INTRODUCTION

The use of dietary folic acid has been studied on the basis of performance in laying hens; however, previous studies have shown lack of dietary folic acid (FA) positivity and production performance (House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Dickson et al., 2010). In support of this, a 2- to 2.5-fold increase in the level of folate concentration in the eggs has been reported in laying hens supplemented with 4 mg of FA per kg of diet compared with hens fed the basal diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). In addition, investigations on the effects of FA on the level of homocysteine in laying hens have reported a decreased level of plasma homocysteine concentration with increased dietary FA supplementation (Tactacan et al., 2012). Furthermore, the role of folate in biochemical reactions is well documented (Selhub and Rosenberg, 1996), and dietary FA supplementation has also been shown to be essential for immunity and is associated with improved immunocompetence with increasing age.

ABSTRACT Folic acid plays a key role in nucleic acids and protein synthesis, and has been associated with anti-inflammatory effects in LPS-induced infections. To this end, a study was conducted to investigate the effects of dietary folic acid (FA) supplementation in older laying hens (58 to 66 wk of age) challenged with Escherichia coli lipopolysaccharide (LPS). A total of 24 Shaver White laying hens at 58 wk were fed 2 diets. The diets were wheat-soybean-based, with either 0 or 4 mg of supplemental FA per kg of diet. After 8 wk of feeding and at 66 wk, the hens were injected intravenously with 8 mg of LPS or saline per kg of BW. Four hours after injection, blood was collected and hens were euthanized to obtain spleen and cecal tonsils. The T cell subsets in the blood and the spleen (CD4+ and CD8+), total IgG, and biochemical constituents (total protein, albumin, globulin, and fibrinogen) were not influenced (P > 0.05) by dietary FA supplementation. However, LPS injection decreased (P < 0.05) biochemical constituents, CD4+, and CD8+ cells in the blood, whereas CD4+:CD8+ ratio and total IgG increased (P < 0.05), and fibrinogen was not influenced. Gene expression in the spleen and cecal tonsils was not influenced by dietary FA supplementation except a diet × challenge interaction for interleukin (IL)-8 in the spleen; IL-8 decreased in FA-fed hens that were treated with LPS. Also, FA supplementation decreased the expression of IL-8 in cecal tonsils. Relative to saline-injected hens, expression of IL-13, interferon-γ, and IL-10 increased in the LPS-injected hens in the spleen and cecal tonsils, IL-8 increased in LPS-injected hens only in the cecal tonsils, whereas Toll-like receptor 4, IL-4, IL-17, and IL-18 increased in the LPS-injected hens only in the spleen; however, LPS decreased expression of IL-13 in the cecal tonsils. In conclusion, FA did not affect inflammatory responses in older laying hens; more studies are required to investigate possible protective effects of FA in laying hens.

Key words: folic acid, Escherichia coli, lipopolysaccharide, inflammatory response, laying hen

Response of older laying hens to an Escherichia coli lipopolysaccharide challenge when fed diets with or without supplemental folic acid

P. M. Munyaka,* G. Tactacan,* M. Jing,* Karmin O,*† J. D. House,*‡ Michael St. Paul,§ Shayan Sharif,§ and J. C. Rodriguez-Lecompte*1

*Department of Animal Science, †Department of Physiology, and ‡Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2; and §Department of Pathobiology, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

INTRODUCTION

The use of dietary folic acid has been studied on the basis of performance in laying hens; however, previous studies have shown lack of dietary folic acid (FA) positivity and production performance (House et al., 2002; Hebert et al., 2005; Roth-Maier and Böhmer, 2007; Bunchasak and Kachana, 2009; Dickson et al., 2010). In support of this, a 2- to 2.5-fold increase in the level of folate concentration in the eggs has been reported in laying hens supplemented with 4 mg of FA per kg of diet compared with hens fed the basal diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). In addition, investigations on the effects of FA on the level of homocysteine in laying hens have reported a decreased level of plasma homocysteine concentration with increased dietary FA supplementation (Tactacan et al., 2012). Furthermore, the role of folate in biochemical reactions is well documented (Selhub and Rosenberg, 1996), and dietary FA supplementation has also been shown to be essential for immunity and is associated with improved immunocompetence with increasing age.

