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Distinct functional responses to stressors of bone marrow derived dendritic cells from diverse inbred chicken lines

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ABSTRACT

Differences in responses of chicken bone marrow derived dendritic cells (BMDC) to *in vitro* treatment with lipopolysaccharide (LPS), heat, and LPS + heat were identified. The Fayoumi is more disease resistant and heat tolerant than the Leghorn line. Nitric Oxide (NO) production, phagocytic ability, MHC II surface expression and mRNA expression were measured. NO was induced in BMDC from both lines in response to LPS and LPS + heat stimulation; Fayoumi produced more NO with LPS treatment. Fayoumi had higher phagocytic ability and MHC II surface expression. Gene expression for the heat-related genes *BAG3*, *HSP25*, *HSPA2*, and *HSPH1* was strongly induced with heat and few differences existed between lines. Expression for the immune-related genes *CCL4*, *CCL5*, *CD40*, *GM-CSF*, *IFN-γ*, *IL-10*, *IL-12β*, *IL-1β*, *IL-6*, *IL-8*, and *iNOS* was highly induced in response to LPS and different between lines. This research contributes to the sparse knowledge of genetic differences in chicken BMDC biology and function.

1. Introduction

Dendritic cells (DC) are the key types of antigen presenting cells (APC). Immature DC are present on all surfaces of peripheral tissues of the body, such as intestinal mucosa or skin epithelia. The DC's major function is to monitor presence of antigens, which are then captured and processed. DC primed with antigen migrate to secondary lymphatic organs, where they present antigens to naïve T cells, which activates them towards Th1/Th2 polarization (Banchereau and Steinman, 1998). As such, DC are considered to bridge innate and adaptive immune responses. In chickens, several classes of DC have been identified [reviewed by Nagy et al., 2016; Wu and Kaiser, 2011]; intestinal (mucosal) DC (Del Cacho et al., 2012), interdigitating DC (Del Cacho et al., 2009), follicular DC (Del Cacho et al., 2008) and epidermal DC (Igyarto et al., 2006). For research purposes, DC can be also be generated *in vitro*, by differentiating bone marrow (BM) cells into DC lineage with GM-CSF and IL-4 cytokines (Wu et al., 2010a). In this manner, a population of bone marrow derived dendritic cells (BMDC) can be used to demonstrate their unique properties in response to environmental and inflammatory stressors.

BMDC can be primed *in vitro* with lipopolysaccharide (LPS), which triggers changes in their phenotype from immature to mature DC by modulating expression of surface molecules. It has been reported that the maturing BMDC under fever-like conditions triggers release of different levels of the cytokines than those that respond to an inflammatory stressor only (Tournier et al., 2003). Thermal stress, which is usually associated with inflammation (local hyperthermia) or fever (systemic hyperthermia), delivers additional inflammatory cues to the immune system [reviewed by

Abbreviations: APC, antigen presenting cells; DC, dendritic cells; BMDC, bone marrow derived dendritic cells; LPS, lipopolysaccharide; GALT, gut-associated lymphoid tissue; BM, bone marrow; IL-4, interleukin 4; GM-CSF, granulocyte-macrophage colony-stimulating factor; NO, nitric oxide; BAG3, BCL2 associated athanogene 3; CASP9, caspase 9; CCL4, chemokine (C-C motif) ligand 4; CCL5, chemokine (C-C motif) ligand 5; CD40, cluster of differentiation 40; HSP25, heat shock protein 25; HSPA2, heat shock protein family A; HSPH1, heat shock 105 kDa/110 kDa protein 1; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; IL-4, interleukin 4; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; IL-12β, interleukin 12 beta; IL-15, interleukin 15; iNOS, inducible nitric oxide synthase; MHC-IIβ, major histocompatibility complex class II beta chain; TGFβ2, transforming growth factor, beta 2; TLR4, Toll-like receptor 4; UBB, ubiquitin B; H6PD, hexose-6-phosphate dehydrogenase; RPL4, ribosomal protein L4.

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Evans et al., 2015]. Temperature elevated above physiological level is sensed as a danger signal, which enhances DC function including antigen uptake, activation-associated migration, antigen presentation, T cell activation and cytokine release [reviewed by (Ostberg and Repasky, 2006)].

Chickens farmed in commercial settings face many inflammatory and environmental stressors including infectious diseases, intensive vaccination programs and management procedures. The latter are often influenced by the seasonal changes, such as waves of elevated ambient temperatures in the summer. Heat stress in farmed chickens leads to an immediate decrease in performance parameters and reproduction (Mashaly et al., 2004; Sandercock et al., 2001). Heat stress negatively influences intestinal microbiota composition and gut barrier function. As a consequence, it causes increased permeability of the junctional complexes in the intestines, known as "leaky gut" (Lambert, 2009). Intra-abdominal influx of intestinal microbiota and gut-derived endotoxins leads to systemic endotoxemia, i.e. presence of endotoxins in the blood, which activates potent inflammatory responses and is a major cause of death during heat stroke (Leon and Helwig, 2010). The intestinal DC population, present in the lamina propria of the gut-associated lymphoid tissue (GALT), samples, processes and presents antigens that were transferred across intestinal epithelium. In this way, intestinal DC take part in immune responses to heat stress-related endotoxemia and transfer the danger signals to secondary immune organs (Fujita et al., 2006).

Chicken breeds with distinct genetic background, such as Leghorn and Fayoumi, may represent different levels of adaptation to pathogenic and environmental stressors. Leghorn is a major commercial egg laying breed, whereas Fayoumi represents a wild-type strain of chicken, and originated in Egypt. Multiple studies indicate that Fayoumi is a hardier genetic line than Leghorn, with a higher level of resistance to infections with Marek's disease (Lakshmanan et al., 1996), coccidiosis (Pinard-Van Der Laan et al., 1998), Newcastle disease (S. Lamont, personal communication), avian influenza (Wang et al., 2014), and *Salmonella* (Redmond et al., 2011) compared to Leghorns. Both genetic lines used in this study, Leghorn (Ghs-6 line) and Fayoumi (M-5.1 line), are highly inbred (99.9%) (Zhou and Lamont, 1999), and they have been used as an excellent discovery platform in numerous studies on avian immunogenetics and disease resistance (Coble et al., 2011; Kim et al., 2008; Redmond et al., 2009). We hypothesize, therefore, that due to the distinct genetic background of the Fayoumi and Leghorn chicken lines, respective BMDC will express differences in biological responses to complex environmental stimuli. In this study, we address the questions of whether, and to what extent, genetic background influences inflammatory responses in chicken BMDC induced by heat and LPS. To our knowledge, this is a first report to provide functional comparisons between cellular and molecular responses to LPS, heat, and LPS + heat in chicken BMDC derived from distinct genetic lines. As such, this research contributes to the sparse knowledge generated by using *in vitro* assays to explore the complex interaction between genetic background, immune stimulation, and environmental stress on innate immune cells *in vitro*. The genes identified in the results of this study may serve as biomarkers to breed more disease and/or heat tolerant birds.

2. Materials and methods

2.1. Animals

All animal maintenance and handling was approved by the Institutional Animal Care and Use Committee at Iowa State University: Log #4-03-5425-G. The highly inbred (99.9%) chicken lines Fayoumi (M-5.1) and Leghorn (Ghs-6) (Deeb and Lamont, 2002) were used in the current study. Adults were maintained in single bird cages and were artificially inseminated for production of fertile eggs that were incubated to produce embryos.

2.2. Generation of bone marrow derived dendritic cells (BMDC)

BM was collected from day 18 embryos of chicken inbred lines Fayoumi and Leghorn. BM was isolated and cultured as previously described (De Geus et al., 2012 and Wu et al., 2010a). Under sterile conditions, embryos were decapitated then femurs and tibias were removed using sterile instruments and placed into cold PBS. The ends of the bones were cut and BM was flushed through the bones using a syringe and needle with PBS, and the BM was pooled within chicken line. Debris was removed from BM using a 70 μm cell sieve. Cells were centrifuged for 10 min at 450 × G. The supernatant was discarded; then cell number and viability was determined using a hemocytometer with the trypan blue exclusion assay. Cells were frozen in 30% FBS and 10% DMSO in RPMI 1640 at a concentration of 1×10^7 viable cells/mL and stored at −70 °C.

Cells were seeded at 1×10^6 cells/mL in 6-well tissue culture plates in pre-warmed RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% chicken serum (Sigma Aldrich, St. Louis, MO, USA), 1% L-glutamine (Gibco, Carlsbad, CA, USA), 1 U/mL penicillin and 1 μg/mL streptomycin (Gibco, Carlsbad, CA, USA) (complete medium), and supplemented with 10 ng/mL recombinant GM-CSF (Cat#RP0290C-025; KingFisher Biotech, St. Paul, MN, USA) and 10 ng/mL IL-4 (Cat#RP0110C-025; KingFisher Biotech, St. Paul, MN, USA), for 6 days at 41.5 °C and 5% CO_2. Both recombinant, chicken-specific proteins (GM-CSF and IL-4) have been produced in yeast and therefore are naturally folded and post-translationally modified (based on manufacturer's declaration). On day 3 of culture, three quarters of the medium was removed from each well, to remove non-adherent cells, and replaced with fresh complete medium containing recombinant cytokines. On day 5 of culture, cells were harvested using 0.25% trypsin-EDTA for 10 min (Gibco, Carlsbad, CA, USA) followed by trypsin deactivation with complete medium, centrifuged for 10 min at 450 × G, and reseeded into 24-well tissue culture plates at a final concentration of 1×10^6 cells/mL with complete medium containing no recombinant cytokines. Cells re-adhered to tissue culture plates for 24 h before experimental treatments were started, which were conducted on day 6 of culture.

