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Kinetic studies on clinical and immunological modulations by intramuscular injection of *Escherichia coli* LPS in laying hens

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Abstract

The present study investigated clinical and immunological modulations due to intramuscular injection of *Escherichia coli* LPS in 49-wk-old laying hens over 48 h post injection (p.i.). LPS induced characteristic sickness behavior but no significant body temperature alterations ($P > 0.05$). During experimental period decreases in blood albumin, calcium, phosphorus and tryptophan concentrations, hyperglycemia, increased plasma nitrite concentrations, leucopenia, decreased thrombocyte counts, lymphopenia, heterophilia and an increased heterophilic granulocyte/lymphocyte (H/L) ratio were observed after LPS administration. Time-dependent effects were shown on T and B cell subsets in caecal tonsils (CT) and on splenic $CD3^+/CD4^+/CD8^+$ proportions, on IL-1β and -10 and inducible NO synthase mRNA expression in peripheral blood lymphocytes (PBL), liver, spleen and CT, and on the mRNA expression of the TLR4 in PBL, liver and spleen p.i. ($P < 0.05$). The main responding period of mentioned alterations due to LPS appears to include the period from 2 until 8 h p.i. According to the H/L ratio, the most stressful phase was 5 h p.i. T and B cell subsets in CT, the IL-1β and TLR4 mRNA expression in liver and plasma nitrite concentrations seemed to be affected for a longer period.

Keywords

Acute phase response, cytokines, laying hens, lipopolysaccharides, T and B cells

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Introduction

Bacterial LPS, which are present in the cell envelopes of Gram-negative bacteria, are known as potent stimulators of the innate immune system and inducers of inflammation and sickness behavior in mammals, but also in chickens.[1] For some time, LPS has attracted less attention in poultry studies because there was a dominant view that, compared with mammals, poultry are more resistant to these chemical compounds. Today, it is known that there are some similarities in the response of mammals and poultry to LPS, and they are used in several studies and bacterial infection models with chicken as an immune stimulator and inducer of the acute phase response.[2–5] However, previous studies also revealed some differences in the acute phase response induced by LPS, especially between positive and negative acute phase reactants.[3]

In previous studies dealing with the effect of LPS on chicken, investigations were conducted at large intervals, or at selective time points, which could hide crucial time points for LPS responses and impede the

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comparability of results. Especially, the period immediately after LPS injection has received less attention in the past, although clinical symptoms induced by LPS injection are observed already within 1 h post administration.[4,6] Therefore, the present study aimed to investigate the effects of LPS on clinical and immunological traits of laying hens at different short time intervals from 0.5 h to 48 h after LPS administration in comparison to a physiological value.

Materials and methods

The present experiment was performed in accordance with the guidelines of the German animal protection law on the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health in Brunswick, Germany. The experimental conditions and procedures were approved by the responsible authority, Lower Saxony State Office for Consumer Protection and Food Safety, LAVES, Germany (registration number: 33.92-42502-04-13/1186).

Animals and husbandry

The present study was performed with 36 49-wk-old laying hens (7th laying mo) (Lohmann Brown). Before the beginning of the study, the birds were housed in groups under conventional conditions in floor pens. Birds had an average initial body mass (BM) of 1.9 kg, and an initial laying intensity of 93.4%. The initial average egg mass was 63.0 g. From wk 49 onwards, the animals were kept in single-cages (42 cm × 35 cm × 42 cm). The cages were integrated in a climate controlled room with a constant temperature of 18°C. The illumination period lasted from 4.00 a.m. to 8.00 p.m. (16 h light: 8 h dark). Each cage was equipped with a feeding and a water trough, which enable an *ad libitum* access to feed and water throughout the entire experimental period. Hens were fed a commercial grain-soybean meal diet for high-performing laying hens.

Experimental design

After changing of the housing conditions, a 3-d adaptation period was granted. On d 4, at the start of the experimental day (6.30 a.m.), the general conditions of the animals were assessed by a previously described scoring system according to their body posture (0 = normal – 4 = lateral position), behavior (0 = normal – 4 = comatose), plumage (0 = normal – 4 = feather loss, initiating alopecia), respiratory rate (0 = normal – dyspnea, beak respiration) and color of wattles, combs, conjunctiva and legs (0 = normal – 4 = necrotic).[6] Furthermore, the animals were weighed, and rectal body temperature was measured. Initially (7.15 a.m.), three animals were slaughtered by mechanical stunning and exsanguination. At 8.00 a.m. the remaining 33 birds were treated intramuscularly (*Musculus pectoralis*) with 2 mg LPS (from *Escherichia coli* O111:B4; Merck KgaA, Darmstadt, Germany) per kg BM. At 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24 and 48 h post injection (p.i.) three animals per time point were slaughtered as described above. Approximately 15 min before slaughtering, the general conditions were assessed by the above described scoring system. BM and rectal body temperature were measured immediately before slaughtering.

Sample collection

During slaughtering, blood samples were collected into commercial serum and heparin tubes. Heart, liver with bile bladder, kidneys, spleen, and caecal tonsils (CT) were dissected and weighed. The spleen was cut into two pieces. One section of the spleen and the right CT (only at time points 0, 2, 4, 6, 8, 12, 24 and 48 h p.i.) were stored in PBS on ice until further analyses. At the time points 0, 2, 4, 6, 8, 12, 24 and 48 h p.i. liver, the remaining spleen section and the left CT were snap-frozen in liquid nitrogen and stored at –80°C.

Blood analyses

Heparinized blood was collected in a micro-hematocrit tube and centrifuged at 12,000 *g* for 5 min by a hematocrit (HCT) centrifuge (Haematokrit 210; Hettich Lab Technology, Tuttlingen, Germany) for determination of HCT.

According to the recommendations of Pendl, two blood smears per bird were prepared, stained by Pappenheim solution and subsequently counted.[7] Leucocytes and thrombocytes were counted in 20 fields of vision, and at least 200 leucocytes were differentiated according to their morphological characteristics at 1000 × magnification.

Albumin, total protein, glucose (Glc), cholesterol, γ-glutamyltranferase, bilirubin, triglycerides, urea, β-hydroxybutyrate and non-esterified fatty acids were analyzed in serum blood samples by an automatic clinical chemistry analyzer (Eurolyser CCA180; Eurolyser Diagnostica GmbH, Salzburg, Austria). Tryptophan, nicotinamide and kynurenine were measured in serum by HPLC (Shimadzu, Kyoto, Japan) according to methods described by Hüther et al.[8] Calcium and phosphorus concentrations in plasma were determined spectrophotometrically (Unicam UV/Vis Spectrometer UV4; Unicam, Kassel, Germany) using commercial kits of Greiner Diagnostic GmbH (Bahlingen, Germany).

Ferric acid reducing ability of plasma (FRAP) was determined according to the methods of Benzie and

Strain.[9] In brief, 300 µl warm FRAP reagent (37°C) containing acetate buffer (on base of sodium acetate trihydrate (Merck KGaK; Darmstadt, Germany), acetic acid and distilled water), iron-chloride-hexahydrate (Merck KGaK; Darmstadt, Germany) and 2,4,6- tripyridyl-striazine (TPTZ; Merck KGaK; Darmstadt, Germany) were added to wells of a microtiter plate containing 10 µl of the plasma sample and 30 µl H_2O. The absorbance change (ΔA) was measured at a wavelength of 593 nm every 36 s for 15 min by a microplate reader (Infinite® M200; Tecan Trading AG, Männedorf, Switzerland).

The concentration of nitrite in plasma was analyzed by using the Griess reaction and a Cayman Assay kit (Cayman Chemical; Ann Abor, MI). The absorbance was measured at a wavelength of 540 nm by a microplate reader also used in the FRAP analyses.

PBL isolation

Initially, PBLs were isolated by different wash steps and the use of Biocoll separation solution (Biochrom AG, Berlin, Germany) according to methods described by Liermann et al.[10] Part of the received cell solution was used for flow cytometric analyses. The remaining part of the cell suspension was centrifuged at 250 *g* for 5 min at 4°C. The pellet was frozen and stored at –80°C until further analyses.

Isolation of leucocytes from spleen and CT

Spleen and the right CT were rinsed, and surrounding tissues were cut. Leucocytes were isolated according to methods of Liermann et al.[10] Briefly, tissues were transferred into a Petri dish containing 5 ml PBS and cut lengthwise. Cells were released by gentle scraping. The cell suspension was sieved using CellTrics® (mash size 50 µm) (Partec GmbH, Görlitz, Germany). While the cell suspension of caecal tonsils contained almost no erythrocytes, the cell suspension of spleen cells have to undergo an additional lysis step. Therefore, 1000 µl of the cell suspension was re-suspended in 1000 µl of an ammonium chloride lysis buffer containing potassium bicarbonate and EDTA (pH 7.4) and centrifuged at 250 *g* for 5 min at 4°C. The samples of caecal tonsils and spleen were subsequently washed by Roswell Park Memorial Institute medium (RPMI-1640; Biochrom GmbH, Berlin, Germany) and centrifugation at 250 *g* for 5 min at 4°C. The pellet was re-suspended in RPMI-1640 and stored on ice until flow cytometric measurements.

