Disturbances of homeostasis by infection, trauma, or immunological disorders activate leukocytes, fibroblasts, and endothelial cells. When activated, these cells release cytokines such as interleukins (ILs), interferons (IFNs), and tumor necrosis factor-α (TNF-α), which act on specific receptors within or on the surface of the targeted cells leading to inflammation. Cytokines are key molecules that participate in the inflammatory response and act as mediators to facilitate the communication network of the immune system. Research on the biology of cytokines and their roles in the regulation of inflammatory responses in avian systems has fallen behind the work in mammals. While not well characterized, studies suggest that chicken cytokines possess biological activity and function similar to those described for mammalian species.

Lipopolysaccharide (LPS) is the primary component of the cell wall of gram-negative bacteria and serves as a potent stimulator of inflammation. It has been commonly used to model bacterial infection and induce an acute immune challenge in poultry. It initiates inflammatory responses via Toll-like receptor 4 (TLR4) expressed on the cell surface of leukocytes such as monocyte or macrophages and heterophils. This receptor-ligand interaction activates local macrophages and other leukocytes to

OBJECTIVE
To investigate inflammatory responses to lipopolysaccharide (LPS) injection in layers.

ANIMALS
33 40-week-old laying hens were used.

METHODS
30 laying hens were divided into 2 groups: the first group was injected with 8 mg/kg LPS, while the second group was injected with sterile saline. At the start of the study, 3 birds served as a baseline and were used as the time 0 controls for both the saline and LPS-treated groups. Blood and spleen tissues were collected at 0 (before) and 1, 2, 3, 4, and 6 hours after injection.

RESULTS
LPS administration increased splenic mRNA levels of IL-1β, IL-2, IL-6, IL-8, IL-10, interferon-γ, and tumor necrosis factor-α (P < .001) and serum IL-6 levels (P < .01) compared to saline injection. The mRNA expression of most cytokine genes increased rapidly toward peak values within 2 hours after the LPS injection, and then the difference between the saline and LPS treatments got smaller as time went on; serum IL-6 reached its highest concentration 2 hours after LPS administration. The magnitude of LPS-induced upregulation of gene expression was the highest for IL-6, followed by IL-1β and IL-8, and tumor necrosis factor-α was the least affected.

CLINICAL RELEVANCE
The temporal and quantitative profile of these inflammatory mediators generated from this study provides valuable information in identifying the optimal time window and appropriate biomarkers for LPS-induced inflammation, which has significant implications in evaluating the effects of interventions on the immune system of chickens.

Keywords: immune challenge, cytokines, spleen, serum, laying hens
produce and secret cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, and IFN-γ. These cytokines exert pro- and anti-inflammatory effects and serve as biomarkers for many diseases; therefore, quantification of cytokines can be used to monitor the state of the immune response and provides insights into physiological and pathological processes. It has been demonstrated that the production of cytokines is generally enhanced by LPS administration in chickens (predominantly in broilers); however, most studies have only examined the effect of LPS at one endpoint, and there exists temporal variation in the expression of those critical inflammatory mediators. As such, optimizing the duration of LPS challenge is warranted for accurate quantification of cytokines. Additionally, there is a lack of research simultaneously examining multiple immunoregulatory molecules in response to a bacterial infection over various time intervals in poultry particularly in laying hens. As early events in the process of inflammation are the most critical for the immune system to effectively combat microbial infections, the focus of this study was on the early, acute phase of the inflammatory response to LPS in laying hens, which was assessed by determining the protein or mRNA levels of certain inflammatory mediators including IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ, and TLR4 at specified time points (0, 1, 2, 3, 4, and 6 hours) post-LPS injection. Knowledge generated from this study should provide important information on the creation of LPS-induced inflammation models in layers to study the effects of nutrients, management, and other exogenous treatments on immune function, and help advance health monitoring and wellness of poultry flocks.

Methods

Experimental animals

A total of 33 Dekalb White layers, 39 weeks of age, were obtained from the main flock of the Poultry Barn, University of Manitoba. The birds were housed individually in cages (38 cm wide × 52 cm deep, providing 1,976 cm² area space per bird), complete with independent feeders, nipple drinkers, and perches. The birds were exposed to 16 hours of light and 8 hours of dark per day, with feed and water available ad libitum. All experimental procedures involving animal usage and care were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee (F18-025 [AC11383]), and the hens were managed according to the recommendations established by the Canadian Council on Animal Care.