2.3. Cell viability assays

Viabilities for BMDC were assessed before and after treatment by using trypan blue exclusion. For the trypan blue exclusion assay, the average viability of BMDC from 3 samples from each treatment group was determined. Additionally, cell viability was assessed after treatment by the addition of Propidium Iodide (PI) to each sample assessed in flow cytometry. For the flow cytometry samples, PI was used as a viability marker in which only live cells were gated in the flow cytometry analyses and then utilized for downstream analyses.

2.4. Determination of BMDC maturation status prior to treatment

The mRNA expression of CCR6, CCR7, and DC-LAMP were quantified, using RT-PCR, within each breed to determine the BMDC maturation status prior to treatments. These genes were chosen based on prior reports (CCR6 and CCR7, Wu et al., 2011, and DC-LAMP, Wu et al. 2010b) that indicate the mRNA expression of these genes are markers of maturation in chicken BMDC. Isolated RNA was used from cells at day 7 of culture, in thermal neutral conditions, without LPS in the media, at the start of the stimulations. A total of 6 individual samples were randomly chosen from

each line, Fayoumi and Leghorn, that represented 2 technical replicates from three individual experimental days. Samples were run in triplicate. The housekeeping gene 28S was used to normalize starting concentration of RNA. The mRNA expression levels were calculated as the mean adjusted Ct values of each triplicate sample.

RT-PCR was conducted using QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA, USA). The adjusted cycle threshold (Ct) values were calculated using the equation: 40 − [Ct sample gene mean + (Ct 28S median − Ct 28S mean) (slope of sample gene/slope of 28S)]. JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA) was used for the statistical analysis with the option of Student's T test to calculate the difference in means between breeds.

2.5. Treatment with LPS, heat, and LPS + heat

A full factorial design, containing the factors of genetic line (Fayoumi and Leghorn), and treatment (control, LPS, heat, and LPS + heat) was used for all assays. The time post treatment that samples were assessed was dependent upon the assay. NO production was assayed at 24 h, phagocytosis ability and MHC II surface expression was assayed at 4 h, and cells for mRNA expression were harvested at 2 h, 4 h, and 8 h post stimulus. On day 6 of culture, the medium was removed from wells and replaced with complete medium containing 200 ng/mL LPS (Sigma Aldrich, St. Louis, MO, USA), or medium alone as a control. Cells were immediately placed in an incubator at 45 °C with 5% CO_2 (heat), or at 41.5 °C with 5% CO_2 as a thermoneutral control (TN), for 2 h. Therefore, the treatment groups include medium alone in TN conditions (control), LPS treatment in TN conditions (LPS), medium alone in heat conditions (heat), and LPS treatment in combination with heat conditions (LPS + heat).

Cells were harvested immediately (2 h post treatment), or placed in an incubator at 41.5 °C with 5% CO_2 for a temperature-recovery time. Supernatant (NO production assay) or cells (flow cytometry assays and RNA isolation) were collected for downstream analyses at different time points post-treatment (details presented below). Cells were harvested using 0.25% trypsin-EDTA for 10 min (Gibco, Carlsbad, CA, USA) followed by trypsin deactivation with complete medium. The cells were centrifuged then for 5 min at 200 × G and resuspended in FACS buffer (PBS supplemented with 0.5% BSA and 0.05% NaN_3) or cell lysis buffer from RNAqueous® Total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) for flow cytometry or RNA isolation, respectively. Three technical replicates (wells) were assayed per genetic line and treatment, and the experiment was replicated on three independent experimental days.

2.6. Nitric oxide production assay

BMDC were incubated for 24 h post treatment (control, LPS, heat, and LPS + heat) at 41.5 °C and 5% CO_2, then NO production in the cell supernatant was determined using the Griess reagent kit (Molecular Probes, Carlsbad, CA, USA). Supernatant was mixed with Griess reagents and incubated for 30 min at room temperature in dark conditions, and then measured at 540 nm on a spectrophotometer. The absorbance values were compared to the sodium nitrite standard curve to determine nitrite concentrations (μM).

2.7. Phagocytosis assay and MHC II surface expression

BMDC were incubated for 4 h post treatment (control, LPS, heat, and LPS + heat) at 41.5 °C and 5% CO_2, then assessed for phagocytic ability using 1 μm carboxylate-modified crimson beads (Molecular Probes, Carlsbad, CA, USA). Beads were suspended in pre-warmed complete medium, with or without 200 ng/mL LPS, and then BMDC were given 1 ml/well resulting in a final concentration of 20 beads/cell, then cells were harvested for flow cytometry at 4 h post treatment. An ice control was used to inhibit phagocytosis. Briefly, cells were cooled on ice for 30 min, then given 20 beads/cell in pre-cooled complete medium, and then harvested at 4 h post treatment. For both the treatment group and ice control, cells were harvested then washed twice in pre-cooled PBS and centrifuged at 450 × G for 5 min at 4 °C. Cells were placed into 96 well round bottom plates and stained with MHC II-PE antibody (Southern Biotech, Birmingham, AL, USA) for 30 min, washed 3 times by 400 × G for 5 min centrifugation at 4 °C, and resuspended in FACS buffer. Cells were assessed for phagocytic ability using the FACS-Canto machine (BD Biosciences, Palo Alto, CA, USA) at the Flow Cytometry Facility at Iowa State University.

2.8. mRNA expression

mRNA expression in BMDC stimulated with nothing (control), LPS, heat, and LPS + heat was analyzed after 2 h, 4 h and 8 h post treatment. Total RNA was isolated using RNAqueous® Total RNA Isolation Kit (Ambion, Carlsbad, CA, USA). Gene expression analysis was performed using microfluidic Reverse Transcription quantitative PCR (RT-qPCR) (Fluidigm Corporation, San Francisco, CA, USA). All procedures were conducted according to manufacturer's recommendations, unless otherwise noted. Briefly, 50 ng of the total RNA was reverse transcribed using the Fluidigm Reverse Transcription Master Mix (Fluidigm Corporation, San Francisco, CA, USA). cDNA was pre-amplified with PreAmp Master Mix (Fluidigm Corporation, San Francisco, CA, USA), using 14 cycles of pre-amplification. Exonuclease I (New England Biolabs, UK) treatment was applied to remove unincorporated primers. Pre-amplified and purified cDNA samples were diluted 10× in TE buffer and stored at −20 °C until further analyses. RT-qPCR analysis was done for 22 target genes and 2 reference genes, listed in Table 1. A 192 × 24 Integrated Fluid Circuits (IFCs) (Fluidigm Corporation, San Francisco, CA, USA) was used to perform RT-qPCR reactions. Sample assay included 1.35 μl of pre-amplified and Exo I treated cDNA, 1.5 μl of the SsoFast™ EvaGreen®Supermix with Low ROX™ (2×) (Bio-Rad) and 0.15 μl of the 192.24 Delta Gene Sample Reagent (Fluidigm Corporation, San Francisco, CA, USA). Primer assays were prepared as 20 μl stock by mixing 1 μl of each primer (100 μM) with 10 μl of the 2× Assay Loading Reagent and adjusted to 20 μl with DNA suspension buffer (low EDTA TE buffer). The samples, assays and the loading reagents were then loaded onto IFCs microfluidic channels using the RX loading station (Fluidigm Corporation, San Francisco, CA, USA). RT-qPCR was performed on the Biomark™ HD (Fluidigm Corporation, San Francisco, CA, USA) using the fast program that consisted of an incubation step at 95 °C for 60 s followed by 30 cycles: 96 °C for 5 s and 60 °C for 20 s. Fluorescence emission was recorded after each cycling step. Upon RT-qPCR completion, melting curves were generated by increasing temperature from 60 to 95 °C, followed by continued fluorescence acquisition.

2.9. Statistical analyses

Mean values of NO production, phagocytic ability, and MHC II surface expression were compared between the Fayoumi and Leghorn BMDC for all treatment groups using Students' *t*-test ($P < 0.05$) with correction for multiple comparisons, implemented in JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). RT-qPCR data were analyzed as follows: raw qPCR data were analyzed and checked for quality using Real-Time PCR Analysis Software (Fluidigm Corporation, San Francisco, CA, USA). Main effects of the stimulation of the BMDC were estimated using least square means method implemented in JMP Pro 10.0.2 software (SAS Institute,

Table 1
Primer sequences used in the gene expression study.