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*Corresponding author: JC_Rodriguez-Lecompte@umanitoba.ca
Inflammatory studies have also demonstrated the role of FA supplementation in preventing the age-associated decline in the production of interferon (IFN)-γ in the spleen by unstimulated cells, and the decrease in Th1/Th2-type responses after stimulation with phorbol myristate acetate and ionomycin (Field et al., 2006). Elsewhere, FA has also been shown to promote anti-inflammatory responses induced by lipopolysaccharide (LPS)-related infections (Wang et al., 2005; Feng et al., 2011).

To investigate whether the addition of FA in laying hen diet has an impact on inflammatory responses, we previously studied the immunomodulation effects of dietary FA supplementation at 4 mg/kg of diet in young laying hens (24 to 32 wk of age) under acute conditions of LPS (Munyaka et al., 2012). We reported that FA influenced some immunological parameters; however, it is not known whether dietary FA will have an impact on older laying hens challenged with LPS at a later stage of production. This study was therefore designed to investigate the effects of dietary FA on the inflammatory responses in older laying hens (58 to 66 wk of age) subjected to acute LPS challenge. The T lymphocyte subsets in the blood and the spleen, serum biochemical constituents, total IgG, and immune-related genes were examined.

**MATERIALS AND METHODS**

**General**

A total of 24 Shaver White laying hens (Manitoba Perfect Pullets, Winnipeg, Manitoba, Canada) at 58 to 66 wk of age were used in this study. Hens were placed based on their performance (≥95% egg laying) and were housed in a confined area under semi-controlled environmental conditions in individual cages (25.4 × 40.6 cm to provide 1,032 cm² space per bird) equipped with individual feeders, a nipple waterer, and a perch and were exposed to 16 h of light. Water and feed were provided ad libitum. Animal usage and care approval was received from the University of Manitoba Animal Care Protocol Review Committee, and the hens were managed in accordance with the recommendations established by the Canadian Council on Animal Care (1984).

**Experimental Design**

The hens were assigned on a study design based on a 2 × 2 factorial arrangement of main factors. The main factors were 1) diet: basal laying hen diet with no supplemental FA (n = 12) and basal laying hen diet + 4 mg/kg of supplemental FA (n = 12), and 2) immunological challenge: injection with LPS or saline. The basal diet was a wheat-soybean-based ration, formulated to meet or exceed the requirements of laying hens consuming 100 g of feed per day (NRC, 1994; Table 1). The basal diet contained 1.76 mg/kg of total folate (from natural folate in feed ingredients) per kg of diet. The birds were maintained on the dietary treatments for 8 wk (the first 2 wk served as adaptation period) after which 6 hens were randomly selected from each dietary treatment and injected intravenously with either 8 mg/kg of BW of *Escherichia coli* LPS (serotype O111:B4, Sigma Aldrich Inc., St. Louis, MO) or sterile saline. Feed was withdrawn after injection.

**Blood and Other Tissue Sampling**

Four hours after LPS or saline injection, blood samples were collected from the wing vein of each hen. Blood samples were divided into 3 aliquots (2 to 3 mL each); one 4-mL serum Vacutainer tube and two 4-mL Vacutainer tubes coated with K$_2$EDTA (BD Biosciences, Franklin Lakes, NJ). The blood samples contained in K$_2$EDTA tubes were kept on ice during collection, whereas the blood samples for serum were clotted at room temperature for approximately 2 h. Blood samples were then centrifuged at 12,000 × g for 5 min, and plasma and serum were obtained and stored at −80°C until analysis. One K$_2$EDTA tube containing blood was retained for flow cytometry analysis. After the blood collection, birds were euthanized by cervical dislocation.