Experimental protocol

After 1-week adaptation, 30 birds (40 weeks old) were divided into 2 groups. The first group was injected IV in the brachial wing vein with 8 mg/kg body weight Escherichia coli LPS (serotype 0111: B4; Sigma Aldrich), while the second group was injected with sterile saline. In addition to the 30 injected birds, 3 birds were nontreated and served as the control animals, which were used as the time 0 (baseline) for both the saline and LPS-treated groups. Immediately following injection, the general behavior of each bird was observed. At 0 (before) and 1, 2, 3, 4, and 6 hours after LPS or saline administration, the rectal body temperature was measured; approximately 5 mL of blood was drawn from the brachial wing vein, the birds were then euthanized by CO2 asphyxiation, and the spleens were collected from all birds. Blood was collected into a serum separator tube and allowed to clot for 2 hours at room temperature; then, serum was separated by centrifugation for 20 minutes at 1,000 X g, and samples were stored at −80°C until assayed. Spleen samples were flash frozen in liquid nitrogen after excision and stored at −80°C until assayed for gene expression.

The dosage of LPS used in this study was based on the previous experiments from our group and other researchers. Given the issue that venipuncture, often from the brachial vein, causes hematomas at the site of blood collection, making multiple blood sampling difficult or impossible, the serial sacrificing of birds at various intervals was employed in the current study. This method, as one of the alternatives for serial blood sampling, has been widely used for research in chickens. Moreover, for the improvement of animal welfare, the number of animals used should be minimized, 3 birds were therefore allotted to each time point, which is justified from a statistical standpoint. The same number of animals has also been reported in other chicken studies.

Relative mRNA expression of inflammation regulatory genes in spleen

RNA isolation and cDNA synthesis—Total RNA was extracted from approximately 10 mg of spleen with the commercial kit (RNeasy Mini kit; Qiagen) according to the manufacturer’s protocol, including on-column digestion of genomic DNA (RNase-Free DNase Set for use with RNeasy/QIAamp Columns; Qiagen). Total RNA concentration was measured at an optical density of 260 nm on a UV-Vis spectrophotometer, and RNA purity was verified by the ratio of absorbance at 260 and 280 nm. Reverse transcription of 1 μg of total RNA in a 20-μL reaction was conducted using cDNA synthesis kit (SuperScript VILO; Invitrogen). The generated cDNAs were stored at −20°C and used as the templates in the following quantitative real-time PCR (qRT-PCR) analysis.

qRT-PCR analysis—qRT-PCR was performed to detect relative mRNA expression levels of targeted genes including IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and TLR4. Primer sequences are listed (Supplementary Table S1). The specificity of the primers was confirmed with NCBI BLASTN. qRT-PCR was performed on a real-time PCR system (StepOne Real-Time PCR System; Applied Biosystems), using real-time PCR SYBR master mix (Fast SYBR Green Master Mix; Applied Biosystems). The amplification conditions were as follows: 95°C for 20 seconds and 40 cycles of denaturation at 95°C for 3 seconds, and combined annealing and extension at 61°C for 20 seconds; 60°C for 30 seconds; 72°C for 30 seconds. The cycle melt curve was run from 65°C to 95°C. The comparative cycle threshold (ΔΔCT) method was used to calculate the relative gene expression levels.
30 seconds, followed by a 3-segment cycle (95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds) of the melting curve. Samples were run in duplicate, and the mean was used for further analysis. The expression of target genes was analyzed by the 2−ΔΔCt method with β-actin as reference. The expression levels of β-actin remained constant through the analysis of its delta cycle threshold (ΔCt) values among the samples used (data not shown).

Serum IL-6 concentrations
Serum concentrations of IL-6 were measured by the ELISA (chicken IL-6 ELISA kit; LSBio) following the user manual. Standard curves and samples were run in duplicate.