Gene	Entrez ID	Forward	Reverse	Ref[a]
BAG3	423931	ACCACAACAGCCGAACCA	GATGGGCCATTTGCTGATGAC	A
CASP9	426970	TTTCAGGTCCCTGTGCTTCC	TTCCGCAGCTCCACATCAA	A
CCL4	395468	CCTCATCCAGAGGCACTACA	GCTTGACGCTCTGCAGGTA	A
CCL5	417465	CTGCCCCAGAATCATGTGAA	CAGCTCCAGGAAGTTGATGTA	A
CD40	395385	AGCCTGGTGATGCTGTGAA	CTCACAGGGTGTGCAGACA	A
GM-CSF	416325	CCTGGAAGAAATAACGAGTCACTTG	ACAGGTTTTATCCCTGATGTCCAT	B
HSP25	428310	GCACGCAGAGACCATCTTCA	TGCTCGAAGCTGCTCATGAA	A
HSPA2	423504	CCACCATTCCCACCAAACAA	ATACACCTGGACGAGGACAC	A
HSPH1	418917	GTAGTTTCGTTCGGCTCCAA	CTGTGTTGTGGGCATGAGTAA	A
IFN-γ	396054	AACCTTCCTGATGGCGTGAA	GCTTTGCGCTGGATTCTCAA	A
IL-1β	395196	TGCTTCGTGCTGGAGTCAC	GGCATCTGCCCAGTTCCA	A
IL-4	416330	AACATGCGTCAGCTCCTGAAT	TCTGCTAGGAACTTCTCCATTGAA	B
IL-6	395337	GCTCGCCGGCTTCGA	GGTAGGTCTGAAAGGCGAACAG	C
IL-8	396495	CCCCACTGCAAGAATGTTGAAA	GTGCCTTTACGATCAGCTGTAC	A
IL-10	428264	CATGCTGCTGGGCCTGAA	CGTCTCCTTGATCTGCTTGATG	D
IL-12β	404671	CTTCTGGAAGCACAGTGGAAAC	AGCTGGTGTCTCATCGTTCC	A
IL-15	395258	TAGGAAGCATGATGTACGGAACAT	TTTTTGCTGTTGTGGAATTCAACT	E
iNOS	395807	GGACCGAGCTGTTGTAGAGATA	AGCAGCTGAGTGATGATCCA	A
MHCII-β	693256	GTGCAGAGGAGCGTGGAG	CGTTCAGGAACCACTTCACC	F
TGFβ2	421352	CGTGCTCTAGATGCTGCCTA	GCCAAGATCCCTCTTGAAGTCA	A
TLR4	417241	CCTGCTGGCAGGATGCA	TGTTCTGTCCTGTGCATCTGAA	A
UBB	396190	GGGGCGTCGGAGGATATATAA	CACAACAGACCAAACCCGTTA	A
H6PD[b]	428188	ATGTACCGGGTGGACCACTA	AACTGACGGTTCTGATCTCGAAA	A
RPL4[b]	415551	TTCTGCCTTGGCAGCATCA	AGGAAGTTCTGGGATCTCCTCA	A

[a] References: A. (unpublished data, Slawinska et al.); B. (Avery et al., 2004), C. (Kaiser et al., 2000), D. (Rothwell et al., 2004), E. (Kaiser et al., 2003), and F. designed in house.
[b] Reference genes.

Cary, NC, USA). Chicken line (Fayoumi and Leghorn), treatment (control, LPS, heat, and LPS + heat) and time post treatment (2 h, 4 h, and 8 h) as well as the interaction between line and treatment were fitted in the model. Analyses were performed separately for each line, gene, and treatment using dCt values (Ct target − Ct reference). To determine the relative gene expression, the ddCt method was used (Livak and Schmittgen, 2001). Delta Ct values were obtained by normalizing the Ct values of the target genes with the geometric mean of the two reference genes (*H6PD* and *RPL4*). Fold induction of the gene expression was estimated as 2^{-ddCt}. Untreated (control) samples were used as calibrators.

3. Results

3.1. BMDC culture and viability

The average viability of BM cells after isolation was 90% and the number of viable cells obtained from each embryo averaged 1.5×10^7. In general, BMDCs reported in the current study displayed typical morphology similar to previous reports (Rajput et al., 2013; Wu et al., 2010a), with the exception of Day 3 of culture. At Day 3 in the current experiment, the BMDCs do not yet display clear aggregates with a veiled appearance as those reported by Wu et al., 2010a. This may be due to sourcing the BM from D18 embryos in the current study compared to 4-12 week-old-chickens used in Wu et al., 2010a. Pictures of the growth and treatment morphology are found in Fig. 1. On Day 3 and Day 6 of culture, small and bright granules are present in the cytoplasm, termed intracytoplasmic circular granules. These granules of the cultured cells are a phenotypic characteristic of monocyte-derived dendritic cells (Grassi et al., 1998), compared to the typical Birbeck granules that are observed in Langerhans cells. Generally, the morphological characteristics of the two chicken lines were very similar to each other during both growth and treatment.

Viabilities for BMDC were assessed before treatment by using trypan blue exclusion and after treatment by using both trypan blue exclusion and PI (Table 2). On day 6, prior to treatment, BMDC were assessed for viability using trypan blue exclusion. On average, 88% and 89% of Fayoumi and Leghorn cells were viable, respectively. After treatments, BMDC were assessed for viability using trypan blue exclusion. On average for Fayoumi and Leghorn, respectively, cell viabilities were: 100% and 92% for TN_Media, 93% and 88% for TN_LPS, 93% and 87% for HS_LPS. The viability determined using PI after treatments was in general similar to that calculated with trypan blue, with the exception of HS_LPS treatment which resulted in 51% and 67% for Fayoumi and Leghorn, respectively. The decrease in viability in this treatment group may have been effected by the antibody staining procedures. However, only viable cells were used in the functional analyses.

3.2. BMDC maturation status prior to treatment

The results for the mRNA expression of CCR6, CCR7, and DC-LAMP along with primer sequences are presented in Table 3. The results show that the mRNA expression of CCR6 and DC-LAMP are not significantly different between breeds, $P = 0.26$ and $P = 0.87$, respectively. The mRNA expression of CCR7 was moderately significantly different ($P = 0.03$) between breeds. These results suggest that the two breeds were at a similar maturation stage prior to stimulation.

3.3. Nitric oxide assay

The results of the NO assay are found in Fig. 2. In thermoneutral temperature with medium alone (control), both chicken lines had detectable levels of NO production but the levels did not differ statistically. LPS treatment increased NO production in both lines compared to controls, and Fayoumi produced more NO compared to Leghorn. Heat treatment alone had no effect on NO production in either chicken line compared to control. Treatment with LPS + heat increased NO production in both lines compared to control, with no difference between lines was detected.

3.4. Flow cytometry, phagocytic ability and MHC II staining

The general gating strategy for cells is found in Fig. 3a. Cells were

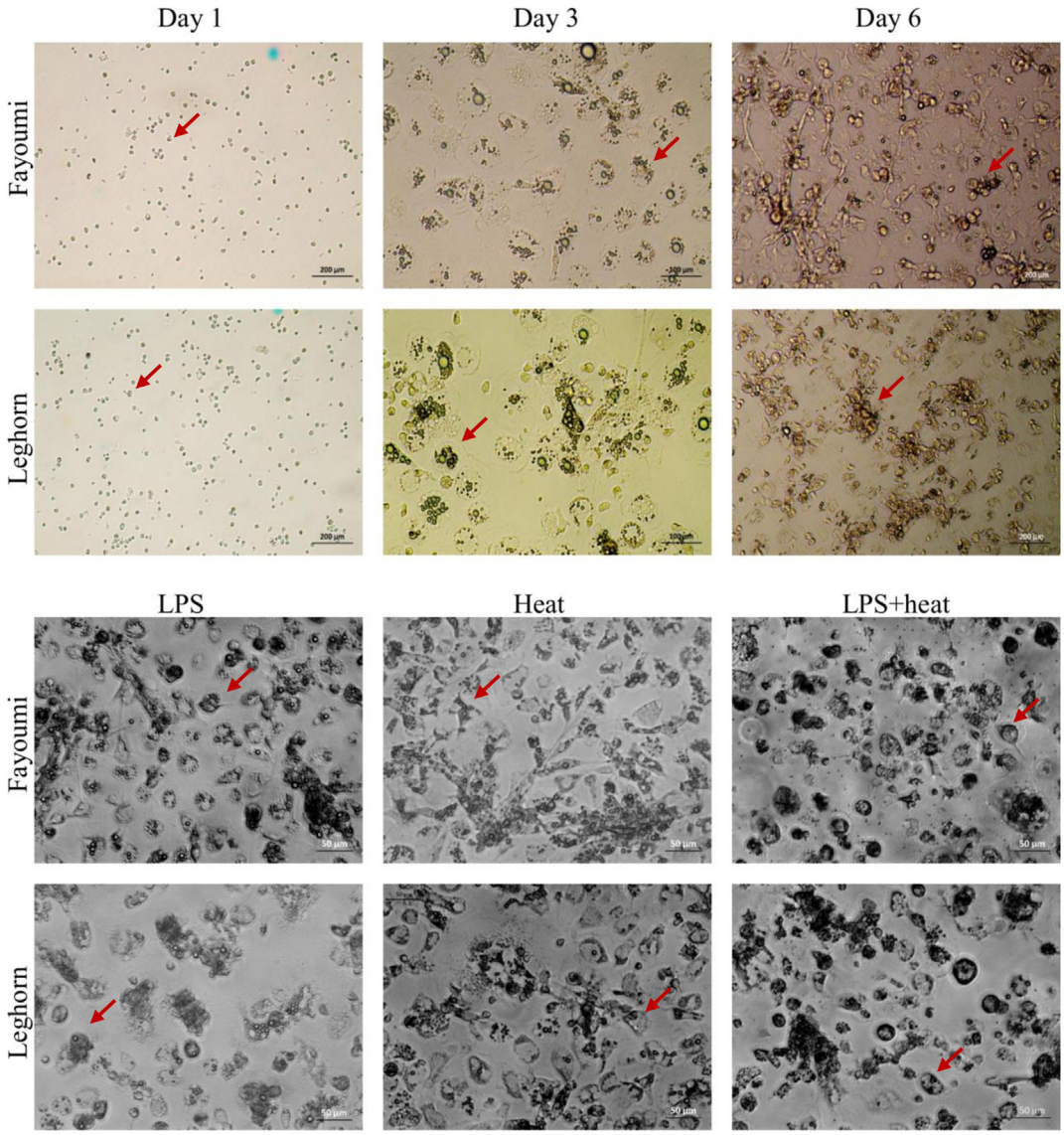


Figure 1. Growth and treatment pictures of chicken bone marrow derived dendritic cells (BMDC). Cells differentiated from bone marrow of chicken day 18 embryos displayed typical growth and morphology (day 1, 3, and 6) for Fayoumi and Leghorn BMDC. Arrows mark BMDCs. On day 6 of culture, cells were stimulated with various treatments. Treatments of cells included thermal neutral (41.5°) and medium alone (control), thermal neutral with 200 ng/mL lipopolysaccharide in the medium (LPS), heat for 2 h at 45 °C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery (LPS + heat). Pictures are representative examples of all experiments.