**Table 1. Composition of the basal wheat/soybean-based laying hen diet**

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat (13.5% CP)</td>
<td>59.26</td>
</tr>
<tr>
<td>Soybean meal (45.8% CP)</td>
<td>22.30</td>
</tr>
<tr>
<td>Vegetable oil (8,800 kcal/kg of ME)</td>
<td>5.22</td>
</tr>
<tr>
<td>Limestone (38% Ca)</td>
<td>9.90</td>
</tr>
<tr>
<td>Biophos (21/17)</td>
<td>1.61</td>
</tr>
<tr>
<td>Vitamin premix$^1$</td>
<td>1.00</td>
</tr>
<tr>
<td>Minerals premix$^2$</td>
<td>0.50</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.14</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.06</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Nutrient composition

- ME, kcal/kg: 2,850
- CP, %: 18.5
- Calcium, %: 4.2
- Total P, %: 0.45
- Methionine, %: 0.43
- Methionine + cysteine, %: 0.80
- Lysine, %: 0.95
- Threonine, %: 0.70
- Linoleic, %: 3.23
- Folate, mg/kg: 1.76

1Provided per kilogram of diet: 11,000 IU of vitamin A, 3,000 IU of vitamin D$_3$, 20 IU of vitamin E, 3 mg of vitamin K$_3$ (as menadione), 0.02 mg of vitamin B$_12$, 6.5 mg of riboflavin, 10 mg of calcium pantothenate, 48.1 mg of nicin, 0.2 mg of biotin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1,000 mg of choline, and 125 mg of ethoxyquin (antioxidant).

2Provided per kilogram of diet: 66 mg of Mn (as manganese oxide), 70 mg of Zn (as zinc oxide), 80 mg of Fe (as ferrous sulfate), 10 mg of Cu (as copper sulfate), 0.3 mg of Na (as sodium selenite), 0.4 mg of I (as calcium iodate), and 0.67 mg of iodized salt.
and spleens were aseptically obtained and cut into 2 pieces. Cecal tonsils were also aseptically collected and together with one-half of the spleen were immediately snap frozen in liquid nitrogen for 2 min and stored at –80°C until analysis. One half of the spleen was kept on ice in 1 × PBS until extraction of splenocytes for flow cytometry analysis.

**Flow Cytometry Analysis**

The percentages of CD4+, and CD8+ cells in peripheral blood mononuclear cells and in the spleen were analyzed by flow cytometry. Mononuclear cells were isolated by gradient using Ficoll-Paque Plus (GE Health Care, Baie-d’Urfé, QC, Canada) following the manufacturer’s procedure and were prepared as described previously (Gehad et al., 2002; Shini and Kaiser, 2009) with minor modifications. Briefly, the blood from K2EDTA Vacutainer tubes was diluted 1:1 with 1 × PBS and held on ice. Each single suspension of splenocytes was prepared in 5 mL of 1 × PBS by mashing the tissue using the end of a syringe plunger through a 100-μm nylon strainer (BD Biosciences). The blood and spleen suspensions were carefully layered in a tube containing equal volume of Ficoll to form a discrete layer above the Ficoll. Tubes were centrifuged at 220 × g for 30 min at room temperature and the mononuclear layers (buffy coat) were removed, transferred to different centrifuge tubes, and washed twice in 1 × PBS. The cells were counted on a hemocytometer using trypan blue exclusion assay (Sigma Chemical Co., St. Louis, MO) and the cell suspensions were adjusted in 1 × PBS to 1 × 10⁶ viable cells per mL.

Fluorochrome conjugated monoclonal antibodies were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL), including mouse anti-chicken CD4-PE, and mouse anti-chicken CD8-PE-CY5. Using 96-well round bottom plates, 100 μL of each cell suspension was plated in duplicate. Each of the fluorochrome-labeled antibodies was added to respective wells and the stained cells were incubated for 30 min at 4°C in the dark. The cells were washed twice in 1 × PBS to remove any traces of unbound antibodies and transferred to 5-mL polystyrene round-bottom tubes for analysis. In all, 10,000 cells per sample were analyzed using a BD FACS Diva software (Becton Dickinson Immuno cytometry Systems, San Jose, CA) and evaluated using Flowjo Software (v.1.1.1, CyFlo Ltd., Turku, Finland).