Statistical analyses
All data were analyzed as a completely randomized design with individual birds as experimental units. There were 11 treatments, including baseline (time 0; preinjection) and 1, 2, 3, 4, and 6 hours after LPS and saline injection. mRNA expression of genes due to the time and injection was analyzed as 1-way ANOVA using the PROC MIXED procedure of SAS, version 9.4 (SAS Institute Inc.). For serum IL-6, data in the saline-treated groups that served as the baseline controls, saline-injected and LPS-injected groups, respectively. The mRNA expression of cytokines including IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNF-α was increased (P < .001) in the spleens of LPS-injected groups compared with the saline-injected groups; as expected, there were no differences between baseline and the saline-injected groups (P > .05; Table 1). Overall, the difference between the LPS and saline treatment was the largest within 2 hours postinjection, and this difference became smaller as time went on. At 1 hour, immediately after the LPS injection, IL-1β mRNA expression reached the maximum and then declined but still maintained higher until 6 hours compared to the saline control. IL-2, IL-6, and IL-8 were altered only through 3 hours post-LPS injection, with the greatest changes found

Table 1—Spleenic mRNA expression profile of inflammation regulatory genes at different time points following lipopolysaccharide (LPS) or saline administration in layers.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-6</th>
<th>IL-10</th>
<th>INF-γ</th>
<th>TNF-α</th>
<th>TLR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline 0 (h)</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Hour postsaline injection 1</td>
<td></td>
<td>11.80 ± 0.39</td>
<td>15.97 ± 0.89</td>
<td>13.71 ± 1.67</td>
<td>10.05 ± 1.13</td>
<td>11.51 ± 1.23</td>
<td>11.80 ± 1.22</td>
<td>6.20 ± 0.48</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1.23</td>
<td>1.16</td>
<td>1.64</td>
<td>0.93</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>11.63 ± 0.77</td>
<td>15.91 ± 0.32</td>
<td>12.73 ± 1.99</td>
<td>9.97 ± 0.88</td>
<td>12.33 ± 1.09</td>
<td>12.64 ± 0.48</td>
<td>6.75 ± 0.38</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.38</td>
<td>1.74</td>
<td>1.74</td>
<td>0.52</td>
<td>1.03</td>
<td>0.73</td>
<td>0.91</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>12.10 ± 0.90</td>
<td>16.18 ± 0.37</td>
<td>14.85 ± 1.45</td>
<td>10.43 ± 1.05</td>
<td>12.10 ± 0.58</td>
<td>12.33 ± 0.20</td>
<td>6.54 ± 0.16</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>1.00</td>
<td>0.64</td>
<td>0.53</td>
<td>1.26</td>
<td>0.62</td>
<td>1.11</td>
<td>0.84</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>11.89 ± 0.77</td>
<td>16.54 ± 0.35</td>
<td>13.47 ± 4.64</td>
<td>10.65 ± 0.21</td>
<td>13.04 ± 0.55</td>
<td>13.21 ± 0.58</td>
<td>6.87 ± 0.29</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.15</td>
<td>1.38</td>
<td>1.08</td>
<td>0.32</td>
<td>0.61</td>
<td>0.67</td>
<td>0.88</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>12.37 ± 0.16</td>
<td>16.17 ± 0.30</td>
<td>15.03 ± 1.85</td>
<td>9.98 ± 0.20</td>
<td>12.94 ± 0.13</td>
<td>12.18 ± 0.29</td>
<td>6.60 ± 0.19</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>0.82</td>
<td>0.64</td>
<td>0.47</td>
<td>1.72</td>
<td>0.34</td>
<td>1.24</td>
<td>0.81</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>1.65</td>
<td>1.26</td>
<td>1.36</td>
<td>1.32</td>
<td>1.34</td>
<td>1.24</td>
<td>0.84</td>
</tr>
<tr>
<td>Hour post-LPS injection 1</td>
<td></td>
<td>3.96 ± 0.44</td>
<td>11.58 ± 1.26</td>
<td>3.63 ± 0.51</td>
<td>2.75 ± 0.89</td>
<td>8.00 ± 1.42</td>
<td>8.12 ± 2.19</td>
<td>5.44 ± 0.77</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>281.4</td>
<td>15.50</td>
<td>1256.5</td>
<td>257.8</td>
<td>10.59</td>
<td>20.52</td>
<td>1.79</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>5.93 ± 0.50</td>
<td>12.20 ± 0.70</td>
<td>3.18 ± 1.10</td>
<td>6.30 ± 0.15</td>
<td>6.20 ± 1.12</td>
<td>7.21 ± 0.52</td>
<td>4.35 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>71.82</td>
<td>10.09</td>
<td>1725.0</td>
<td>21.96</td>
<td>36.66</td>
<td>38.58</td>
<td>3.82</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>3.90 ± 0.78</td>
<td>14.31 ± 1.10</td>
<td>7.99 ± 0.51</td>
<td>7.92 ± 1.53</td>
<td>9.85 ± 1.20</td>
<td>9.40 ± 0.25</td>
<td>4.30 ± 0.47</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>18.24</td>
<td>62.68</td>
<td>17.21</td>
<td>7.15</td>
<td>2.93</td>
<td>8.47</td>
<td>3.96</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>8.03 ± 0.30</td>
<td>14.85 ± 0.55</td>
<td>9.78 ± 1.07</td>
<td>8.31 ± 0.19</td>
<td>9.25 ± 2.15</td>
<td>10.14 ± 0.85</td>
<td>4.65 ± 0.20</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>16.64</td>
<td>1.60</td>
<td>17.67</td>
<td>5.46</td>
<td>4.43</td>
<td>5.06</td>
<td>3.12</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>9.82 ± 1.25</td>
<td>14.84 ± 0.42</td>
<td>11.26 ± 0.45</td>
<td>8.38 ± 0.80</td>
<td>11.66 ± 0.65</td>
<td>11.78 ± 0.65</td>
<td>5.50 ± 0.46</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>4.83</td>
<td>1.62</td>
<td>6.33</td>
<td>5.02</td>
<td>0.84</td>
<td>1.62</td>
<td>1.72</td>
</tr>
<tr>
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<td></td>
<td>&lt;.01</td>
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<td>&lt;.01</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