Table 2
BMDC viability values measure before and during experimental procedures using trypan blue exclusion assay and Propidium Iodide (PI) assay for flow cytometry.

Day	Treatment	Breed	
		Fayoumi	Leghorn
Day 6	None	88	89
Day 7 (trypan blue)	TN_Media	100	92
	TN_LPS	93	88
	HS_Media	93	87
	HS_LPS	93	96
Day 7 (PI)	TN_Media	88	87
	TN_LPS	79	80
	HS_Media	87	88
	HS_LPS	51	67

stained with PI as a viability marker and the average percent live cells was 78%. Gating for live cells and identified as BMDC were utilized for downstream analyses. The gating strategy used to determine phagocytic ability (percentage of bead + cells) and MHC II surface expression (percentage of MHC II + cells) is found in Fig. 3b. The phagocytic ability of cells is quantified in Fig. 3c. The percentage of bead + cells in the ice control was not different between lines (data not shown). Generally, treatment had little effect on phagocytic ability of BMDC. For the treatment groups of control, LPS, and heat, a larger percentage of Fayoumi cells were bead + compared to Leghorns. This relationship is reversed in the LPS + heat treatment group.

The surface expression of MHC II is quantified in Fig. 3d. Treatment had no effect on the surface expression of MHC II in the Leghorn line, but in the Fayoumi line, LPS increased the surface expression of MHC II. In all treatment groups, Fayoumi had statistically higher surface expression of MHC II.

Table 3
BMDC maturation status prior to treatments RT-PCR of CCR6, CCR7, and DC-LAMP.

Gene Forward primer Reverse primer	Fayoumi [(40-Ct) ± SEM]	Leghorn [(40-Ct) ± SEM]	P-value
CCR6[a] Fwd 5′: GCCAGCCGCAGAAGAATGTA Rev 5′: TGTGGAGAAGAGTTTCAGAATGCT	4.02 ± 0.69	5.09 ± 0.56	0.2575
CCR7[a] Fwd 5′: CATGGACGGCGGTAAACAG Rev 5′: TCATAGTCGTCGGTGACGTTGT	5.71 ± 0.26	6.93 ± 0.38	0.0255
DC-LAMP[b] Fwd 5′: CACACTTGGTCTTGCTAGCCTTT Rev 5′: TGACACCCAGGGCCACTT	13.71 ± 0.28	13.63 ± 0.41	0.8743

References:
[a] Wu et al. 2011.
[b] Wu et al. 2010b.

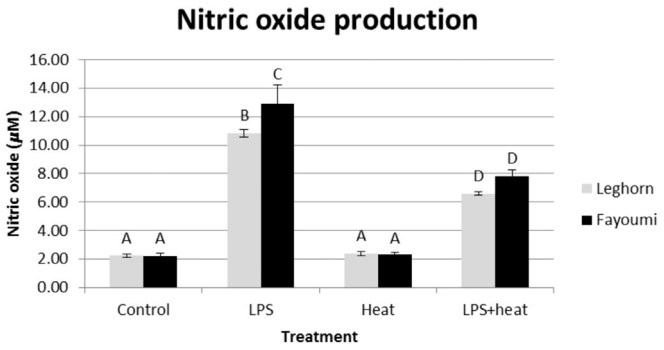


Figure 2. Nitric oxide production in chicken bone marrow derived dendritic cells (BMDC). Nitric oxide was measured using the supernatant of BMDC 24 h post treatment. Comparative analysis between BMDC from Fayoumi (black bars) and Leghorn (grey bars). Treatments of cells included thermoneutral and medium alone (control), thermoneutral with 200 ng/mL LPS in the medium (LPS), heat for 2 h at 45 °C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery (LPS + heat). Data are depicted as mean ± SEM; N = 7–9 from 3 independent experimental days. Different letters indicate significance with multiple testing correction $P < 0.05$.

3.5. mRNA expression in BMDC

Table 4 presents statistical significance of the effects in the gene expression study. The main effects that were tested included breed (Leghorn vs. Fayoumi), treatment (control, LPS, heat, and LPS + heat) and time point post-stimulation (2 h vs. 4 h vs. 8 h). Treatment had a highly significant effect on all the genes analyzed ($P < 0.0001$). Breed had a significant effect on all genes except *HSPH1*, *IFN-γ*, *IL-10*, *IL-12β* and *IL-6* ($P < 0.05$). Time had a significant effect on all genes, except *IL-12β* ($P < 0.05$). The interaction of breed and treatment had a significant impact on mRNA expression of *CASP9*, *CCL4*, *GM-CSF*, *HSP25*, *HSPH1*, *IL-15*, *IL-8*, *MHCII-β*, *TLR4* and *UBB* ($P < 0.05$) and a highly significant effect on *iNOS* ($P < 0.0001$). The interaction of breed and time had no significant effects on any gene tested and was, therefore, removed from the model.

3.5.1. Gene regulation in response to LPS

Relative expression of the analyzed target genes is presented in Figs. 4–6. Immune related genes are found in Fig. 4. In BMDC from both chicken lines the genes with the highest level of expression in response to LPS treatment alone were *IL-10*, *IL-6*, *CCL4*, *IL-1β* and *iNOS* ($P < 0.05$). Their expression peaked at the 2 h time point (reaching a fold change between approximately 300 and 2000), with exception of *iNOS* which had the highest mRNA fold change at the 4 h time point. The expression of *IL-10*, *IL-6*, *IL-1β* and *iNOS* was significantly higher in BMDC of Fayoumi than Leghorn ($P < 0.05$). The second set of genes that were also significantly up-regulated upon LPS treatment, but with lower fold change values (approximately between 10 and 70), included *CCL5*, *CD40*, *GM-CSF*, *IFN-γ*, *IL-12β* and *IL-8*. In general, induction of those genes (except *CCL5*) was also higher in BMDC from Fayoumi compared to Leghorn. Genes slightly up-regulated in response to LPS included *TGFβ2* (across all time points) as well as stress response genes (*BAG3*, *HSP25*, *HSPH1* and *HSPA2*) at 4 h time point. LPS induced a clear down-regulation of some genes involved in immune (*IL-15*, *TLR4*) and stress response (*CASP9*, *HSPA2* and *UBB*). *MHC-IIβ* and *IL-4* mRNA expression in response to LPS was regulated inversely in Leghorns and Fayoumis ($P < 0.05$); both genes were up-regulated in Leghorns but down-regulated in Fayoumis.

3.5.2. Gene regulation in response to heat

Heat-regulated genes are presented in Fig. 5. Heat treatment induced mRNA expression of the stress response genes, including anti-apoptotic *BAG3* and heat stress response genes (*HSP25*, *HSPA2* and *HSPH1*). The highest expression of those genes was detected directly after heat treatment (2 h); however, *HSP25* continued to be highly up-regulated also at later time points. The panel of immune response genes was not strongly induced by heat alone. Their expression slightly fluctuated, but typically did not exceed a fold change between 0.5 and 2.

3.5.3. Gene regulation in response to LPS + heat

The synergistic effects of LPS combined with heat were demonstrated by a down-regulation of the immune-related genes (*CD40*, *GM-CSF*, *IFN-γ*, *IL-12β*, *IL-15*, *IL-4*, *MHCIIβ* and *TLR4*) and the stress response genes *UBB* and *CASP9* ($P < 0.05$). This inhibition of mRNA expression was more evident at later time points, peaking at 8 h (fold induction between 0.23 and 0.01). Down-regulation of the immune-related genes was in most cases stronger in BMDC derived from Fayoumis than Leghorns ($P < 0.05$). Expression of the heat response genes (*HSPH25*, *HSPA2* and *HSPH1*) maintained a similar profile to heat treatment alone. However, genes that were highly up-regulated in response to LPS alone (*CCL4*, *CCL5* and *IL-10*) were still induced by the double stressor, but on much lower level.

3.5.4. Differences in mRNA expression in BMDC derived from Fayoumi and Leghorn

The relative gene expression of the inflammatory mediators upon LPS treatment was, in most cases, similar in Fayoumis and Leghorns. Overall, up-regulation of the Heat Shock Protein (HSP) genes upon heat treatment was similar between BMDC derived from both chicken lines. The major differences in response to LPS

stimulation were determined for *iNOS* expression, that was higher in Fayoumi (4 h − $P = 0.57$, 8 h − $P < 0.05$), which corresponds with the increased NO production at 24 h. The LPS treatment induced four times higher mRNA expression of anti-inflammatory *IL-10* in Fayoumi than Leghorn ($P < 0.05$). In Leghorn, LPS induced higher mRNA abundance of *CCL5*, *GM-CSF* and *IL-4* ($P < 0.05$).

The main differences attributed to the genetic component occurred in response to combined treatment with LPS + heat. Stress response genes, such as *HSPH1* and *BAG3* were up-regulated in Leghorns ($P < 0.05$). The inflammatory mediators *IL-1β*, *iNOS*, *IL-8*, *CCL4* and *CCL5* and markers of maturation *CD40* and *TGFβ2* were higher in Leghorn stimulated with LPS + heat, especially at 8 h time point ($P < 0.05$). At the same time, there was a very consistent and strong mRNA down-regulation in Fayoumi stimulated with LPS + heat in respect to maturation signatures (*MHCII-β*, *CD40*, *IL-15*), immune-related genes (*TLR4* and *IL-4*) and stress response genes (*CASP9* and *UBB*) ($P < 0.05$) in comparison to Leghorn.