Flow cytometric measurements

Isolated PBLs and cells isolated from spleen and CT were incubated with mAbs against CD3 (T cells) (mouse anti-chicken CD3: PE; Southern Biotech; Birmingham, AL), CD4 (T helper cells) (mouse anti-chicken CD4: FITC; Southern Biotech) and CD8 (T cytotoxic cells) (mouse anti-chicken CD8: Cy5; Southern Biotech) for 30 min at room temperature in the dark. Further samples were incubated with an Ab against Bu1 (B cells) (mouse anti-chicken Bu-1: FITC; Southern Biotech) under conditions similar to those described above. Samples intended for corresponding isotype controls were incubated with either mouse IgG1 negative control: PE; mouse IgG1 negative control: FITC or mouse IgG1 negative control: Cy5 (Southern Biotech). Thereafter, 1000 µl HEPES-buffered saline was added and the samples were centrifuged at 250 *g* for 5 min at 4°C. Subsequently, the T and B cell subsets were measured by FACS Canto II (BD Bioscience, San Jose, CA). At least 10,000 cells were counted. The BD FACSDiva™ Software (BD Biosciences) was used to evaluate results and compensate non-specific signals indicated by isotype controls.

Gene expression analyses

The methods for the extraction of RNA from the PBL cell pellet and tissues, cDNA synthesis and quantitative Real-Time PCR were described by Bühler et al.[11] and Drong et al.[12]

Briefly, extraction of total RNA was performed according to the manufacturer's protocol using a chaotropic ions buffer system and a silica membrane (NucleoSpin® RNA II; Macherey Nagel, Düren, Germany). Contaminating DNA was digested on-column. RNA extracted from PBLs and tissues was eluted with H_2O. A Nano Drop® ND-1000 (NanoDrop, Wilmington, DE) was used to assess RNA concentration and quality. The integrity of the RNA was verified by 1.1% agarose gel electrophoresis. RNA was stored at –80°C until further analyses.

A total of 1000 ng RNA was reverse-transcribed into cDNA using a qScript™ cDNA Synthesis Kit (Quanta Biosciences™, Inc., Gaithersburg, MD) in a thermal cycler (Biometra GmbH, Göttingen, Germany). For subsequent analysis, the cDNA thus obtained was diluted with H_2O and frozen in aliquots at –20°C.

Gene-specific primer pairs were designed using Primer-BLAST and are summarized in Table 1.

The quantitative Real-Time PCR was conducted in duplicate on a CFX96™ Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA) using 5 µl of cDNA (corresponding to 25 ng RNA) and a iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in a final volume of 15 µl. After an initial denaturation step at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s and annealing as well as elongation for 30 s at 60°C

Table 1. Characteristics of primers used in real-time PCR.

Gene	Forward primer (fw) (5′-3′) Reverse primer (rev) (5′-3′)	PCR product size (bp)	Accession number
Reference genes			
ACTB	ATGATATTGCTGCGCTCGTTG CATACCAACCATCACACCCTGA	134	NM_205518.1
H6PD	GGACCACTACCTTGGCAAACA AACTGGTGCGGCCTTTAGCA	152	XM_425746.5
RPL4	GAGTGACTACAACCTGCCGA TTTCAGGACTCTGCGGTGAA	124	NM_001007479.1
GAPDH	CCGTCCTCTCTGGCAAAGTC ACAGTGCCCTTGAAGTGTCC	115	NM_204305.1
TBP	CTCTGGGATAGTGCCACAGC AGCAGCAAAACGCTTGGGAT	124	NM_205103.1
HMBS	TGGCTGTAGTGTCCCTGTTG GGTCCATCTTCATTCCGGTGT	150	XM_417846.5
Target genes			
IL1	TGCCTGCAGAAGAAGCCTCG CTCCGCAGCAGTTTGGTCAT	137	NM_204524.1
IL10	GCTGAGGGTGAAGTTTGAGGAA AGACTGGCAGCCAAAGGTC	121	NM_001004414.2
TLR4	CTGGATCTTTCAAGGTGCCACA GTGCTGGAGTGAATTGGCAG	102	NM_001030693.1
NOS2	TCTCCACTTCTGTTTCCAGGC TGTGTGATGTGGGAACGCTT	89	NM_204961.1

followed. After the last amplification cycle, a melting curve analysis from 60°C to 95°C in 0.5°C increments was performed to ensure generation of single PCR products. C_q-Values of target and reference genes were obtained by the CFX Manager™ Software 3.1 (Bio-Rad). The genes β-actin (ACTB), GAPDH, hexose-6-phosphate dehydrogenase/Glc 1-dehydrogenase (H6PD) and ribosomal protein L4 (RPL4) exhibiting a mean reference target stability M value of 0.85 were identified as the optimal reference genes in PBLs by the qBase$^+$ software 2.5 (Biogazelle NV, Zwijnaarde, Belgium) (Table 1). The optimal reference genes in liver and spleen were RPL4, GAPDH and TATA-box binding protein (TBP) (mean reference target stability M value liver = 0.29; spleen = 0.27) and in CT RPL4, hydroxymethylbilane synthase (HMBS) and TBP (mean reference target stability M value = 0.23).

Calculations and statistical analyses

The sum of cells of counted fields of vision of a blood smear was multiplied by the factor 875 according to recommendations of Pendl[7] to calculate the total counts of leucocytes and thrombocytes. All animals had a HCT lower than 35%; therefore, cell counts had to be corrected by the following equation:[7]

Corrected cell counts [cells/µl blood] = Σ of cells of 20 fields of vision × 87 5× HCT [%]/45%.

The H/L ratio was calculated by dividing heterophilic granulocytes by lymphocytes counted in a blood smear.

For statistical analyses, variance analysis of the SAS Enterprise guide 6.1 was used, which considered the fixed effect "time". Time-dependent effects and differences between parameters estimated at various time points after LPS administration and the physiological value before LPS injection were assessed as statistically significant at $P < 0.05$. The SAS Enterprise Guide 6.1 was also used to estimate Pearson correlation coefficient; r. Correlations were assessed as significant at P values < 0.05.

Results

Clinical symptoms and body temperature

First noticeable clinical symptoms were observed at 0.45 h p.i., as indicated by changes in behaviour such as reduced activity and slightly or moderately ruffled plumage. One animal showed slight alterations of wattles and comb color. Alterations in behaviour, activity, plumage and wattles and comb color rapidly exacerbated within 1.45 h p.i. and persisted until 5.45 h p.i. Additionally, at 3.45 h p.i. some animals showed alterations in respiratory rate. After 6.45 h p.i. the clinical symptoms began to regress. Alterations in the respiratory rate were no longer apparent from

7.45 h p.i. At 8 h p.i. almost all clinical symptoms were completely reversed. At no time of the experimental period changes in the color of conjunctivae or legs were observed. No premature animal losses occurred during the experiment.

The rectal body temperature varied between the minimum of 40.3°C and the maximum of 41.4°C. There were no significant differences in the rectal body temperature of animals slaughtered before LPS injection and animals slaughtered at different time points after LPS injection ($P > 0.05$). Only 0.5 h p.i. the body temperature (mean = 40.8°C ± 0.1) seemed to be slightly reduced compared with animals slaughtered before LPS injection (mean = 41.2°C ± 0.1).

BM and visceral organs

BM at the beginning of the experiment (data not shown) and immediately before slaughtering (Table 2) did not differ between animals slaughtered before or at various time points after LPS injection ($P > 0.05$). Furthermore, there were no significant differences between visceral organ masses of animals slaughtered before LPS injection and animals slaughtered at various time points after LPS injection. However, a time-dependent effect was detected for the masses of spleen ($P = 0.018$).

Clinical chemistry, calcium, phosphorus, tryptophan, kynurenine and niacin

During the experiment, a time-dependent effect on albumin and Glc was observed ($P < 0.05$) (Table 3). The concentration of albumin increased numerically from 0 until 0.5 h p.i. and decreased numerically thereafter until 5 h p.i. At 48 h p.i. the albumin concentration was numerically higher compared with the concentration measured in animals slaughtered before LPS injection. Animals slaughtered 3, 4, 5 and 6 h p.i. showed significantly higher Glc concentration in blood serum than animals slaughtered before LPS injection ($P < 0.05$). The highest concentration of Glc was measured 6 h p.i. No effect of time was observed on other parameters of clinical chemistry.

A time-dependent effect was detected on blood concentrations of calcium, phosphorus and tryptophan ($P < 0.05$) but not on blood niacin concentrations ($P > 0.05$) (Table 4). Significant differences between these concentrations in animals slaughtered before or after LPS injection were not observed. However, calcium concentrations of animals slaughtered 2 h p.i. were significantly higher compared with concentrations of animals slaughtered 24 h p.i. There was a significant positive correlation between calcium concentrations and the blood albumin concentrations ($r = 0.522$, $P = 0.001$). Animals slaughtered 12 h p.i. showed significantly higher concentrations of phosphorus compared with animals slaughtered 5 and 48 h p.i. Compared with animals slaughtered before LPS injection animals slaughtered at 2, 3, 4, 5, 6, 8, 24 and 48 h p.i. had only numerically but markedly lower phosphorus concentrations in blood. The tryptophan concentrations were slightly increased at 0.5 h p.i. Thereafter, the concentrations decreased significantly from 2 h until 5 h p.i. After 6 h p.i. the concentrations increased once again. The concentrations of tryptophan differed no longer from values measured 0.5 h p.i. from 24 h p.i.

Table 2. BM and various visceral organs after LPS injection (means; $n = 3$).