**Total IgG Determination**

Serum samples obtained from the blood were used to measure the concentration of IgG antibody via sandwich ELISA using chicken IgG ELISA quantitation and Starter Accessory Kits (Bethyl Laboratories, Montgomery, TX) following the manufacturer’s procedure. A microtiter plate reader (SoftMax Pro 3.1.1, Molecular Devices, Abingdon, Oxfordshire, UK) was used to measure the absorbance at 450 nm and a 4-parameter logistic curve fit was developed using the chicken reference serum absorbance.

**Clinical Biochemistry Analysis**

Serum total protein, albumin, and globulin, and plasma fibrinogen were determined using an automated analyzer (Cell-Dyn 3500 system, Abbott Laboratories, Abbott Park, IL) at the Manitoba Veterinary Services Laboratory (Winnipeg, Manitoba, Canada).

**Total RNA Extraction and Reverse Transcription**

Total RNA was extracted from the spleen and cecal tonsil sections using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) following the manufacturer’s protocol. Briefly the ileal and cecal samples were removed from −80°C storage and they were kept on ice until completely thawed. About 80 to 100 g of tissue was added to 1 mL of ice-cold Trizol reagent (Invitrogen Canada Inc.) and homogenized with a homogenizer at full speed for about 1 min. After extraction, the pellet was left to dry for 5 min and was dissolved in 300 μL of nuclease-free water. Total RNA concentration was determined at an optical density at 260 nm and RNA purity was verified by evaluating the ratio of optical density of 260 nm to optical density at 280 nm. Total RNA was diluted to 2 μg/μL in nuclease-free water. Reverse transcription was done using the high-capacity cDNA reverse Transcription kit (Applied Bio systems, Mississauga, Ontario, Canada) following the manufacturer’s protocol, and the cDNA was stored at –20°C.

**Quantitative Real-Time PCR**

Quantitative real-time PCR was performed using the Step One Thermo Cycler (Applied Biosystems). One microliter of cDNA was added to each well of a 48-well plate. Next, 11.5 μL of real-time PCR master mix containing AmpliTaq Gold DNA Polymerase LD SYBR Green I, dNTP with dTTP/dUTP (Applied Biosystems), and nuclease-free water were added to each well to a final volume of 12.5 μL as outlined by Parvizi et al. (2009). Primer sequences for β-actin, Toll-like receptor (TLR)-4, interleukin (IL)-4, IL-8, IL-10, IL-1β, IL-13, IL-17, IL-18, and IFN-γ were obtained from GenBank (Table 2).

Primer concentrations were optimized depending on standard curve using a gene target or endogenous gene previously cloned as a control for PCR efficiency, and different thermal cycling parameters were used for each target gene. Each reaction was run in duplicate using Step One Software (Applied Biosystems).
**Quantitative Real-Time PCR Analysis**

Relative expression was calculated using Pfaffl’s formula (Pfaffl, 2001) as described previously (Parvizi et al., 2009). Briefly, relative expression ratio of all genes was calculated based on the expression of the housekeeping gene, β-actin. Absolute quantification of β-actin expression was estimated using the Step One Software (Applied Biosystems). The absolute expression of all genes tested was then normalized to the expression PCR efficiency: 

\[ E = 10^{-\frac{1}{\text{slope of standard curve}}} \]

with the gene of interest as the target and β-actin as the reference, the relative expression ratio \( R \) was determined as follows:

\[ R = \frac{(E_{\text{target}}) \Delta CP_{\text{target}} \text{(calibrator – sample)}}{E_{\text{ref}} \Delta CP_{\text{ref}} \text{(calibrator – sample)}} , \]

where \( E_{\text{target}} \) and \( E_{\text{ref}} \) are the efficiencies of the target gene and β-actin, respectively, and the \( \Delta CP \) is the difference of crossing points between calibrator and samples. The calculated \( R \) was used to determine differences in gene expression among different treatment groups.

**Statistical Analysis**

A completely randomized design with 2 dietary treatments and 2 levels of immunological challenge in a 2 × 2 factorial arrangement was used. To test for the effects of each treatment combination, values were subjected to ANOVA using the PROC GLM procedure of SAS software (SAS Institute Inc., Cary, NC). Differences between means were determined using Tukey’s procedure. Significance statements were based on \( P < 0.05 \).