4. Discussion

In the current study, we characterized the response of BMDC derived from unique and distinct inbred chicken lines to an inflammatory stimulus (LPS), an environmental stressor (heat), and the combination of both (LPS + heat). We chose a variety of *in vitro* assays (NO production, phagocytic ability, MHC II surface expression, and mRNA expression) to characterize the impact of genetics on the biological functions of chicken BMDC. Because BMDC in chicken have been previously characterized as antigen presenting cells (Wu et al., 2010a), we chose to focus on the responses of these cells to stimulations, and to quantify the differences between unique breeds of chickens. We anticipated BMDC from the Fayoumi line to be more responsive to stimuli compared to Leghorn, including higher phagocytic ability, more NO production, higher surface expression of MHC II, and differences in RNA-expression. The treatments in the current study were chosen to represent important abiotic (high temperature) and biotic (LPS, a bacterial component immune stimulant) stressors frequently encountered in poultry production, and to better understand the interaction of the combined stressors.

4.1. Nitric oxide assay

There are two categories of NO: calcium-dependent constitutive NO that is produced by endothelial and neural cells, and calcium-independent inducible NO (iNOS) produced by mononuclear phagocytes (Gross et al., 1995; MacMicking et al., 1997a). The production of iNOS is an important host defense against invading bacteria (Hibbs et al., 1988). Production of NO inhibits microbial proliferation and when NO production is interrupted, microbial burden increases (MacMicking et al., 1997b; Stenger et al., 1996). In humans, increased NO production results in better clinical outcomes during malaria infection (Anstey et al., 1996), supporting the hypothesis that higher production of NO is indicative of a more effective immune function. In chickens, there is a strong genetic component in the amount of NO production and this is associated with enhanced immunocompetence. For instance, NO production is higher in macrophages derived from chickens selected for high antibody response (Guimarães et al., 2011). Also, embryo fibroblasts from chicken lines genetically resistant to Marek's disease, generate higher NO levels upon LPS stimulation compared to a susceptible line (Xing and Schat, 2000).

In the current study, BMDC derived from Fayoumi birds produced significantly more NO compared to Leghorns in response to LPS stimulation. This difference in NO production was a modest amount and the relevance *in vivo* remains to be elucidated. However, previous studies have observed similar results as those reported here. Lines divergently selected for response to phytohemagglutinin-P show differences in resistance to *E. coli* attributed to Th1 activity levels (Sundaresan et al., 2005), and isolated PBMCs stimulated with Salmonella in the high selection line produced more NO, but this was not significant at 24 h post stimulation (Singh et al., 2012). Additionally, studies of chickens differing in susceptibility to Marek's disease herpes virus show that the resistant birds produce more NO compared to susceptible birds, 8.8 ± 2.0 and 2.3 ± 1.6 μM, respectively (Djeraba et al., 2002), but this large difference occurs after stimulation *in vivo* and a subsequent stimulation with IFN-gamma *in vitro*. When evaluating the genetic differences without *in vivo* stimulation and only *in vitro* stimulation with IFN-gamma, the lines show modest differences (4.9 ± 1.9 and 6.2 ± 1.7 μM). Another study using lines divergent for response to Marek's found subtle differences in NO production in the spleen when stimulated with LPS; the resistant line had 3.3 μM and the susceptible line had 1.3 μM NO production (Xing and Schat, 2000). Therefore, the degree of NO difference is dependent up the type of stimulation and time post stimulation.

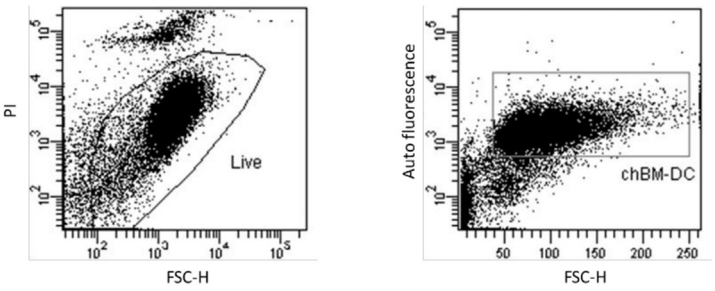
Inducible differences in NO were identified between chicken lines in response to LPS stimulation, but not in response to heat or the combination of LPS + heat. In humans, heat stroke patients have higher levels of plasma NO levels (Alzeer et al., 1999). The increase in NO *in vivo* could be attributed to "leaky gut" syndrome which occurs during periods of heat stress, and is caused by disruption of tight junctions in gut, allowing intestinal bacteria or LPS to enter into the blood stream (Dokladny et al., 2006). The current study produced no evidence that heat alone increases production of NO *in vitro*. However, the double stimulus (LPS + heat) resulted in decreased NO production in both chicken lines compared to LPS alone.

4.2. Phagocytic ability

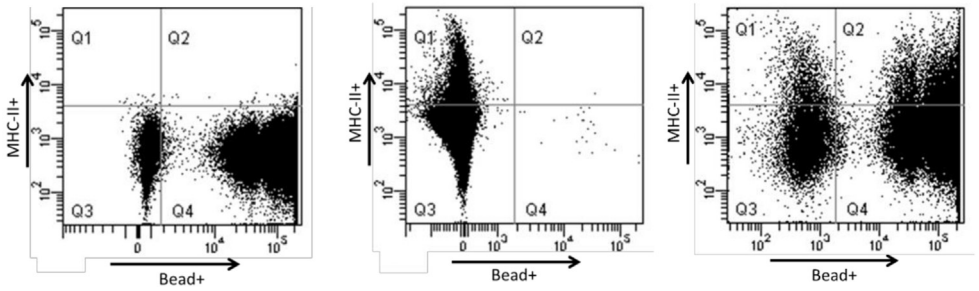
Assessing phagocytic ability is one way to determine the efficiency of the host at removing pathogens during an infection. DC endocytose through both non-specific mechanisms, such as micropinocytosis, and specific mechanisms, such as receptor-mediated endocytosis and phagocytosis (Trombetta and Mellman, 2005). Upon maturation, APC alter chemokine and cytokine expression patterns and also undergo various morphological and functional changes, including changes in phagocytic ability (Guermonprez et al., 2002). Phagocytic ability is higher in macrophages from chickens selected for high antibody response (Guimarães et al., 2011), indicating genetic control of this function exists and also that chickens with a "primed" immune system phagocytize more. However, the primary role of specialized APC such as DC is not only to engulf, but also to process the antigens for presentation and subsequent activation of the adaptive immune system. Therefore, a critical function of APC is that upon maturation, phagocytic ability decreases so the cells can move to sites of T cells for antigen presentation (Sallusto et al., 1995). However, in the current study we chose to assay phagocytic ability of BMDC upon immediate exposure to LPS to determine how "primed" the BMDC were upon initial interaction with the PAMP rather than switching from immature to mature phenotype, in which we would anticipate a decrease in phagocytic ability similar to what has been shown by Wu et al. 2010a.

Phagocytic ability was determined using fluorescently labeled beads and quantified using flow cytometry. The BMDC from Fayoumis had significantly higher percent bead + cells compared to Leghorns under control conditions, LPS, and heat. Few differences in phagocytic ability were identified due to treatment. In conclusion, BMDC from Fayoumis phagocytosed more efficiently,

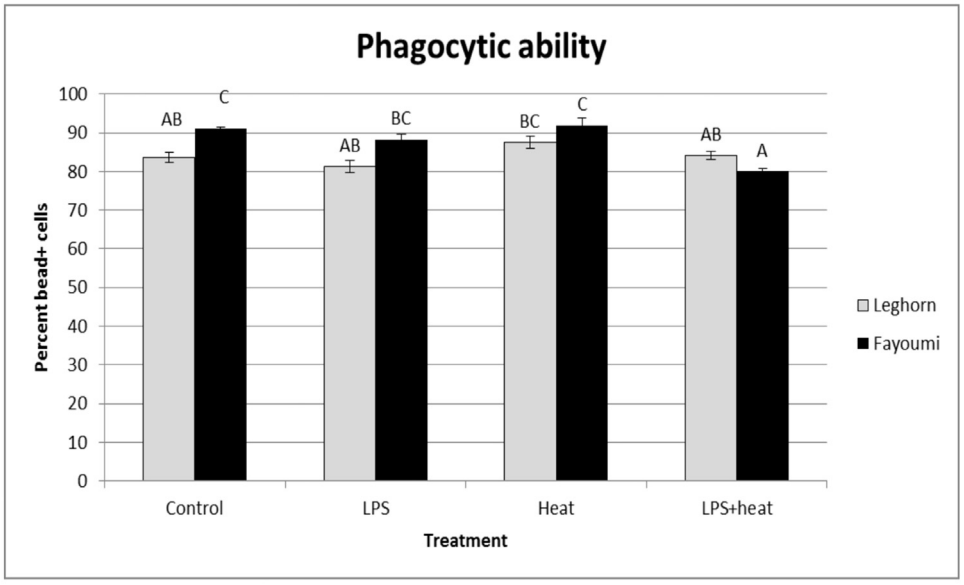
a)


PI
Live
FSC-H
Auto fluorescence
chBM-DC
FSC-H

b)


Q1
Q2
Q3
Q4
MHC-II+
Bead+

c)


Phagocytic ability
Percent bead+ cells
AB
C
AB
BC
BC
C
AB
A
Control
LPS
Heat
LPS+heat
Treatment
Leghorn
Fayoumi

d)

