty et al., 2005a). More importantly the increased resistance against SE is accompanied by increased mRNA expression levels of pro-inflammatory cytokines and chemokines (Ferro et al., 2004).

Economic pressure on the poultry industry has directed genetic selection to develop broilers that grow rapidly while consuming less feed. However, selection based heavily on growth characteristics and other phenotypic traits can adversely affect immune competence thereby leaving chickens and turkeys more susceptible to disease (Bayyari et al., 1997; Beaumont et al., 1999; Han and Smyth, 1972; Janss and Bolder, 2000; Nestor et al., 1996). The acquired immune response may be adversely affected by modern selection pressures while cell-mediated and inflammatory responses are improved (Cheema et al., 2003). This study is based on a comparison of a 2001 broiler to a 1957 broiler where a decrease in antibody response accompanied with an increase in the toe-web inflammatory response was observed following exposure to a T cell mitogen. It is possible that the increased toe-web response observed in the 2001 population was due to an enhanced chemotactic response, possibly driven by CXCL12. Cheema et al. (2003) did not address the role of the innate immune response with regard to cytokine and chemokine responses and our current study did not address the antibody response or the T cell response. Since the innate immune response is directly involved in coordinating the acquired immune response it is possible that the antibody-mediated response of the broilers used in the present study might also be enhanced. Addressing the humoral response is outside the scope of the current study, but should be evaluated in future studies if this selection method is adopted by the poultry industry.

The fact that the innate immune response directs the acquired response (Parish and O’Neill, 1997) supports efforts for selecting poultry with an efficient early innate immune response. Microbial recognition by cells of the innate response activates intracellular signaling pathways that result in the (a) activation of microbicidal killing mechanisms, (b) release of cytokines and chemokines, and (c) production of co-stimulatory molecules required for antigen presentation to the acquired immune system (Medzhitov and Janeway, 1997a,b; O’Connell et al., 2005; Romagnani, 1992; Takeuchi and Akira, 2007). In poultry, previous reports have shown that strong pro-inflammatory cytokine and chemokine responses are associated with increased resistance against disease (Coussens et al., 2004; Ferro et al., 2004; Heinrich et al., 2001; Sebastiani et al., 2002; Withanage et al., 2004). Heterophils migrate to the liver and intestinal villi of newly hatched chickens infected with Salmonella typhimurium (ST), accompanied by elevated levels of pro-inflammatory cytokine in the tissues (Withanage et al., 2004), indicating a potential role of the acute inflammatory response in neonatal chickens. In vitro stimulation of macrophages isolated from Salmonella-resistant chickens results in a significant increase in pro-inflammatory cytokine and chemokine mRNA expression levels, including IL-1β, IL-6, and CCL2 (Wigley et al., 2006). Collectively, the data presented herein are in agreement and further support the important role of pro-inflammatory cytokine and chemokine responses.

The mRNA expression levels of the pro-inflammatory chemokine CXCL2 was up-regulated in PBL from progeny of high sires compared to the levels observed in progeny from low sires. In addition to an effective pro-inflammatory cytokine response, a strong chemotactic response may also influence susceptibility of chickens to pathogens. Chemokines are small, structurally related chemoattractant molecules that regulate movement of leukocytes (Zlotnick and Yoshie, 2000). Both IL-1β and IL-8 (CXCL2) are found in the gut of newly hatched chickens and mRNA expression levels continue to increase the first week post-hatch, indicating their importance (Bar-Shira and Friedman, 2006). IL-8 (CXCL2) mRNA expression is up-regulated in Salmonella-resistant chickens (Sadeyen et al., 2004). CXCL2 and CCL2 mRNA expression levels did not change in broilers following an SE infection while IL-1β and IL-6 mRNA expression did increase (Cheeseman et al., 2007). Clearly there is evidence for a strong innate cytokine response and this is likely very important in determining the response against Salmonella in poultry. Now that the baseline data has been confirmed, future in vivo challenge studies using the high and low selected birds should be conducted to confirm the findings presented herein.

These data indicate the mRNA expression levels of the innate immune pro-inflammatory cytokines and
chemokines evaluated are influenced, in part, by the levels observed in the sires. Our initial study evaluating a parental pair and their F1 reciprocal crosses shows the sire is more influential in determining in vitro heterophil functional efficiency which then translates to increased resistance against organ invasion by SE (Ferro et al., 2004; Swaggerty et al., 2003b). In the present study, the IL-6 and CCLi2 mRNA expression levels from high progeny were higher than the levels observed for the high sires; however, the average IL-1β and CXCLi2 levels were lower for the high progeny compared with their sires. In all instances the low progeny values were higher than the low sires used to produce them. It should be noted that we did not evaluate the hens in this study. Future studies should consider the role of the hen and the contribution and/or dampening affect on the cytokine and chemokine profile observed in progeny.

With the present selection trial, we have moved beyond theoretical and into practical application. Currently, we have: (1) identified sires from a broiler population with higher and/or lower-than-average pro-inflammatory cytokine and chemokine mRNA expression levels and (2) utilized small numbers of high and low responding sires to produce progeny with increased or decreased, respectively, pro-inflammatory cytokine and chemokine profiles. We believe this novel approach will allow us to improve broiler breeding stock by improving the immunological responsiveness. The desired growth qualities would be in place but now the population would also have an effective pro-inflammatory innate immune response which should improve resistance against diverse pathogens, improve responses to vaccines and increase livability.

Acknowledgments

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