Time after LPS injection (h)	BM (kg)	Heart (g/kg BM)	Liver (g/kg BM)	Kidney (g/kg BM)	Spleen (g/kg BM)	Caecal tonsils (g/kg BM)
0	1.8	4.1	22.9	5.6	0.8	0.3
0.5	2.0	3.9	21.6	5.6	0.9	n.m.[a]
1	1.9	4.1	23.8	5.8	1.0	n.m.
2	1.8	3.9	23.8	6.2	0.8	0.4
3	1.8	3.6	21.0	6.2	0.8	n.m.
4	1.8	4.2	21.0	7.2	0.9	0.3
5	1.8	4.0	21.6	6.6	1.0	n.m.
6	1.7	3.6	21.4	6.9	0.7	0.3
8	1.8	4.0	20.7	6.7	1.2	0.3
12	1.8	3.5	22.2	6.3	1.2	0.3
24	1.9	4.2	18.1	6.7	0.9	0.3
48	1.6	4.2	18.7	6.1	1.2	0.3
P Value	0.201	0.859	0.207	0.601	0.018	0.814
PSEM	0.1	0.4	1.4	0.6	0.1	0.04

BM: body mass measured immediately before slaughtering; PSEM: combined standard error of means.
[a]Not measured.

Table 3. Traits of clinical chemistry after LPS injection (means; $n = 3$).

Time after LPS injection (h)	Albumin (g/l)	Globulin (g/l)	Albumin/ globulin ratio	Total protein (g/l)	Glc (mg/dl)	Cholesterol (mg/dl)	γ-GT (U/l)	Bilirubin (mg/dl)	Triglycerides (mg/dl)	Uric acid (mg/dl)
0	21.5	23.4	0.99	44.8	227.2^{c}	122.0	28.6	<d.l.	631.0	3.82
0.5	22.8	22.8	1.00	45.5	280.8^{abc}	108.3	23.8	0.10	460.2	3.90
1	22.6	29.7	0.98	52.4	282.6^{abc}	96.1	26.7	0.13	613.0	2.53
2	20.7	17.8	1.18	38.5	259.0^{abc}	94.0	27.5	0.13	1027.3	2.07
3	18.7	38.4	0.93	57.1	293.0^{ab}	89.1	23.5	0.17	781.1	2.13
4	18.7	25.0	1.04	43.7	298.9^{ab}	87.6	23.9	0.16	1316.1	2.75
5	18.6	11.7	1.60	30.3	297.6^{ab}	79.5	23.0	0.17	1073.9	3.55
6	18.8	20.8	1.00	39.6	311.0^{a}	86.5	26.1	0.15	1532.1	4.84
8	18.8	15.7	1.22	34.5	276.9^{abc}	94.0	23.9	0.17	1322.6	4.83
12	21.5	18.3	1.20	39.9	260.3^{abc}	90.2	20.8	0.12	1008.4	5.49
24	20.0	18.3	1.09	38.3	237.8^{bc}	68.5	24.5	0.12	847.0	4.31
48	24.4	23.4	1.08	47.8	249.5^{abc}	63.7	21.9	0.10	926.3	6.09
P Value	0.032	0.753	0.615	0.726	0.002	0.690	0.810	0.572	0.308	0.418
PSEM	1.3	8.5	0.20	8.9	12.8	18.2	3.0	0.03	286.3	1.27

γ-GT: γ-glutamyltransferase; PSEM: combined standard error of means; d. l.: detection limit.
[a,b,c]Different superscripts mark significant differences between time points within a column ($P < 0.05$).

Table 4. Blood concentrations of calcium, phosphorus, tryptophan and niacin after LPS injection (means; $n = 3$).

Time after LPS injection (h)	Calcium (mg/dl)	Phosphorus (mg/dl)	Tryptophan (μg/ml)	Niacin (μg/ml)
0	18.1^{ab}	5.2^{ab}	9.4^{abcd}	0.70
0.5	17.5^{ab}	5.9^{ab}	13.5^{a}	0.81
1	17.7^{ab}	5.1^{ab}	9.0^{abcd}	0.63
2	18.7^{a}	3.7^{ab}	7.5^{cd}	0.64
3	16.8^{ab}	4.4^{ab}	6.3^{d}	0.64
4	17.3^{ab}	3.3^{ab}	5.6^{d}	0.67
5	16.4^{ab}	2.7^{b}	5.5^{d}	0.54
6	15.9^{ab}	3.8^{ab}	5.8^{d}	0.57
8	15.9^{ab}	3.7^{ab}	6.7^{d}	0.72
12	15.7^{ab}	6.9^{a}	8.3^{bcd}	0.51
24	14.7^{b}	3.7^{ab}	12.6^{ab}	0.53
48	16.9^{ab}	2.5^{b}	11.8^{abc}	0.85
P Value	0.034	0.013	< 0.001	0.318
PSEM	0.7	0.8	1.0	0.10

PSEM: combined standard error of means.
[a,b,c,d]Different superscripts mark significant differences between time points within a column ($P < 0.05$).

Kynurenine concentrations in blood were less than or equal to the detection limit of 0.04 μg/ml in 31 out of 36 animals. The other animals showed blood concentrations between 0.06 and 0.09 μg/ml (data not shown).

Hematology

There was a significant effect of time on the HCT value ($P < 0.001$) (Table 5). Immediately after LPS injection (0.5 h), HCT increased numerically and decreased thereafter until 4 h p.i. At 48 h p.i. the HCT increased significantly once again ($P < 0.05$). There were no significant differences between HCT values of animals treated with LPS and animals without LPS treatment. However, the HCT values of animals slaughtered at 0.5 and 48 h p.i. were significantly higher compared with animals slaughtered between 2 and 24 h p.i.

A time-dependent effect was also observed on total counts of leucocytes and thrombocytes as well as on differentiated white blood cell populations ($P < 0.05$).

While total counts of thrombocytes of animals slaughtered before LPS injection and animals slaughtered after LPS injection did not differ significantly ($P > 0.05$), total counts of leucocytes were significantly decreased in animals slaughtered 2, 3, 4 and 12 h p.i. compared with animals slaughtered before LPS injection ($P < 0.05$).

The proportions of lymphocytes increased numerically 0.5 h p.i. and decreased thereafter until 5 h p.i. before they increased once again. The proportions of lymphocytes were significantly lower in animals slaughtered between 3 and 12 h p.i. compared with the animals slaughtered 0 h p.i. ($P < 0.05$). The proportions of heterophilic granulocytes showed an opposite development compared with lymphocyte populations. Initially, proportions of this cell population decreased significantly compared with animals slaughtered before (0.5 h p.i., $P = 0.026$) and increased thereafter until 5 h p.i. From 3 h p.i. onwards, the proportions of heterophilic lymphocytes were significantly higher than proportions determined before LPS injection ($P = 0.01$). After 5 h p.i. these proportions decreased until the end of the experiment. From 24 h p.i. on,

Table 5. Hematocrit (HCT) and blood cell counts after LPS injection (means; $n = 3$).

Time after LPS injection (h)	HCT (%)	Leucocytes (cells/μl)	Thrombocytes (cells/μl)	Lymphocytes (%)	Heterophilic granulocytes (%)	H/L ratio	Eosinophilic granulocytes (%)	Basophilic granulocytes [%]	Monocytes [%]
0	25.9^{ab}	27361^{ab}	10882	65.0^{ab}	32.2^{ef}	0.50^{d}	0.5^{cd}	2.0^{c}	0.7^{abc}
0.5	29.7^{a}	13788^{bcd}	12707	81.3^{a}	6.8^{g}	0.09^{d}	4.7^{a}	6.5^{ab}	0.7^{abc}
1	27.5^{ab}	16906^{abcd}	5321	68.5^{ab}	20.5^{efg}	0.32^{d}	2.3^{b}	6.8^{a}	1.8^{ab}
2	25.1^{b}	4524^{d}	8502	73.7^{ab}	20.0^{efg}	0.29^{d}	0.7^{cd}	3.7^{abc}	2.0^{a}
3	24.4^{b}	3388^{d}	3289	31.7^{de}	65.2^{bcd}	2.31^{cd}	0.7^{cd}	1.7^{c}	0.8^{abc}
4	23.4^{b}	7886^{cd}	2140	9.7^{ef}	88.5^{ab}	11.31^{ab}	0.2^{d}	1.3^{c}	0.3^{bc}
5	25.4^{b}	20352^{abc}	3525	6.3^{f}	92.2^{a}	16.81^{a}	0.0^{d}	0.5^{c}	1.0^{abc}
6	23.6^{b}	22245^{abc}	7402	13.0^{ef}	85.0^{ab}	7.55^{bc}	0.2^{d}	1.3^{c}	0.5^{abc}
8	24.8^{b}	17587^{abcd}	8015	24.3^{def}	73.8^{abc}	3.48^{cd}	0.5^{cd}	0.8^{c}	0.5^{abc}
12	26.3^{b}	11669^{cd}	3962	39.0^{cd}	59.3^{cd}	2.85^{cd}	0.7^{cd}	0.8^{c}	0.2^{c}
24	24.9^{b}	21505^{abc}	10615	52.5^{bc}	42.0^{de}	0.95^{d}	1.8^{bc}	3.0^{bc}	0.7^{abc}
48	29.9^{a}	31391^{a}	13252	77.5^{a}	17.7^{fg}	0.23^{d}	1.8^{bc}	2.2^{c}	0.8^{abc}
P Value	< 0.001	< 0.001	0.022	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.006
PSEM	0.8	2862	2354	4.6	4.6	1.12	0.3	0.7	0.3

H/L ratio: heterophilic granulocytes/lymphocyte ratio; PSEM: combined standard error of means.
^{a,b,c,d,e,f}Different superscripts mark significant differences between time points within a column ($P < 0.05$).

differences between animals slaughtered before and after LPS injection were no longer significant ($P > 0.05$). The opposite development of lymphocytes and heterophilic granulocytes resulted in a significant increase of the H/L ratio in animals slaughtered 4, 5 and 6 h p.i. compared with animals slaughtered before LPS injection ($P < 0.05$).