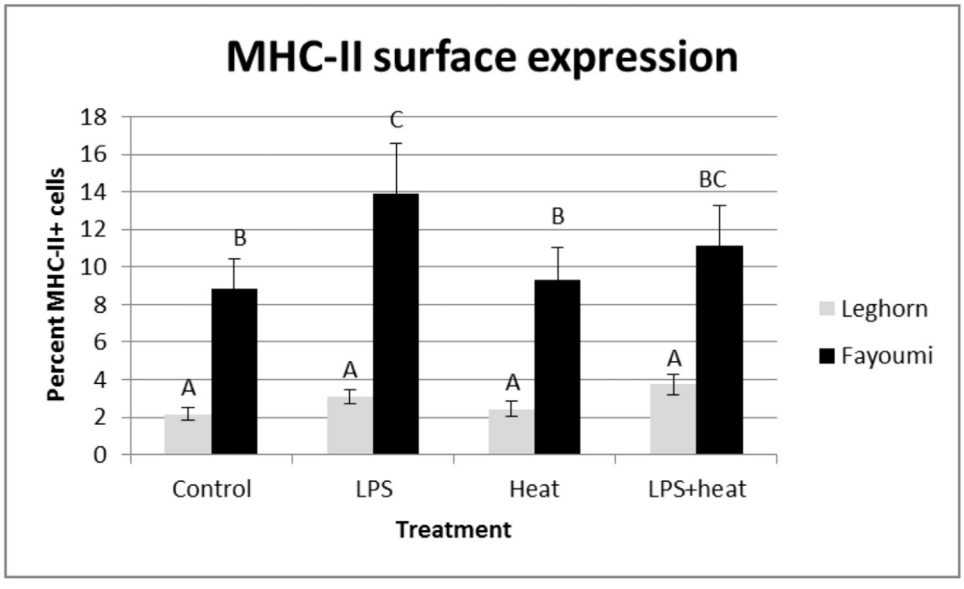

MHC-II surface expression
Percent MHC-II+ cells
A
B
A
C
A
B
A
BC
Control
LPS
Heat
LPS+heat
Treatment
Leghorn
Fayoumi

Table 4
Significant effects of treatment on mRNA expression of chicken bone marrow derived dendritic cells (BMDC).

Gene	Line	Treatment	Time	Line*treatment
IL-1β	<0.0001	<0.0001	<0.0001	0.3239
IL-6	0.6212	<0.0001	0.0024	0.0842
IL-8	<0.0001	<0.0001	<0.0001	0.0261
IL-10	0.6262	<0.0001	<0.0001	0.3876
iNOS	<0.0001	<0.0001	<0.0001	<0.0001
CCL4	0.0365	<0.0001	<0.0001	0.0009
CCL5	<0.0001	<0.0001	0.0009	0.0522
TLR-4	<0.0001	<0.0001	0.0005	0.0059
IL-4	<0.0001	<0.0001	0.0147	0.0523
GM-CSF	<0.0001	<0.0001	<0.0001	0.0023
HSP25	<0.0001	<0.0001	<0.0001	0.0045
HSPA2	0.0018	<0.0001	<0.0001	0.424
HSPH1	0.2599	<0.0001	<0.0001	0.0216
BAG3	0.0001	<0.0001	<0.0001	0.4347
CASP9	<0.0001	<0.0001	0.0051	0.008
UBB	<0.0001	<0.0001	<0.0001	0.0014
MHC-IIβ	<0.0001	<0.0001	<0.0001	0.0008
CD40	<0.0001	<0.0001	<0.0001	0.0939
IFN-γ	0.0998	<0.0001	<0.0001	0.7693
IL-1β	0.521	<0.0001	0.8066	0.4405
IL-15	0.0116	<.0001	<0.0001	0.0002
TGFβ	0.0024	<0.0001	<0.0001	0.3365

Twenty two genes were tested for mRNA expression levels. Chicken line (Fayoumi and Leghorn), treatment (control, LPS, heat, and LPS + heat) and time post treatment (2 h, 4 h or 8 h) as well as the interaction between line and treatment were fitted in the model. Analyses were performed separately for each line, gene, and treatment using dCt values (Ct target − Ct reference). To determine the relative gene expression, ddCt method was used. All treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. Significance is considered if $P \leq 0.05$.

compared to Leghorns. Sun et al. (2008) suggested that phagocytic ability *in vitro* is a good predictor of disease resistance in chickens, which may indicate that BMDC from Fayoumis are more potent in generating innate immune responses (i.e. phagocytosis) that those of Leghorn.

4.3. MHC II surface expression

Surface expression of MHC II is limited to a small number of cells including thymic epithelial cells, B cells, macrophages, and DC (Pieters, 2000). Expression of MHC II is an intrinsic characteristic of APC, which allows them to present foreign antigens to T cells and subsequently activate the adaptive immune response (Steinman, 1991). Maturation of APC by an immune stimulus, such as LPS, or pathogen challenge results in a redistribution of MHC II proteins from the intracellular compartments to the cell surface, resulting in an increase in MHC II surface expression (Cella et al., 1997; Pierre et al., 1997; Turley et al., 2000). However, few studies to date have identified the effect of high temperature on APC activity and maturation (Ostberg and Repasky, 2006). The cellular origin and thermal stress conditions are determining factors in whether there is an increase in MHC II surface expression (Ostberg et al., 2003), or no change (Tournier et al., 2003).

BMDC from the Fayoumi responded to treatment with LPS and LPS + heat by increasing surface expression of MHC II, which was the expectation based on previous reports (discussed above). No difference in MHC II surface expression in BMDC from Leghorn was identified among any treatment groups, which was an unexpected outcome. BMDC from Leghorn had significantly lower surface expression compared to Fayoumi. Because MHC II surface expression was only assayed at one time point (4 h post stimulation), the Leghorn may simply be slower at moving MHC II to the cellular surface upon stimulation compared to Fayoumis. Another explanation for the observed difference in MHC II surface expression is that the genetic lines may have different avidity for the monoclonal antibody utilized to quantify the expression. We included MHC II beta in the mRNA expression portion of the experiment, and both lines significantly differed in their expression at all time points and treatments. However, the mRNA expression and surface expression studies were contradictory. The Fayoumi displayed significant down-regulation in all treatments, except heat at 4 h, whereas the direction of change due to treatment in the Leghorn was time and treatment dependent. Both lines showed extreme downregulation of MHC II mRNA expression when treated with LPS + heat.

In conclusion, BMDC from the Fayoumi had inherently higher levels of MHC II surface expression, in control and all treatment groups, compared to the Leghorn. The MHC II surface expression of BMDC from Fayoumi were responsive to treatments that included LPS, but not to heat alone While MHC II surface expression from BMDC from Leghorn did not respond to any treatment at the times assayed. The ability to rapidly change MHC II surface expression upon stimulation indicates a higher potency of Fayoumi in activating adaptive immune responses by mature DC.

4.4. mRNA expression in BMDC treated with LPS, heat, and LPS + heat

4.4.1. Up-regulation of the inflammatory mediators with LPS

Endotoxemia activates immature DC and rapidly modulates their gene expression towards secretion of inflammatory signals. Inflammatory DC migrate to spleen, where they regulate adaptive immunity through recruitment of the microbe-specific T cells (Sallusto and Lanzavecchia, 2000). In our study, LPS treatment induced high levels of pro-inflammatory cytokines (*IL-1β*, *IL-6*) and chemokines (*CCL4*, *CCL5* and *IL-8*) as well as inducible nitric oxide synthase (*iNOS*) in BMDC. Expression of these inflammatory mediators belongs to the core function of mature DC (Foti et al., 2006). Chemotaxis initiated by inflammatory chemokines aims to attract other cells with chemokine receptors, such as monocytes/macrophages, T and B cells, NK and immature DC to the inflammation site. DC are also involved in the migratory pattern that is an intrinsic feature of DC (Dieu-Nosjean et al., 1999). Furthermore, LPS-activated BMDC expressed a high level of the anti-inflammatory cytokine, *IL-10*, which alleviates the potentially harmful effects of prolonged inflammation by immunosuppressive activity via

Figure 3. Flow cytometry gating strategy of chicken bone marrow derived dendritic cells (BMDC). a) General gating strategy to identify BMDCs. Cells were stained with PI as a marker of viability and gating was done on live cells. Using the live cell population, BMDC were identified based on forward scatter and side scatter (auto fluorescence) characteristics, then used for downstream analyses. Flow cytometry plots are representative samples from all experiments. b) Gating strategy to determine phagocytic ability (bead+), MHC-II surface expression (MHC-II+), and an illustration for samples which were both bead+ and MHC-II+. c) Phagocytosis ability. Fluorescently labeled polystyrene beads were administered at 20 beads/cell to BMDC to compare phagocytic ability differences between treatment and genetic line. Treatments of cells included thermoneutral and medium alone (control), thermoneutral with 200 ng/mL LPS in the medium (LPS), heat for 2 h at 45 °C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery (LPS + heat). Data are depicted as mean ± SEM; N = 7 from 3 independent experiments. Different letters indicate significance with correction for multiple testing; $P < 0.05$. d) MHC-II surface expression. Cells were stained with MHC-II-PE antibody after treatments. Treatments of cells included thermal neutral and medium alone (control), thermal neutral with 200 ng/mL lipopolysaccharide in the medium (LPS), heat for 2 h at 45 °C followed by temperature recovery and medium alone (heat), and 200 ng/mL lipopolysaccharide in combination with heat treatment for 2 h at 45 °C followed by temperature recovery (LPS + heat). Data are depicted as mean ± SEM; N = 6–7 from 3 independent experiments. Different letters indicate significance with correction for multiple testing; $P < 0.05$.