### RESULTS

Among all parameters that were measured, significant diet × challenge interactions was found only for the expression of cytokine IL-8 in the spleen. All the other parameters are therefore discussed on the basis of the main effects of either LPS or FA.

**T Lymphocyte Subsets in the Blood and the Spleen**

To establish the percentages of T lymphocytes in circulation and in the spleen, CD4+ and CD8+ T cells

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**Table 2. Toll-like receptor and cytokine primer sequences**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–3′)</th>
<th>GenBank accession no.</th>
<th>Annealing temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>chTLR-4</td>
<td>F: AGTCTGAAATTGCTGAGCTCAAAT</td>
<td>AY064697</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: GCGACGTAAAGCCATGGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chIL-13</td>
<td>F: GTGAGCTTCAACATTTGCGCTGA</td>
<td>Y15006.1</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>R: TGTCAGGCAGTTGAAGATGAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chIL-4</td>
<td>F: TGTGGTCTGACAGCTCAGTG</td>
<td>AJ621249.1</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>R: ACCATGTTGAGGAAAGAACAG</td>
<td>AJ609800</td>
<td>57</td>
</tr>
<tr>
<td>chIL-8</td>
<td>F: CCAAGTGAGGACCTGTTCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCAAGGGTAGGACCTGTTCAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chIL-10</td>
<td>F: AGCAAGTCAAGGAGGACCTTC</td>
<td>AJ621614</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R: ATCAGACAGTACCTCAGATG</td>
<td>AJ62150.1</td>
<td>56</td>
</tr>
<tr>
<td>chIL-13</td>
<td>F: ACTTGTCGCAAGCTGAGAGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TCTGGACGCTGTCAGTTGTGC</td>
<td>AJ493595.1</td>
<td>60</td>
</tr>
<tr>
<td>chIL-17</td>
<td>F: GCCTGCAGCACTGGACACCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GGCTGCGTCAGCTGCTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chIL-18</td>
<td>F: GAAAGCTCATAAGAGCCATTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TCCCATGCTTCTTTCTCAAAACA</td>
<td>NM204608.1</td>
<td>53</td>
</tr>
<tr>
<td>chIFN-γ</td>
<td>F: CTGAAAGACTGGAACAGAGAG</td>
<td>X99774</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: CACGACCTTCTGTAAGATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chβ-Actin</td>
<td>F: CAACACAAGCTGCTGTGGTCTGA</td>
<td>X00182</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R: ATCAGACAGTACCTCAGATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1F = forward; R = reverse.
were measured. Supplementation with dietary FA did not influence the percentage of T cell subsets in the blood as well as in the spleen (P > 0.05). Injection with LPS significantly influenced the amount of T cells in circulation, but no significant effect was found on the level of T cells in the spleen. Compared with the saline group, LPS-injected hens had lower percentages of CD4+ and CD8+ T cells (P = 0.0004, and P = 0.0003 respectively); however, the ratio of CD4+:CD8+ significantly increased following LPS challenge (P = 0.0376) (Table 3).

**Total IgG**

The amount of total IgG was not influenced by dietary FA supplementation; however, relative to the hens that were injected with saline, the LPS-injected hens had higher amount of IgG (P = 0.0054; Figure 1).

**Clinical Biochemistry**

The levels of total protein, albumin, globulin, as well as the albumin:globulin ratio in serum, were not influenced by dietary FA supplementation. However, there was a significant reduction in the levels of total protein (P = 0.0006), albumin (P < 0.0001), globulin (P = 0.0030), and albumin:globulin ratio (P = 0.0049) in the LPS-treated group compared with the saline group. No significant difference was found on the level of fibrinogen among the treatment groups (Table 4).