Table data are presented as the mean of ΔCt ± SD (n = 3). ΔCt = Ct (target gene) – Ct (β-actin), where target gene refers to IL-1β, IL-2, IL-6, IL-8, IL-10, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and Toll-like receptor 4 (TLR4); β-actin is used as an internal control for gene expression normalization; and Ct is the number of cycles required for the fluorescent signal to cross the threshold (ie, exceeds background level).

Values with different superscript letters in the same column indicate significant (\( P < .05 \)) differences in the measurement; means among the 11 treatment groups were compared using Tukey’s multiple range test.

Italic values are the fold changes of mRNA expression of genes from the saline and LPS-treated groups relative to the baseline group, calculated based on the 2−ΔΔCt method.

Results
As stated above, 3 birds were not treated and served as the baseline, which was used as the time 0 control for both the saline and LPS-treated groups. No deleterious effects of LPS on the bird’s behavior were observed. Also, there was no significant difference (\( P > .05 \)) in the rectal temperature among the birds, with an average of 40.7, 40.3, and 40.1°C for baseline controls, saline-injected and LPS-injected groups, respectively. The mRNA expression of cytokines including IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNF-α was increased (\( P < .001 \)) in the spleens of LPS-injected groups compared with the saline-injected groups; as expected, there were no differences between baseline and the saline-injected groups (\( P > .05 \); Table 1). Overall, the difference between the LPS and saline treatment was the largest within 2 hours postinjection, and this difference became smaller as time went on. At 1 hour, immediately after the LPS injection, IL-1β mRNA expression reached the maximum and then declined but still maintained higher until 6 hours compared to the saline control. IL-2, IL-6, and IL-8 were altered only through 3 hours post-LPS injection, with the greatest changes found
The expression of IL-10, INF-γ, and TNF-α was changed through 4 hours after LPS administration; however, the biggest alterations were detected at 2 hours. Together, the magnitude of LPS-induced increase in gene expression was the highest for IL-6 (1,725-fold), followed by IL-1β (281-fold) and IL-8 (258-fold), and then by IFN-γ (39-fold), IL-10 (37-fold), and IL-2 (16-fold), and TNF-α mRNA expression (4-fold) was lowest induced among the cytokines examined. The TLR4 mRNA levels were not affected by LPS injection ($P > .05$). Additionally, the administration of LPS led to an increase in serum IL-6. Consistent with its mRNA expression pattern in the spleen, serum IL-6 reached its maximum concentration 2 hours after LPS administration and decreased thereafter; however, there were no statistically significant differences among 3, 4, 6, and 0 hours (baseline; $P < .01$; Figure 1).

**Figure 1**—Changes over time in the concentration of serum IL-6 in lipopolysaccharide (LPS) and saline-injected laying hens (means ± SD; $n = 3$).