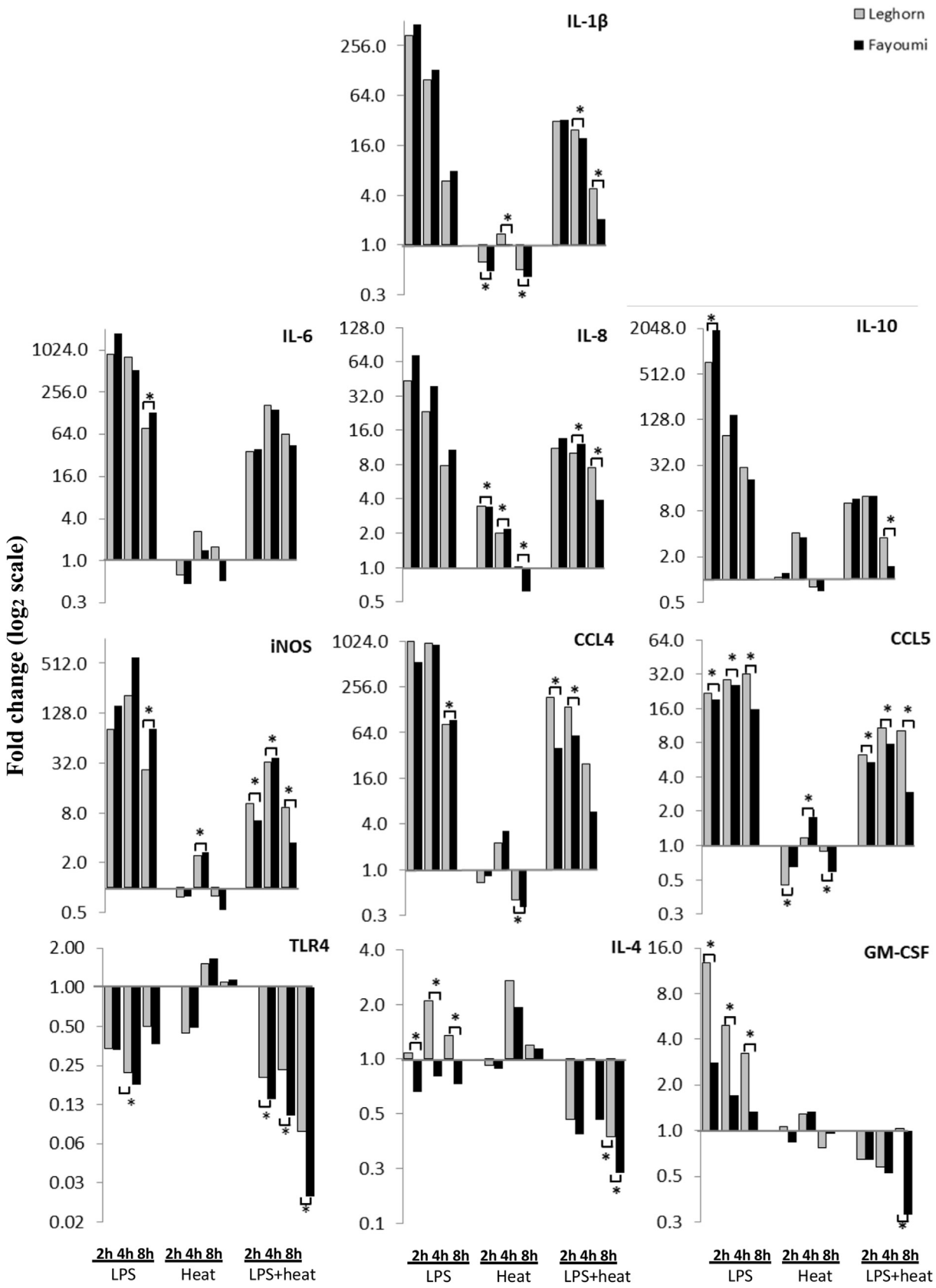


Figure 4. Immune-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC). Ten genes were tested for immune-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2 h at 45 °C followed by temperature recovery (heat), (3) LPS + heat: 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery, and (4) untreated (control) samples were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2 h, 4 h, and 8 h post-treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes (*H6PD* and *RPL4*) (dCt = Ct target − Ct reference). Data are depicted as fold change (FC) of the gene expression. FC was estimated as 2^{-ddCt}. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student's T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.

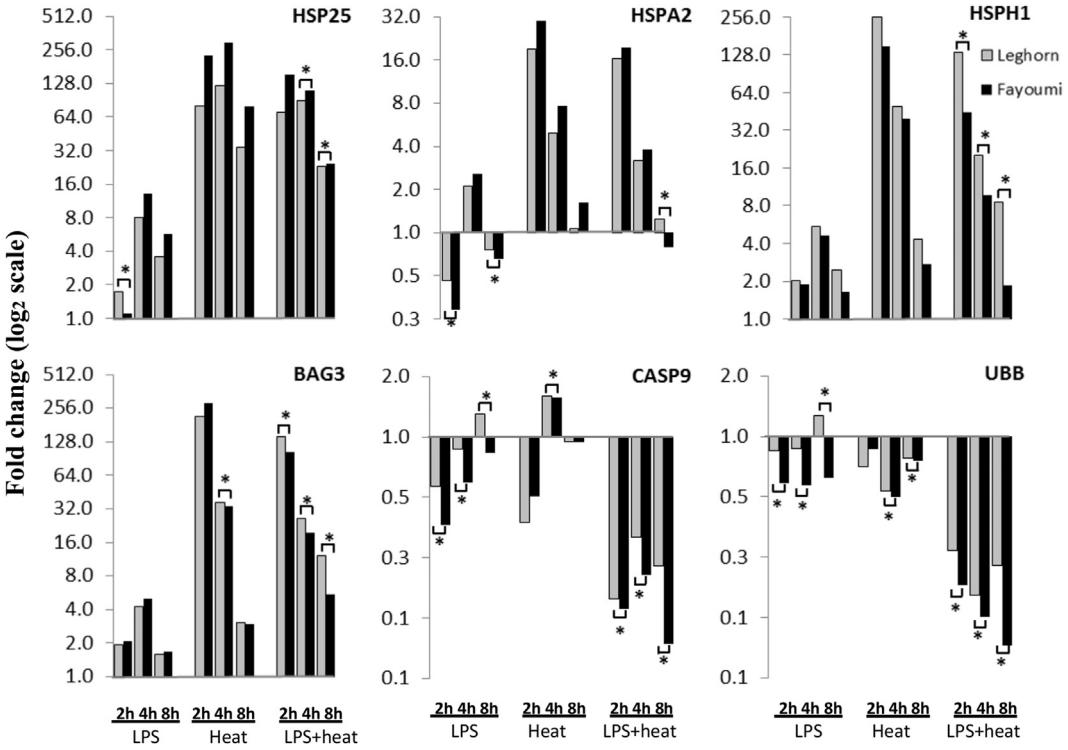


Figure 5. Stress-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC). Six genes were tested for heat-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2 h at 45 °C followed by temperature recovery (heat), (3) LPS + heat: 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery, and (4) untreated (control) samples were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2 h, 4 h, and 8 h post-treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes (*H6PD* and *RPL4*) dCt values (Ct target − Ct reference). Data are depicted as fold change (FC) of the gene expression. FC was estimated as 2^{-ddCt}. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student's T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.

negative signaling of MyD88-dependent signaling (Chang et al., 2009). LPS also induced expression of *iNOS*, which plays an important role in early antimicrobial defense (Serbina et al., 2003). Induction of *iNOS* was in agreement with increased NO production in LPS-activated chicken BMDC in the current study, as discussed earlier. Even though inflammatory responses were up-regulated by LPS, the expression of the TLR4 receptor, which binds LPS, was down-regulated in our study. Such a phenomenon is in agreement with the literature and helps to control the host inflammatory response by preventing excessive production of pro-inflammatory cytokines that lead to endotoxic shock (Liew et al., 2005). However, we did not investigate the surface expression of the TLR4 receptor and cannot conclude that this recycling mechanism was occurring in the current study. It would be useful to analyze the TLR4 surface expression in response to LPS, and to identify if the genetic lines differ in the ability to change the surface expression of the receptor upon stimulation. This study did assay downstream mRNA expression of the activation elicited by TLR4 activation such as *IL-1β*, *IL-6*, *iNOS*, and *INF-γ*, and the breeds exhibited similar expression patterns in these genes in response to stimulation.

4.4.2. Th-1/Th-2 balance in BMDC treated with LPS

An important function of DC, associated with their maturation stage, is polarization of the Th cell-mediated immune responses into Th-1 and Th-2 by secreting specific, immunoregulatory cytokines. Cross-regulatory cytokines *IL-12* and *IL-10* induce polarization of the T cells into Th-1 and Th-2, respectively (Corinti et al., 2001). *IL-12* is a critical cytokines that activates naïve T cells recruitment into Th-1 cells, and therefore triggers cell-mediated immunity (Sartori et al., 1997). In contrast, autocrine *IL-10* expression was shown to inhibit *IL-12* (De Smedt et al., 1997) that is required for *IFN-γ* production in BMDC (Fukao et al., 2001) and, as such, favors Th-2 immunity. In our study, *IL-10* mRNA abundance in LPS-treated BMDC was extremely high. However, there was also a significant up-regulation in expression of *IL-12β* and *IFN-γ*, which suggests the ability of the activated BMDC to induce Th-1 polarization even with the high levels of *IL-10*. However, the surface expression of IL-12β was not assayed in the current study. A commercially available antibody (KingFisher Biotech, Saint Paul, MN, US) is available for IL-12, but this is a polyclonal antibody that also recognizes IL-23 in chicken, thus limiting its usefulness. Autocrine IL-10 prevents spontaneous *in vitro* maturation of the DC and therefore plays an important regulatory role (Corinti et al., 2001). Mature DC can lose sensitivity to autocrine IL-10 through reduction in the activity of the IL-10 receptor on the cell surface (Corinti et al., 2001). In this study, LPS treatment only minimally regulated *IL-4* mRNA expression in BMDC – a cytokine that drives Th-2 responses. BMDC do not produce high levels of *IL-4* in response to LPS in both mammalian (Yao et al., 2005) and avian (Wu et al., 2010a, 2010b) systems. Overall, the current results suggest that there was a bias towards Th-1 cell-mediated immune responses in chicken BMDC derived from both genetic lines in response to LPS.