Proportions of eosinophilic and basophilic granulocytes increased significantly until 0.5 and 1 h p.i., respectively ($P < 0.05$) and decreased thereafter numerically until 5 h p.i. Subsequently, proportions of these cell populations increased slightly without differing from the initial value ($P > 0.05$). Proportions of monocytes in animals slaughtered after LPS injection did not differ significantly compared with animals slaughtered before LPS injection ($P > 0.05$). However, the proportions in animals slaughtered 2 h p.i. were significantly higher compared with proportions in animals slaughtered 4 or 12 h p.i. ($P < 0.05$).

T and B cell subsets

Proportions of $CD3^+/CD4^+/CD8^-$ T cells in blood and spleen were not affected by time ($P > 0.05$) (Figure 1). They differed significantly between blood, spleen and CT ($P < 0.05$). In general, this cell phenotype was most frequent in blood. However, before LPS injection $CD3^+/CD4^+/CD8^-$ T cell subsets of CT were similar to subsets in blood ($P > 0.05$). After LPS injection the $CD3^+/CD4^+/CD8^-$ T cell subsets in CT significantly decreased and did not reach the initial level during the entire remaining experimental period ($P < 0.01$).

The $CD3^+/CD4^-/CD8^+$ T cell subsets in considered localizations were significantly affected by time ($P < 0.001$) except for $CD3^+/CD4^-/CD8^+$ T cell subsets in spleen ($P > 0.05$). Lowest proportions of this cell population which were also significantly lower compared with the proportions in other considered localizations ($P < 0.001$) were found in blood. Immediately after LPS injection, $CD3^+/CD4^-/CD8^+$ T cell subsets in blood decreased until 5 h p.i. The $CD3^+/CD4^-/CD8^+$ T cell subsets of animals slaughtered between 3 and 6 h p.i. were significantly lower compared with the animals slaughtered before LPS injection ($P < 0.01$). From 8 h p.i. on, $CD3^+/CD4^-/CD8^+$ T cell subsets reached the initial level. Interestingly, $CD3^+/CD4^-/CD8^+$ T cells were less frequent (minimum = 0.4% – maximum = 1.7%) in CT of animals without LPS treatment. However, from 2 h p.i., the proportions of this cell population significantly increased ($P < 0.001$) and reached the levels observed in spleen. During the remaining experimental period the splenic $CD3^+/CD4^-/CD8^+$ T cell subsets remained on this level.

$CD3^+/CD4^+/CD8^+$ T cell subsets in all considered localizations changed significantly over the entire experimental time ($P < 0.05$). Proportions in blood peaked 5 h p.i.; in spleen 2 h p.i. and in CT 8 h p.i. Significant differences compared with animals slaughtered before LPS injection were only observed in blood of animals slaughtered 5 and 6 h p.i. ($P < 0.001$).

There was no time-dependent effect on $CD3^+/CD4^-/CD8^-$ T cell subsets in blood and spleen ($P > 0.05$). However, $CD3^+/CD4^-/CD8^-$ T cell subsets in CT differed significantly over the entire experimental

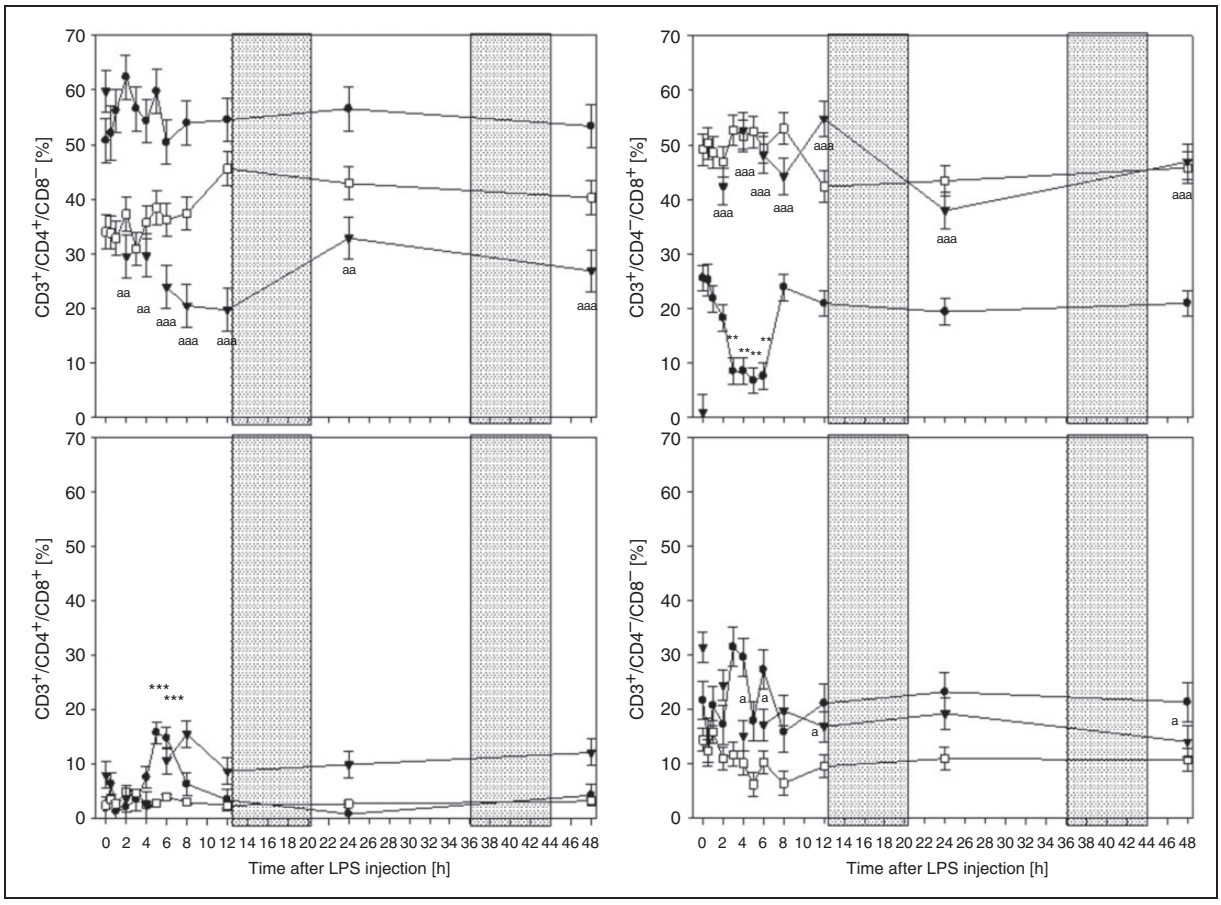

Figure 1. T cell subsets after LPS injection in blood (●), spleen (□) and caecal tonsils (▼) (means ± SE; $n = 3$). *Significant differences of blood cells compared with animals without LPS injection (time point 0) (**$P < 0.01$; ***$P < 0.001$); [a]Significant differences of cells of caecal tonsils compared with animals without LPS injection (time point 0) ([a]$P < 0.05$;[aa]$P < 0.01$; [aaa]$P < 0.001$). Dotted area indicates the dark period.

time ($P = 0.011$). At 0 h p.i. the proportions of this cell population were 31.4 ± 2.8% (means ± SE). Animals slaughtered at 4, 6, 12 and 48 h p.i. showed significantly lower proportions of $CD3^+/CD4^-/CD8^-$ T cells in CT ($P < 0.05$).

Highest proportions of Bu^+ cells were detected in CT and lowest proportions in blood (Figure 2). The proportions of Bu^+ cells varied significantly over the experimental time in blood and CT ($P < 0.001$) but not in spleen ($P > 0.05$). Proportions of Bu^+ cells peaked in blood at 6 h p.i. However, there were no significant differences between proportions of this population in animals slaughtered before LPS injection and animals slaughtered after LPS injection. Initially, the proportions of Bu^+ cells in CT amounted on average to 94.5 ± 5.5% (means ± SE). After LPS injection the proportions were markedly reduced over the entire remaining experimental period. Bu^+ subsets in CT of animals slaughtered 2, 4, 6, 12 and 48 h p.i. differed significantly from subsets in CT of animals slaughtered before LPS injection ($P < 0.05$).

IL-1β and IL-10

The relative gene expression of IL-1β in PBLs and considered tissues differed significantly over the entire experimental period ($P < 0.05$) (Figure 3a and b). While the relative gene expression in liver and CT peaked already at 2 h p.i. that in PBLs peaked at 3 h p.i. and in spleen at 4 h p.i. The relative gene expression in PBLs of animals slaughtered between 1 and 8 h p.i. was significantly higher compared with the expression of animals slaughtered before LPS injection ($P < 0.05$). While relative gene expression of IL-1β in liver differed only 2, 4 and again at 48 h p.i. from that before LPS injection ($P < 0.05$) the relative gene expression in spleen increased significantly at 2 h p.i. and remained on a significantly higher level until 12 h p.i. compared with the initial level ($P < 0.05$).