### Discussion

The use of LPS as a potent proinflammatory agent is a well-known model of inflammation applied in both in vivo and in vitro studies. Its effects include an altered production of a number of mediator molecules from which the cytokines are of pivotal importance. Depending on their role cytokines can be classified as proinflammatory or anti-inflammatory. Proinflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, TNF-α, and INF-γ contribute to the initiation and propagation of autoimmune inflammation, whereas anti-inflammatory cytokines such as IL-6 and IL-10 inhibit inflammation and suppress immune cells. Some cytokines such as IL-6 have both pro- and anti-inflammatory properties. The present experiment was conducted to study the temporal inflammatory profile following an acute immune challenge induced by LPS in the laying hens by determining changes in the serum concentrations or splenic mRNA expression of the above cytokines and TLR4 over 6 hours post-LPS injection. Overall, our results demonstrated that in vivo administration of LPS led to an inflammatory response in the layers, which manifested as increased expressions of cytokines. On the other hand, the current study showed injecting LPS did not affect the rectal thermal temperature of the bird. Leshchinsky and Klasing found a significant increase in the body temperature in layers but not in broilers following LPS injection. Another study showed that the body temperature initially decreased below normal and then later increased above normal after the administration of LPS in broilers. Chickens are generally resistant to the deleterious effects of LPS, and these variations could also be attributable to differences in the ambient temperature, dosage, age, route of administration, or genetics employed in different studies.

TLR4 can recognize gram-negative bacteria and mediate the inflammatory response against these bacteria. It is best known as the primary receptor for LPS. The binding of LPS to TLR4 initiates a signaling cascade that results in the activation of NF-κB, which ultimately leads to the synthesis and release of various cytokines and other inflammatory mediators. Expression of TLR4 mRNA has been reported in chicken tissues. In this study, LPS administration did not lead to changes in TLR4 mRNA compared to the saline control. Similar to our results, Munyaka et al. reported the expression of TLR4 in the spleen and cecal tonsils did not change in the LPS-challenged laying hens, while other studies showed that injecting LPS significantly upregulated the gene expression levels of TLR4 in the spleen and jejunal mucosa of broilers.