### Table 3. The T cell subsets (%) in the blood and the spleen of older laying hens (66 wk of age) fed diets with or without dietary folic acid (FA) for 8 wk and at 4 h postinjection with 8 mg of lipopolysaccharide (LPS) or saline per kilogram of BW1

<table>
<thead>
<tr>
<th>Item</th>
<th>0 mg of folic acid</th>
<th>4 mg of folic acid</th>
<th>SEM</th>
<th>Diet</th>
<th>Challenge</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD8−</td>
<td>10</td>
<td>3.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8+ CD4−</td>
<td>8.1</td>
<td>1.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>1.4</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ CD8−</td>
<td>11</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8+ CD4−</td>
<td>35.5</td>
<td>37.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>0.31</td>
<td>0.26</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

1Data presented as least squares means.
2PBMC = peripheral blood mononuclear cells.

### Gene Expression in the Spleen and the Cecal Tonsils

Dietary FA supplementation did not influence gene expression in the spleen and the cecal tonsils except the expression of IL-8. There was a diet × challenge interaction for IL-8 expression only in the spleen. Expression of IL-8 in the spleen was found to be higher in the FA-supplemented hens that were treated with LPS (P < 0.05). Supplementation with FA also reduced the expression of IL-8 in the cecal tonsils compared with the nonsupplemented hens (P < 0.05); however, expression of IL-8 in the cecal tonsils was significantly higher in the LPS-injected hens compared with the control (P < 0.05; Figure 2). Expression of IL-13, IL-10, and IFN-γ in the spleen and the cecal tonsils was higher in the LPS-injected hens compared with the control, whereas the expression of IL-4, IL-13, IL-17, IL-18, and TLR-4 was significantly higher in the LPS-injected hens only in the spleen; however, the expression of IL-13 in the cecal tonsils decreased in LPS-injected hens (P < 0.05; Figure 2).

**DISCUSSION**

Our aim in this study was to examine the effects of supplementing dietary FA on inflammatory changes and responses in older laying hens (58 to 66 wk of age) challenged with *Escherichia coli* lipopolysaccharide. In our previous work with young laying hens (24 to 32 wk...
Figure 2. Gene expression in the spleens and cecal tonsils of older laying hens (66 wk of age) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h postinjection with 8 mg of lipopolysaccharide (LPS) or saline per kilogram of BW. Gene expression was assessed using quantitative real-time PCR and was calculated relative to the housekeeping gene β-actin. Graphed values are expressed as relative expression. Error bars represent SE. Bars with asterisks show significant \( P < 0.05 \) FA × LPS interactions; letters (a,b) show significant main effects of LPS; and symbols (§,#) show a significant main effect of FA. TLR = Toll-like receptor; IL = interleukin; IFN = interferon.
of age), we found that under acute conditions of LPS, dietary FA supplementation at 4 mg/kg of diet enhanced the levels of total proteins, albumin, globulins, and total IgG, as well as influencing the expression of some genes associated with pro- and antiinflammatory responses. However, FA did not alter the level of T cells in circulation and in the spleen (Munyaka et al., 2012). We therefore surmised that dietary FA supplementation at this level may elicit similar benefits in older laying hens, as observed in young hens, or may be more beneficial with advancing age, thus counteracting the age-associated decline in immune competence.

As previously reported in old mice supplemented with FA (35.7 mg/kg) for 3 wk (Field et al., 2006), the percentages of T cell subsets (CD4+ and CD8+) in the blood and spleen were not influenced by dietary FA supplementation in this study. This may imply that the duration of supplementation that was used in our study and the other study could have been insufficient to elicit noticeable changes because previous studies have demonstrated a slow rate of FA uptake by cells in vivo (Dhur et al., 1991; Kim et al., 2002). Alternatively, although in this study the hens were used at their late stages of production cycle, biologically they may not be considered old and this may partially explain this observation; however, other factors still not considered in our study may be responsible. In addition, the lack of a significant effect in the spleen following LPS challenge has also been reported previously in chickens (Gehad et al., 2002), which could be partly explained by the general resistance of chickens to LPS (Adler and DaM- assa, 1979) or the duration of LPS exposure that was employed in this study. On the other hand, a reduction of T cell subsets in circulation following injection with LPS could be due to LPS toxicity (Gehad et al., 2002; Shini et al., 2008), or induced cell death of T cell subsets and B cells (Richardson et al., 1989; Hotchkiss et al., 1999). However, migration of immune cells from the blood to other immune-related tissues and organs to participate in immune responses could also cause changes in the T cell population in the blood (Nii et al., 2011). A higher CD4+ to CD8+ ratio is associated with improved immunocompetence in chickens (Bridle et al., 2006). In addition, the CD4+ to CD8+ ratio reflects changes in T cell populations evaluated together at a particular time point and can therefore serve as a good indicator of changes in the T cell subsets (Holt et al., 2010). Therefore, the increased CD4+ to CD8+ ratio observed in the blood in this study following LPS challenge could be an indicator of higher amount of CD4+ cells, as well as improved immunocompetence.