The relative gene expression of IL-1β in PBL was significantly negatively correlated with the albumin concentration in blood ($r = -0.494$, $P = 0.002$). Moreover, a significant negative correlation was detected between the

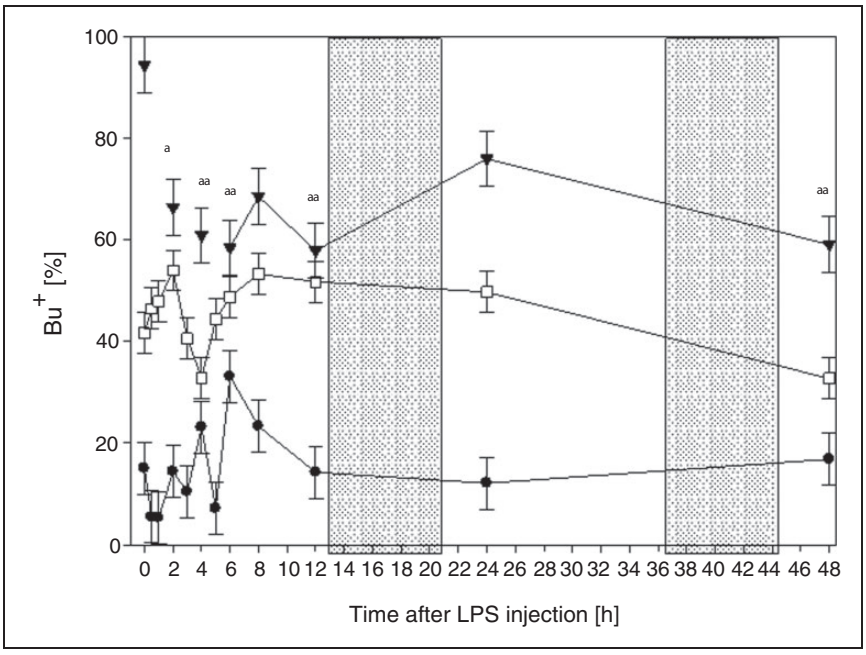

Figure 2. Proportions of Bu^+ cells after LPS injection in blood (●), spleen (□) and caecal tonsils (▼) (means ± SE; $n = 3$). [a]Significant differences of cells of caecal tonsils compared with animals without LPS injection (time point 0) ($^aP < 0.05$; $^aP < 0.01$). Dotted area indicates the dark period.

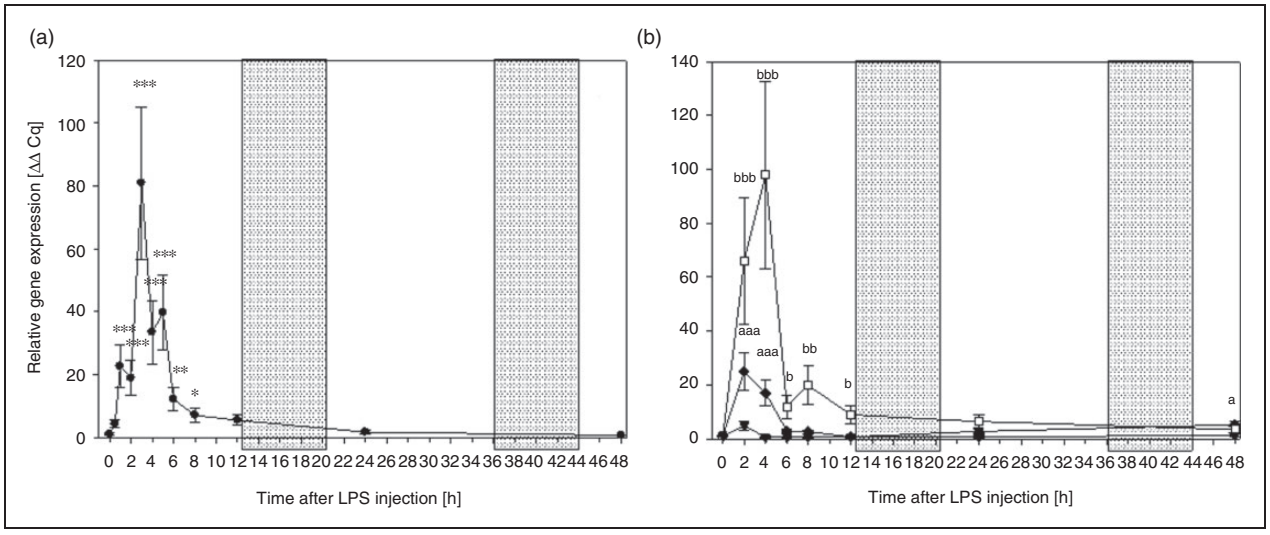

Figure 3. (a) Relative gene expression levels of IL-1β in PBLs (means ± SE; $n = 3$). *Significant differences compared with animals without LPS injection (time point 0) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). (b) Relative gene expression levels of IL-1β in tissues (◆ liver; □ spleen; ▼ caecal tonsils) (means ± SE; $n = 3$). [a]Significant differences of gene expression levels in liver compared with animals without LPS injection (time point 0) ($^aP < 0.05$; $^{aaa}P < 0.001$). [b]Significant differences of gene expression levels in spleen compared with animals without LPS injection (time point 0) ($^bP < 0.05$; $^{bb}P < 0.01$; $^{bbb}P < 0.001$). Dotted area indicates the dark period.

gene expression of IL-1β and blood tryptophan concentrations ($r = -0.613$; $P < 0.001$).

A time-dependent effect was detected on relative gene expression of IL-10 in PBLs and considered tissues ($P < 0.01$) (Figure 4a and b). In all localizations the IL-10 mRNA expression peaked at 2 h p.i. However, the expression at this time was markedly higher in liver and spleen compared with the expression in PBLs or CT. While the relative gene expression in liver was significantly higher ($P < 0.001$) than the initial level only at 2 h p.i. the expression in PBLs and spleen remained at a significantly higher level until 4 h p.i.

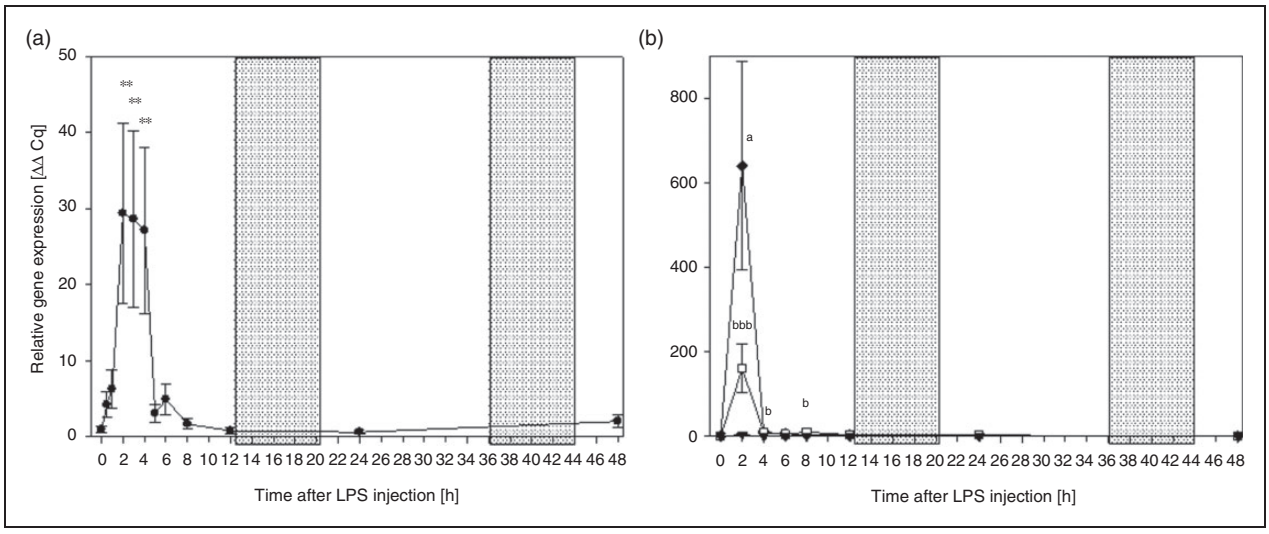

Figure 4. (a) Relative gene expression levels of IL-10 in PBLs (means ± SE; $n = 3$); **Significant differences compared with animals without LPS injection (time point 0) ($P < 0.01$). (b) Relative gene expression levels of IL-10 in tissues (◆ liver; □ spleen; ▼ caecal tonsils) (means ± SE; $n = 3$); [a]Significant differences of gene expression levels in liver compared with animals without LPS injection (time point 0) ($P < 0.05$). [b]Significant differences of gene expression levels in spleen compared with animals without LPS injection (time point 0) ($P < 0.05$). Dotted area indicates the dark period.

($P < 0.05$). Furthermore, the relative gene expression in spleen was significantly higher once again at 8 h p.i. compared with the initial level ($P = 0.027$). The relative gene expression of IL-10 in CT differed significantly over the entire experimental period; however, there were no significant differences between animals slaughtered before or at different time points after LPS injection ($P > 0.05$). Significant positive correlations were noted between relative gene expression of IL-1β and IL-10 in the various considered localizations (PBLs: $r = 0.645$, $P < 0.001$; liver: $r = 0.648$, $P = 0.001$; spleen: $r = 0.789$, $P < 0.001$; CT: $r = 0.524$, $P = 0.010$).