TNF-α, IL-1β, IL-6, and IL-8, usually produced by monocytes and macrophages in the early phase of inflammation, have been implicated as key inflammatory mediators in many animal models. TNF-α is the first cytokine to appear in the process of inflammatory response in mammals and was found to peak in the plasma following LPS injection in mammals. TNF-α activates monocytes or macrophages to release a number of proinflammatory molecules including IL-1β, IL-6, and IL-8 through feedback regulation. During sepsis, TNF-α was the first cytokine to appear 1.5 to 2 hours after LPS injection, followed by IL-1β and IL-6. In the present study, TNF-α mRNA levels in the spleen reached a maximum 2 to 3 hours following the administration of LPS, relatively later than IL-1β and IL-6 mRNA achieving its peak. Gehad et al. reported that plasma TNF-α activity reached a peak at 6 hours post-LPS injection in chickens. Aside from the difference in the studied traits (ie, protein abundance in plasma in the previous studies and mRNA abundance in spleens in the current study), this difference in TNF-α release kinetics could have also been due to the variation between animals. Moreover, the magnitude of LPS-induced increase in the mRNA levels was the lowest for TNF-α among all the studied cytokines, indicating TNF-α may not be a sensitive inflammation biomarker in chickens, and further studies are warranted. IL-1β and IL-8 play a vital role in innate immune responses by recruiting inflammatory cells. Studies have indicated that...
deteriorating performance in chickens challenged with LPS was attributed primarily to the increased release of proinflammatory cytokines such as IL-1β. Exposure of intestinal epithelia to LPS stimulated the secretion of IL-8 leading to enhanced intestinal inflammation.26 IL-6 functions as a proinflammatory and anti-inflammatory cytokine and promotes T helper (Th) 17 cell differentiation.24 IL-6 has also been reported to aid in populations of heterophils that are more capable of responding to and eliminating pathogens.23 The present study showed that splenic mRNA expression of IL-1β, IL-6, and IL-8 reached a maximum level at 1 hour immediately after LPS injection (the IL-6 mRNA was slightly higher at 2 hours than at 1 hour, but the difference was not statistically significant), which confirmed their expression at the early stage of inflammation. However, the difference between the saline and LPS treatments became smaller as time went on. Higher circulating concentrations or mRNA levels of IL-1β, IL-6, and IL-8 have been reported previously at 2 or 4 hours after LPS administration in chickens.35,6,33 Relative to the majority of the studies focusing on one endpoint, very few studies have investigated the time course of IL-1β and IL-6 in chickens inoculated with LPS. The concentration of serum IL-6 was examined chronologically (0 hours to 14 days) by Nakamura et al22 who found that its level was elevated from 1 hour to 2 days and was the highest at 3 hours after LPS administration. De Boever et al28 reported that plasma IL-6 reached its highest concentration 3 hours after LPS administration and returned to baseline levels after 9 hours in broilers. The highest mRNA level of IL-1β in the spleen was found at 2 hours after LPS injection among specified time points (0, 2, 3, 4, 6, 8, 16, and 24 hours).35 Taken together, it is presumed that LPS challenge induces rapid increases in these key inflammatory mediators over a short duration of time; however, the level of stimulation gets smaller as time goes on, indicating the birds are “adapting” or “healing” as a result of the attack on their immune system with a bacterial infection. Furthermore, the current study showed the magnitude of LPS-induced increase in the gene expression of those cytokines was highest for IL-6, followed by IL-1β and IL-8, suggesting that these 3 cytokines, especially IL-6, should be preferably considered to be biomarkers of acute inflammation.23,35 It is necessary to further investigate temporal changes in IL-10 expression due to LPS injections. In conclusion, IV administration of LPS induced an inflammatory response in the laying hens, which manifested as increased expressions of cytokines including IL-1β, IL-2, IL-6, and IL-10. Results of the present study indicated that the expression of cytokines was rapidly increased to maximal levels between 1 and 2 hours following LPS administration and then returned to baseline values by 6 hours, suggesting that the layers are adapting or offsetting the immune challenges associated with bacterial infections as time goes on. This implies that timing is a crucial aspect in the detection of inflammation in the clinical environment and the optimal time frame would be within the first few hours following LPS injection. Furthermore, the magnitude of LPS-induced increase in the transcriptional levels was highest for IL-6 among other cytokines examined, in which IFN-γ mRNA expression was significantly increased from 0 to 1 hour and the level of induction was maintained but decreased at 3 hours post-LPS injection. Consistently, several studies showed increased mRNA abundance or circulating concentrations of IFN-γ and IL-2 when broiler chickens were challenged with LPS for 2 hours.30,31 Sibben et al32 measured splenic mRNA expression of IL-2 and IFN-γ at 0, 2, 3, 4, 6, 8, 16, and 24 hours after LPS injection and found the highest transcription level of the 2 cytokines at 2 hours after injection. Collectively, it can be concluded that the optimal timing for the detection of IL-2 and IFN-γ should be in the first few hours following the administration of LPS. IL-10 acts as an anti-inflammatory cytokine, and its anti-inflammatory properties have been demonstrated in chickens. IL-10 is produced primarily by regulatory T cells and Th2 cells.39 It maintains the immune balance by inhibiting excessive production of proinflammatory cytokines such as TNF-α, IL-1β, IL-2, and IL-6; IL-10 has also been reported to affect the function of Th1 cells by inhibiting IFN-γ production.39 A recent study reported that IL-10 was detected at higher levels in the serum and higher mRNA abundance in the ileum of pigeons that were injected IP with LPS. Our results showed that injecting LPS upregulated the expression of IL-10 in the spleen. The balance between pro- and anti-inflammatory responses to LPS was therefore embodied in this study, which is crucial for maintaining homeostasis and performance of birds. Furthermore, the mRNA expression of IL-10 reached its highest level at 2 hours and then rapidly returned to baseline values at 3 hours after LPS administration, indicating that the induction of IL-10 expression by LPS occurred early and lasted for a short time. Previous studies showed the circulating concentrations of IL-10 were increased 2 hours after LPS administration in broilers. However, Echeverry et al25 recently reported that 4 hours after LPS challenge the mRNA expression of IL-10 was upregulated in chicken B cells (DT 40). Interestingly, our data showed that IL-10 mRNA expression tended to be higher at 4 hours than 3 hours or 6 hours after LPS administration. More studies are needed to further investigate temporal changes in IL-10 expression due to LPS injections.
indicating that IL-6 may serve as a distinct inflammation biomarker in layers, although more research involving larger numbers of animals is necessary. Knowledge generated from this 6-hour-window challenge may find direct clinical application in the identification of multiple inflammatory biomarkers that are needed to evaluate immune activation in chickens and provide important information for studying the effects of exogenous treatments (eg, nutrients, feed additives, immunomodulators, etc) on the immune system of the birds.

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Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org