Toll-like receptor 4 recognizes LPS of gram-negative bacteria such as E. coli and Salmonella and contributes to the initiation of inflammatory responses mediated by proinflammatory cytokines (Kogut et al., 2005). In laying hens, a tissue- and time-dependent expression of TLR-4 in the ovarian follicles and other tissues has been reported in response to LPS challenge (Subedi et al., 2007). The upregulation of TLR-4 may imply a robust inflammatory response and could be associated with the upregulation of IL-13 and IL-8, which was observed in the present study. Interleukin-13, a proinflammatory cytokine, is involved in the stimulation of T cells and macrophages, induction of fever, triggering of acute-phase responses, and activation of the vascular endothelium (Lotz et al., 1988; Diehl et al., 2000; Corwin, 2000); however, most of these activities are also mediated by other proinflammatory cytokines and chemokines (Staeheli et al., 2001). Interleukin-17 is produced by activated and memory T cells and, like IL-13, induces the production of other proinflammatory cytokines (Hong et al., 2006). Interleukin-18, also a proinflammatory cytokine is produced by macrophages, operates in conjunction with IL-12 to induce a cell-mediated immune response following pathogenic insult (Corwin, 2000; Cox et al., 2010). It is mainly involved in the differentiation of Th1-like cells, which in turn secrete IFN-γ that plays an essential role in activating macrophages as well as in regulating the innate and adaptive immune responses (Göbel et al., 2003; Cox et al., 2010). Upregulation of IL-18 observed in this study could therefore be correlated with the increased level of IFN-γ, suggesting stimulation of both innate and cell-mediated immune responses.

Interleukin-8, also known as CXCLi2, is a chemokine produced mainly by macrophages and is an important mediator of the innate immune response (Cox et al., 2010). Its primary role involves acting as a chemoattractant that induces the migration of heterophils (Cox et al., 2010; Redmond et al., 2011), monocytes (Barker et al., 1993), and CD3+ T cells (Min et al., 2001) to the site of inflammation or infection in birds. Increased production of CXCLI2 along with other inflammatory signals by heterophils of some broiler genetic lines is associated with enhanced immune response to Salmonella Enteritidis (Ferro et al., 2004; Swaggerty et al., 2004; Chiang et al., 2008). In this study, dietary FA enhanced expression of IL-8 in the spleen, however, there was a downregulation in the expression of IL-8 in the cecal tonsils, indicating a pleiotropic effect of FA in older laying hens under acute LPS challenge.

Interleukin-4 is a Th2-like cytokine that plays a crucial role in the stimulation of B lymphocytes, proliferation of T lymphocytes, and the differentiation of CD4+ Th cells into Th2 cells (Fietta and Delsante, 2009; Cox et al., 2010). However, the functions of IL-13, also characterized as a Th2 cytokine, overlap considerably with those of IL-4 (Cox et al., 2010). Increased levels of IL-4 and IL-13 in the spleen following LPS injection may suggest enhanced Th2-like adaptive immune responses, although this was not observed in the cecal tonsils. Regulatory IL-10 is also a Th2 cytokine that serves to keep immune responses in check by inhibiting IFN-γ cytokine production by Th1 cells, and downregulating the expression of the major histocompatibility antigens expressed by immune cells (Corwin, 2000; Min et al.,


REFERENCES