TLR4

The relative gene expression of the TLR4 was significantly affected by time in PBLs, liver and spleen ($P < 0.01$) and tended to be affected by time in CT ($P = 0.060$) (Figure 5a and b). Concretely, the gene expression in PBLs decreased numerically at 2 h p.i. and increased thereafter until 4 h p.i. After the peak at 4 h p.i., the relative gene expression of TLR4 decreased until the end of the experimental period. However, there was no significant difference between the TLR4 mRNA expression in PBLs of animals slaughtered before and after LPS injection ($P < 0.05$). The relative gene expression of the TLR4 in liver decreased until 4 h p.i. and increased thereafter until 24 h p.i. The relative gene expression of animals slaughtered 4 h p.i. was significantly lower ($P = 0.006$) and the expression of animals slaughtered 24 h p.i. was significantly higher ($P = 0.019$) than the relative gene expression of animals slaughtered before LPS injection. The relative gene expression in spleen increased numerically 2 h p.i. and decreased thereafter significantly compared with the initial level ($P = 0.007$). After this drop the relative gene expression increased until 8 h p.i. and remained constant thereafter. A dropdown of the relative gene expression of the TLR4 in CT was observed at 2 and 12 h p.i., which was not significant compared with the initial level ($P > 0.05$). After the last drop, the relative gene expression remained on a numerically lower level compared with the gene expression of animals slaughtered before LPS injection.

The expression of TLR4 in PBLs was significantly positively correlated with the expression of IL-1β in blood ($r = 0.395$, $P = 0.017$). Conversely, TLR4 mRNA expression in liver was significantly negatively correlated with the expression of IL-1β in liver ($r = -0.552$, $P = 0.005$). There was no correlation between the relative gene expression of TLR4 and IL-1β in spleen and CT ($P > 0.05$).

Inducible NO synthase and blood nitrite concentration

The relative gene expression of inducible NO synthase (iNOS) in PBLs increased immediately after LPS injection until 3 h p.i., decreased thereafter until 6 h p.i. and remained constant until the end of the experiment (Figure 6). At 2 and 3 h p.i. the relative gene expression of iNOS in PBLs was significantly higher compared with the initial level ($P < 0.05$). The relative gene expression in liver and spleen peaked at 2 h p.i. and

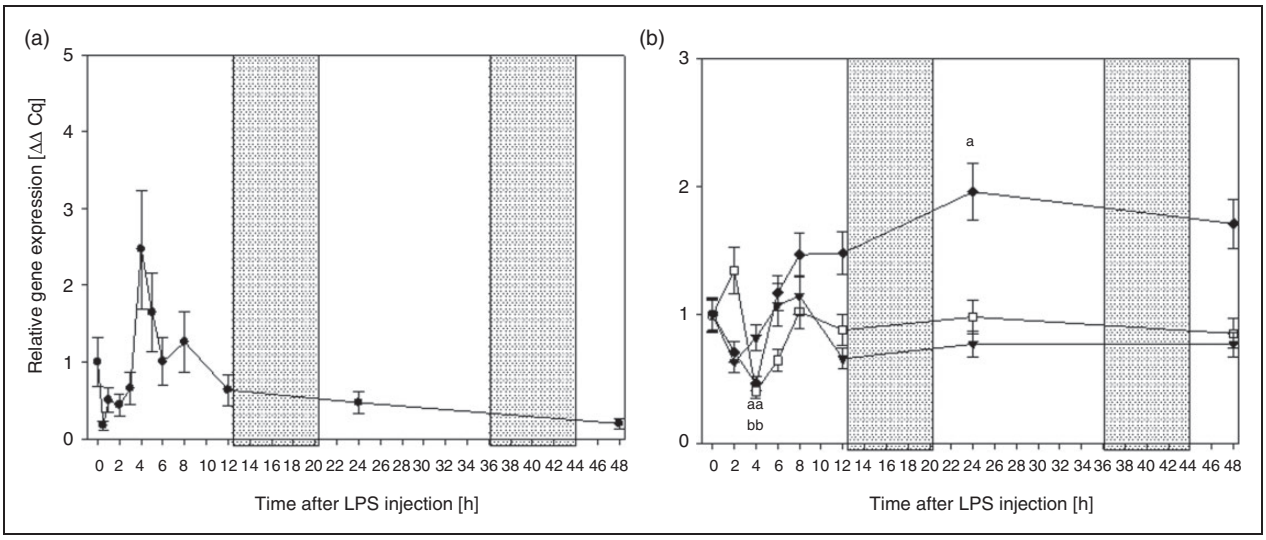

Figure 5. (a) Relative gene expression levels of TLR4 in PBLs (means ± SE; $n = 3$). (b) Relative gene expression levels of TLR4 in tissues (◆ liver; □ spleen; ▼ caecal tonsils) (means ± SE; $n = 3$). [a]Significant differences of gene expression levels in liver compared with animals without LPS injection (time point 0) ([a]$P < 0.05$; [aa]$P < 0.01$). [bb]Significant differences of gene expression levels in spleen compared with animals without LPS injection (time point 0) ($P < 0.01$). Dotted area indicates the dark period.

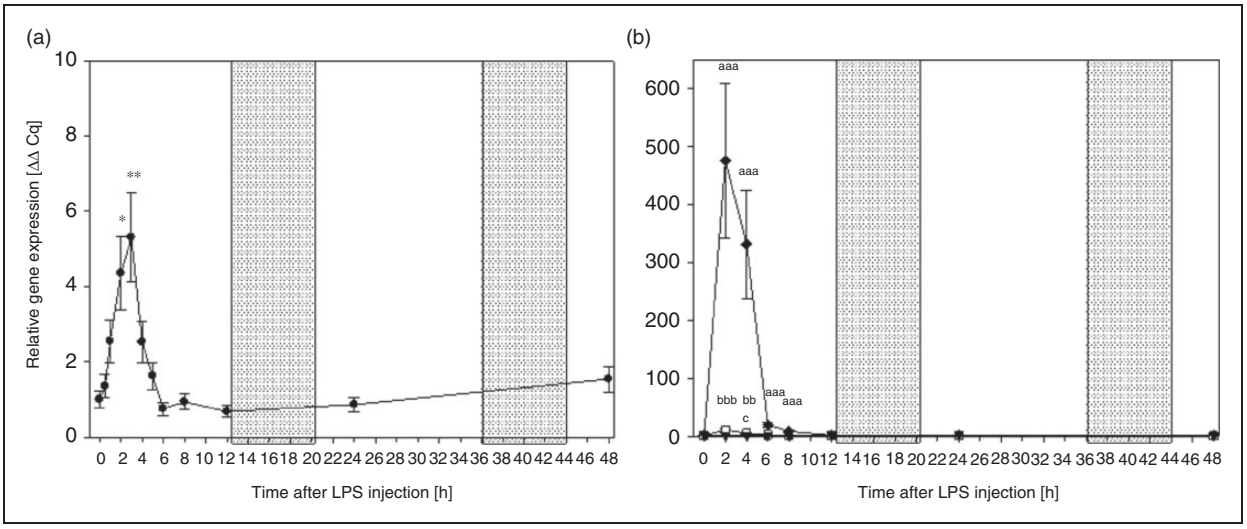

Figure 6. (a) Relative gene expression levels of iNOS in PBLs (means ± SE; $n = 3$). *Significant differences of gene expression levels compared with animals without LPS injection (time point 0) (*$P < 0.05$; **$P < 0.01$). (b) Relative gene expression levels of iNOS in tissues (◆ liver; □ spleen; ▼ caecal tonsils) (means ± SE; $n = 3$); [aaa]Significant differences of gene expression levels in liver compared with animals without LPS injection (time point 0) ($P < 0.001$). [b]Significant differences of gene expression levels in spleen compared with animals without LPS injection (time point 0) ([bb]$P < 0.01$; [bbb]$P < 0.001$); [b]Significant differences of gene expression levels in caecal tonsils compared with animals without LPS injection ([b]$P < 0.05$). Dotted area indicates the dark period.

remained significantly higher in spleen until 4 h p.i. ($P < 0.01$) and in liver until 8 h p.i. ($P < 0.001$) compared with animals slaughtered before LPS injection. In contrast, the relative gene expression in CT was significantly decreased at 4 h p.i. ($P = 0.025$). Thereafter, the expression increased slightly and remained on a constant level below the initial level subsequently.

Plasma nitrite concentrations peaked at 6 and 12 h p.i. and were significantly higher than the initial nitrite concentration at 5, 6 and 12 h p.i. ($P < 0.05$) (Figure 7). Furthermore, the nitrite concentration tended to be higher at 8 h p.i. ($P = 0.055$).

The iNOS mRNA expression in PBLs, liver, spleen and CT correlated significantly with the IL-1β mRNA

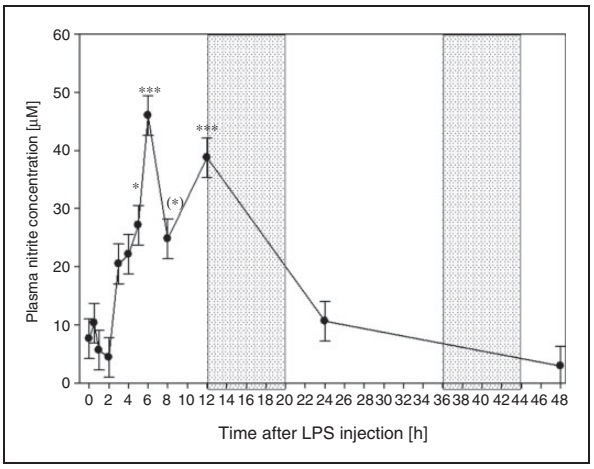

Figure 7. Plasma nitrite concentration after LPS injection (means ± SE; $n = 3$); *Significant differences compared with animals without LPS injection (time point 0) ((*) $P < 0.1$); *$P < 0.05$; ***$P < 0.001$). Dotted area indicates the dark period.

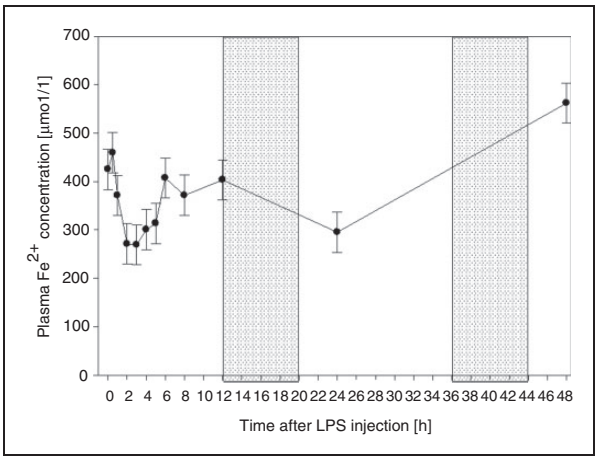

Figure 8. Ferric reducing ability of plasma (FRAP) after LPS injection (means ± SE; $n = 3$). Dotted area indicates the dark period.

expression in the same localization (PBLs: $r = 0.565$, $P < 0.001$; liver: $r = 0.802$, $P < 0.001$; spleen: $r = 0.809$, $P < 0.001$; CT: $r = 0.541$, $P = 0.008$). The plasma concentration of nitrite tended to correlate with the gene expression of IL-1β in PBLs ($r = 0.301$, $P = 0.074$).

Moreover, there was a significant correlation between liver iNOS mRNA expression and liver TLR4 mRNA expression ($r = -0.782$; $P < 0.001$) and between the plasma nitrite concentration and the PBL TLR4 mRNA expression ($r = 0.401$; $P = 0.015$).

A significant correlation between iNOS mRNA expression in liver and blood Glc concentration ($r = 0.470$; $P = 0.020$) as well as between plasma nitrite concentrations and blood Glc concentration was detected ($r = 0.442$; $P = 0.007$). PBL mRNA expression of iNOS and blood Glc concentration did not correlate ($P > 0.05$).

Ferric reducing capacity of plasma

There was a time-dependent effect on FRAP indicated by the Fe^{2+} concentration in plasma over the entire experimental period ($P = 0.001$) (Figure 8). Immediately after LPS injection the Fe^{2+} concentration increased slightly and decreased thereafter until 2 h p.i. After 3 h p.i. until 6 h p.i. the Fe^{2+} concentration increased. The concentration peaked at 48 h p.i. There was no significant difference between animals slaughtered before or after LPS injection ($P > 0.05$).

A significant negative correlation was detected between the FRAP and the iNOS mRNA expression in PBLs ($r = -0.433$; $P = 0.008$) but not between FRAP and the plasma nitrite concentration ($P > 0.05$).

Discussion

In order to examine the response of the innate immune system to dietary interventions, it is important to know the kinetics of an acute phase response in order to record most relevant endpoints and most indicative time points. As high-resolute kinetics of an LPS-induced acute phase response in laying hens are rarely described in the literature, in the present study clinical signs were recorded frequently and a number of endpoints closely associated with the acute phase response were determined.

The used dosage of intramuscularly administered LPS proved adequate to induce a characteristic febrile response and sickness behavior in different genotypes of layer pullets and cockerels in previous studies by Lieboldt et al.[2,6] In the present study, laying hens treated with LPS showed clinical signs indicative of an acute phase response in mammals and birds caused by endotoxins, such as sickness behavior, a decrease in the negative acute phase-protein albumin, a reduction in blood calcium concentration, leucopenia, lymphopenia, and an increase in heterophilic granulocytes as well as an increase in relative gene expression of the pro-inflammatory cytokine IL-1β.[3,13] However, the mentioned alterations were not accompanied by a fever response, which is normally mediated by IL-1β during acute phase response.[3]

Similar to the previous studies of Lieboldt et al.,[2,6] a drop in body temperature was observed after injection of LPS. However, this drop occurred markedly earlier compared with the previous cited studies, and regressed markedly faster. The development of an elevated body temperature after this initial drop, as described by Lieboldt et al.,[2,6] was not observed. In previous studies, it was pointed out that the body temperature response

to LPS depends on dosage, site of injection and genotype.[2,5,6] In the present study, the application of LPS and genotype corresponded to the conditions used in the studies of Lieboldt et al.[2,6] However, a possible explanation for the markedly lower febrile response could be the higher age of animals used in the present study. In studies with broiler chickens, De Boever et al.[14] clearly demonstrated that the body temperature response to LPS decreased with increasing age of birds. Although body temperature was regressed 2 h p.i., the typical clinical symptoms due to LPS administration, as already described in studies by Xie et al.[4] and Lieboldt et al.,[6] persisted until 6.45 h p.i. Studies by Johnson et al.[5] also showed that the behavioral response to LPS lasted longer than the febrile response of animals, which indicates, as also emphasized by Johnson et al.[5], that the development of the behavioral and the pyrogenic response is mediated differently.

Cytokines such as IL-1β trigger the acute phase response, which includes the up-regulation of positive acute phase proteins in liver.[3] As a consequence, the synthesis of negative acute phase proteins decreases.[3] This aspect explains the reduction of blood albumin concentration within 3–8 h p.i. The significant negative correlation between the relative gene expression of IL-1β and the concentration of albumin in blood and PBLs supports the association between this pro-inflammatory cytokine and the acute phase response.

The positive correlation between relative gene expression of IL-1β and iNOS, which is expressed primarily by macrophages, substantiated the key role of IL-1β as an inducer of NO production.[3] The increase in the corresponding plasma nitrite concentration started later and peaked 2 h later than the increase in the relative gene expression of iNOS in PBLs. While the gene expression of iNOS was completely reversed at 6 h p.i., long lasting effects on plasma nitrite concentrations were shown after LPS injection that remained at a significantly higher value until 12 h p.i.

IL-10 is able to inhibit the synthesis of IL-1β.[15] The almost simultaneous up-regulation of IL-1β and IL-10 protects the animal from overproduction of pro-inflammatory cytokines.[15] Approximately 2 h after the reduction of IL-10 mRNA expression, the IL-1β mRNA expression also decreased. In a study by Munyaka et al.,[16] a simultaneous up-regulation of IL-1β and IL-10 was also observed in spleen and CT of LPS-treated laying hens. Interestingly, the mRNA expression of IL-1β was markedly higher in PBLs than in liver, but the mRNA expression of IL-10 was markedly higher in liver compared with PBLs. In contrast to Yang et al.,[17] who observed increased IL-1 expression in PBMCs 24 h p.i., no long lasting effects on IL-1β mRNA expression were noted in the present study in the considered localizations except for the expression in liver. According to Leshchinsky and Klasing,[18] it is assumed that the mRNA expression level of the cytokines correlated with a boost of their biological activity in the considered localizations.

Hypoglycemia after LPS administration, which was reported in a study by Xie et al.,[4] was not noted in the present experiment. Conversely, lower Glc concentrations were detected in animals slaughtered before LPS injection, and in animals slaughtered 24 and 48 h p.i. These findings could be related with the lower feed intake of these animals resulting from the preceding dark period, and an increase of feed intake the longer the illumination period. Nevertheless, the marked increases of Glc concentrations from 0 to 0.5 h p.i. might also indicate an LPS-related effect on this parameter. It was reported that the administration of LPS results in an increase in hepatic gluconeogenesis, which can enhance blood Glc concentrations.[19,20] In recently published studies in dairy cows, Kvidera et al.[21] suggested hepatic glycogenolysis as the primary supporter of the increased Glc concentrations in the blood of cows after LPS treatment. Moreover, it was described that LPS induce insulin resistance, which in turn results in hyperglycemia in rats.[20] To test possible insulin resistance due to LPS in laying hens in further studies, insulin concentrations in blood also have to be considered. Sugita et al.[20] showed clear relationships between endotoxin-induced hyperglycemia and hepatic insulin resistance as well as the protein expression of iNOS. The significant correlations between blood Glc concentrations and iNOS mRNA expression in liver as well as the plasma nitrite concentration might indicate similar relationships in LPS-treated laying hens in the present study.

Xie et al.[4] reported that LPS induces diuresis in broiler chickens. It is known that diuresis increases phosphorus excretion,[22] which might explain the numerical decrease in blood phosphorus concentrations from 2 h p.i. In studies with horses, Toribio et al.[23] found that hypophosphatemia was accompanied by hyperphosphaturia. However, in agreement with the findings of Xie et al.,[4] the phosphorus level increased markedly 12 h p.i. in the present study. Xie et al. [4] ascribed this increase to impaired kidney function due to the acute phase response.

In the present study, blood calcium concentrations seemed to be reduced from 3 h p.i. In general, a considerable proportion of the calcium found in the body is bound to albumin. Therefore, the lower calcium concentration could be related to the decreasing albumin concentration in blood. This suggestion was supported by the significant positive correlation between these two parameters. In studies with pigs and horses, LPS also reduced calcium concentration in the blood.[23,24] Carlstedt et al.[24] suggested that decreases of blood

calcium concentration could be related, in part, with the translocation of albumin in the extracellular space induced by vascular permeabilisation due to endotoxemia.

Tryptophan plays a key role in the regulation of the immune response during inflammation. It is known that the enzyme indoleamine 2,3 dioxygenase 1 catalyzes tryptophan degradation to *N*-formylkynurenine, which can be subsequently converted into kynurenine.[25] Pro-inflammatory cytokines such as IFN-γ, TNF-α, or IL-1 and -2 induce the activity of this enzyme.[25] The significant negative correlation between plasma tryptophan levels and IL-1β in the current study might indicate an LPS-induced degradation of tryptophan by activation of indoleamine 2,3 dioxygenase 1. An indicator for the activity of this enzyme is an increase of the kynurenine/tryptophan ratio.[25] However, making reliable conclusions regarding this ratio was not possible in the present study because of the very low blood kynurenine levels. In studies with pigs, LPS also reduced plasma tryptophan levels, accompanied by increases in kynurenine levels and the kynurenine/tryptophan ratio.[26] In this latter study, the LPS-induced activation of indoleamine 2,3 dioxygenase was proven.

Leucopenia, which became significant 2–4 h p.i. and 12 h p.i. appeared to be based on the leucocyte extravasation.[13] A similar effect on the development of thrombocyte counts after LPS injection as shown in the present study was also detected in mice.[27] Shibazaki et al.[27] clearly showed that these cells will be translocated to lungs, liver and spleen due to LPS injection. Transient enhancement of thrombocytes at 2, 6 and 8 h p.i. might be the result of the recruitment from lungs and bone marrow by homeostatic signals, as suggested by Wang et al.[13] In agreement with the results of Lieboldt et al.,[2] a numerical decrease of eosinophilic and basophilic granulocyte proportions was observed 4 and 8 h p.i.; however, in the present study, immediately after LPS injection, the amount of these cells increased before granulocyte proportions decreased. A similar development of basophilic granulocytes was also shown iby Wang et al.[13] LPS injection-associated monocytosis as described iby Lieboldt et al.[2] was not observed in the present study.

The significant reduction of heterophilic granulocytes in blood could be an indication that these cells migrate already at a very early stage to the site of LPS injection. Chemotaxis of avian heterophilic granulocytes for endotoxins is known.[28] Thereafter, strong recruitment from bone marrow or a marginated pool seemed to follow, which peaked 5 h p.i. and continued until 12 h p.i. In general this recruitment is explainable by the high importance of this cell type in the inflammatory response because of its key functions in clearance of microbial agents by phagocytosis and antimicrobial activities which appear to be stimulated by cytokines and chemokines.[28] In accordance with studies by Gross and Siegel,[29] Lieboldt et al.[2] and Munyaka et al.,[30] LPS treatment resulted in increases of the H/L ratio, which is a reliable stress indicator in chicken.[29] Also in previous studies, H/L ratios >1.0 were detected in chickens after LPS injection. According to the results of the current study, the effect of LPS on lymphocytes and heterophilic granulocytes, as well as the H/L ratio, seemed to be more severe than presented in previous studies. It appears that the period between 4 and 6 h p.i. is much more stressful, which was not considered in previous studies.

Lieboldt et al.[6] observed changes in relative organ masses of spleen; these authors associated the alteration in organ mass with a higher splenic production of immune mediators and cells due to LPS treatment.

In contrast to other organs, the T and B cell subsets in spleen seemed to be stable against LPS treatment, except for $CD3^+/CD4^+/CD8^+$ cells. Although Munyaka et al.[30] exposed laying hens to higher amounts of LPS, they also found no effects on T cell subsets in spleen but there were effects on T cell subsets in blood. Also in previous studies with older laying hens, no effects of LPS were found on splenic T cell subsets.[16]

In the present study, a strong decrease of $CD3^+/CD4^-/CD8^+$ in blood was observed. In contrast, $CD3^+/CD4^-/CD8^+$ T cell subsets of CT simultaneously increased markedly. This aspect clearly indicated the migration of T cells from the circulation in secondary lymphoid organs or other related immune organs, which was already suggested by Munyaka et al.[16,30] Furthermore, in the present study, a shift from T-helper cells ($CD3^+/CD4^+/CD8^-$ T cells) in favor of T-cytotoxic cells ($CD3^+/CD4^-/CD8^+$ T cells) was shown in CT, which was not reversed until the end of the study. Cytotoxic T cells are able to attack directly pathogens. Therefore, a migration and enhancement of these cells in CT could be important for the resolution of inflammation. Also the reduction of double negative T cells could be related with the increase in $CD3^+/CD4^-/CD8^+$ T cell subsets in CT. In contrast, CD4/CD8 double-positive-stained T cells, which are considered as specific memory cells and protective and immune-regulatory functions,[31] increased significantly in blood and slightly in CT. In contrast to the results of Wang et al.,[13] no long lasting effects up to 48 h p.i. on T cell subsets in blood were shown. All effects on T cell subsets appeared to be reversed at 8 h p.i. except for T cell subsets of CT. In the present study, a pronounced and long lasting decrease in Bu^+ subsets in CT was observed after LPS injection. Similar effects were shown in previous studies of Tan et al.[32] in spleen of

broiler chickens. These latter authors suggested a higher production of H_2O_2 by macrophages as a reason for the decreasing effect on Bu^+ subsets in the spleen. In the present study, no effects on splenic Bu^+ subsets were detected; however, similar effects could be possible in CT.

It is known that, in cases of bacterial infection, LPS recognition is mediated via TLR4, which interacts with the LPS receptor CD14 and mediates the activation of macrophages in a time- and tissue-dependent manner.[33,34] In the present study, intramuscular LPS injection seemed to influence the TLR4 in all considered locations. Interestingly, while TLR4 expression was down-regulated at an early stage after LPS injection and peaked at 4 h p.i. in PBLs, expression was significantly down-regulated in liver and spleen at 4 h p.i. Nomura et al.[35] showed that LPS stimulation of murine macrophages can result in a transient reduction in TLR4 mRNA expression. Furthermore, decreased TLR4 expression could be an indication of the development of LPS tolerance. LPS tolerances were especially shown after repeated LPS treatment of murine macrophages in birds.[35,36] Murine macrophages pre-treated with LPS also showed a marked decrease in mRNA expression of TLR4.[35] However, Nomura et al.[35] emphasized that the TLR4 surface expression of macrophages is a more reliable indicator for LPS tolerance than mRNA expression. The development of LPS tolerance may also be a further explanation for the lack of a febrile response, which is known to be abolished after tolerance development in birds.[36] A significant up-regulation of TLR4 was only noted at 24 h p.i. in liver. Up-regulating effects of LPS on TLR4 mRNA expression after 24 h p.i. were also shown by Tan et al.[32] However, these authors reported effects in spleen and CT, locations that were not significantly affected in the present study. In contrast to the present study, Munyaka et al.[16] showed that expression of TLR4 was significantly up-regulated 4 h p.i. in spleen after LPS injection, and accompanied with the up-regulation of the relative gene expression of IL-1β. In the present study, there was no significant correlation between the expression of TLR4 and the expression of IL-1β in spleen, but a significant positive correlation was detected in PBLs, and a negative correlation between both parameters in liver. Dil and Qureshi[34] suggested a strong relationship between TLR4 and iNOS expression of macrophages. Indeed correlations between the gene expression of TLR4 and iNOS were detected only in liver. However, this correlation was significantly negative, which resulted from the opposite kinetics of both parameters. In general, TLR4 signaling results in NF-κB activation, which in turn is essential for the expression of iNOS.[34,37,38] Therefore, in further studies, the expression of NF-κB should also be considered. In PBLs, no correlation between the gene expressions of TLR4 and iNOS was shown, but a positive correlation was seen between relative gene expression of TLR4 and plasma nitrite concentration.

The high production of iNOS contributed to a high release of NO, as indicated by the stable end product nitrite in the present study. However, it is also known that NO, in combination with the superoxide anion, can lead to the formation of peroxynitrite anion and subsequently to the formation of hydroxyl radicals.[39,40] The production of these reactive oxygen species due to LPS-mediated iNOS induction could be an explanation for the reduction of antioxidants in circulation, which was indicated by FRAP in the present study. This hypothesis is supported by the negative correlation between FRAP and iNOS mRNA expression in blood.

In the present study did not include an control group that was treated with NaCl or PBS. However, other studies have shown that there are no obvious time-dependent effects on thrombocyte counts, white blood cell counts, differentiated blood cells, T and B cell subsets and plasma nitrite concentrations.[13,41] Furthermore, in previous studies most parameters known to be influenced by the circadian rhythm, such as body temperature or albumin, show significant changes only in dark periods.[6,42] Previous studies have also shown that plasma concentrations of phosphorus and calcium increase until 12 and 16 h post oviposition.[43] All animals laid an egg immediately before LPS injection or within the 12 h before slaughtering. Therefore, decreases in calcium and phosphorus measured in the present study are not a result of the egg production rhythm, and appeared to be related to the injection of LPS. An influence of this rhythm on the result of the measurements after 24 h and 48 h p.i. cannot be fully excluded. With regard to proven time-indecencies during the daily period of different parameters, and in comparison to results of previous studies, it is suggested that most of the present results were related directly to the injection of LPS.

Conclusion

In conclusion, the intramuscular administration of LPS to older laying hens (7th laying mo) induced alterations such as sickness behaviour, a decrease in the anti-acute phase protein albumin, a reduction of calcium and phosphorus concentration in blood, leucopenia, lymphopenia, heterophilia and increases of IL-1β mRNA expression, which are associated with acute phase response in mammalians and birds; however, no fever response. Possibly, the development of more efficient mechanisms to overcome inflammation or tolerance against LPS by the animals, due to their higher age,

reduced the febrile response. The main response time of the mentioned alterations due to LPS appears to contain the period between 2 and 8 h p.i. According to the H/L ratio, the most stressful period after LPS injection in older laying hens is 5 h p.i. However, alterations in T and B cell subsets in CT as well as IL-1β and TLR4 mRNA expression in liver and plasma nitrite concentrations seemed to be affected for a longer period.

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