4.4.3. Expression of markers of maturation in BMDC

Recognition of PAMPs by TLRs leads to increased expression of

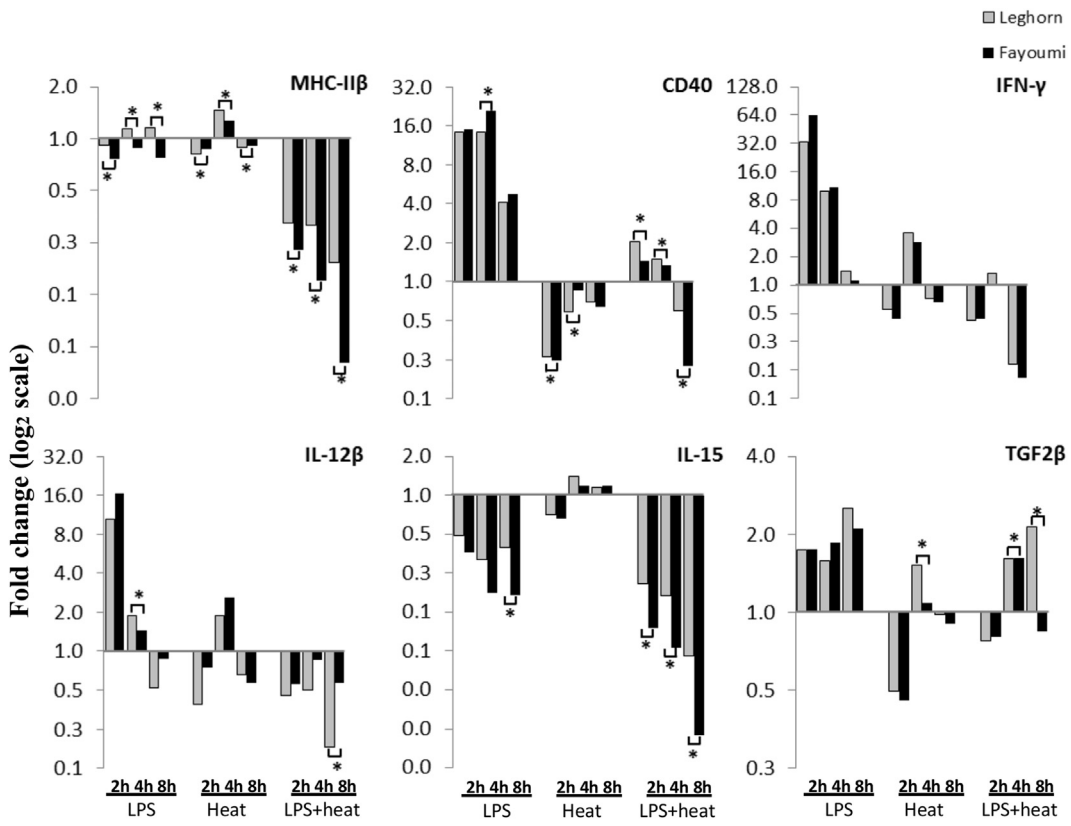


Figure 6. Maturation-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC). Six genes were tested for heat-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2 h at 45 °C followed by temperature recovery (heat), (3) LPS + heat: 200 ng/mL LPS in combination with heat treatment for 2 h at 45 °C followed by temperature recovery, and (4) untreated (control) samples were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2 h, 4 h, and 8 h post-treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes (*H6PD* and *RPL4*) dCt values (Ct target − Ct reference). Data are depicted as fold change (FC) of the gene expression. FC was estimated as 2^{-ddCt}. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student's T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.

the markers of maturation in DC, such as CD40 or MHC II (Liang et al., 2013; Wu et al., 2010a). In this study, significant up-regulation of *CD40* mRNA upon LPS treatment indicates maturation of BMDC. The surface receptor CD40 takes part in a cross-talk between APC and T and B cells (Ma and Clark, 2009). However, mRNA abundance of *MHC-IIβ* was practically unchanged in LPS-stimulated BMDC, which is seemingly in disagreement with increased surface expression of MHC II reported in our study. Inhibition of *MHC-IIβ* mRNA by LPS was previously reported through the MyD88 pathway (Simmons et al., 2012). Regarding increased surface expression of MHC II molecule, it had been demonstrated that immature DC effectively and continuously express pathogen-associated peptide-MHC II complexes and their rapid turnover is regulated by ubiquitination. DC activation with LPS decreases MHC II ubiquitination, endocytosis and degradation and therefore increases retention of existing MHC II molecules (Walseng et al., 2010).

4.4.4. Heat treatment triggers molecular chaperones in BMDC

Heat treatment greatly increased expression of *HSP25* and *HSPH1* and moderately increased *HSPA2* expression in BMDC in both chicken lines. These genes encode ATP-dependent (*HSPA2* and *HSPH1*) and ATP-independent (*HSP25*) chaperones that are responsible for maintaining proper folding of the cytosolic proteins, unfolded during heat stress. Temperature-inducible chaperones are responsible for achieving thermo-tolerance. Induction of HSP by heat stress has been reported by other authors studying chicken using *in vitro* (unpublished data, Slawinska et al.; Sun et al., 2015) and *in vivo* (Wang et al., 2013) models, which indicates their conserved physiological role in heat response. In addition, HSP can modulate the immune system by acting like a cytokine (Ferat-Osorio et al., 2014). DC recognize chaperones that bind to their surface receptors and trigger release of inflammatory cytokines and chemokines. Recently, it has been proposed to use chaperones as vaccine adjuvants to deliver the peptide directly to DC MHC II molecules for T cell priming (McNulty et al., 2013). Here, we report expression of three major HSP genes in chicken BMDC that could be potential targets for vaccine development.

4.4.5. Heat treatment down-regulates LPS-induced immune genes

Heat treatment combined with endotoxemia did not modify the high level of mRNA abundance of the heat response genes, i.e. HSP (*HSP25*, *HSPH1* and *HSPA2*) and anti-apoptotic *BAG3*. In contrast, immune-response genes (*IL-1β*, *IL-6*, *IL-8*, *IL-10*, *iNOS*, *CCL4* and *CCL5*) were expressed at lower level, compared to LPS treatment alone. The most distinct changes in the gene expression under combined LPS and heat treatment resulted in the significant down-regulation of immune- and maturation-related genes *TLR4*, *IL-4* and *GM-CSF*, *MHC-IIβ*, *CD40*, *IFN-γ*, *IL-12β* and *IL-15*, as well as stress-response genes *UBB* and *CASP9*, especially at the later time point

(8 h). The question emerges, whether those changes result from the negative effect of heat treatment on cells or are they rather induced by the protective role of HSP against elevated inflammatory processes. High expression of the molecular chaperones combined with strong down-regulation of the apoptotic gene (*CASP9*) and ubiquitin B gene (*UBB*) involved in the protein degradation, suggests induction of the protective mechanisms in BMDC subjected to biotic (LPS) and abiotic (heat) stresses.

However, HSP are also known to modulate expression of inflammatory mediators (Ferat-Osorio et al., 2014). Ferlito and Maio (2005) reported induction of TNFα in heat-stressed and LPS-treated promonocytic cells, but not in the differentiated macrophage-like cells (Ferlito and De Maio, 2005), suggesting an impact of heat stress temperature, temperature-recovery period and differentiation stage of the cells on the immunomodulatory effect of heat treatment. In our earlier study (unpublished data, Slawinska et al.), a chicken macrophage-like cell line (HD11) was stimulated with LPS, heat, and LPS + heat using identical conditions to the current study. LPS + heat treatment increased expression of inflammatory cytokines in HD11 cells even more than LPS treatment alone. However, BMDC studied here are much more differentiated in comparison to the HD11 cell line. As such, they might have developed distinct HSP mediated regulatory mechanisms to protect cells from endotoxic shock.

4.4.6. Genetic background influences mRNA expression in BMDC under heat and LPS

BMDC analyzed in this study were derived from two distinct chicken genetic lines (Leghorn and Fayoumi), characterized by different immune responses (regular vs. robust). The genetic background of the bone marrow donors was reflected in gene expression of BMDC treated with LPS + heat. Leghorn BMDC were characterized by overall higher inflammatory responses to LPS under heat conditions than Fayoumi BMDC. Intestinal inflammation is one of the major detrimental effects of heat treatment combined with *Salmonella* challenge in poultry (Quinteiro-Filho et al., 2012). It may lead to systemic inflammation, multi-organ failure and sepsis (Leon and Helwig, 2010). In such cases, the ability of the organism to lessen the inflammatory responses may be a pro-survival strategy. The mechanism of this strategy is to reduce LPS signal transduction from the surface receptor TLR4 to the inside of the cell. In Fayoumi BMDC, expression of *TLR4* was significantly lower than the in Leghorn BMDC, followed by decreased expression of inflammatory cytokines and chemokines. We hypothesize that adaptation of Fayoumi chicken line to high ambient temperatures may be based on the ability of the BMDC to efficiently reduce heat induced inflammatory responses. In this basis, DC with their potent immunoregulatory role are a good target of the further evaluation of the genetic adaptation to heat.

5. Conclusions

DC serve as a bridge between the innate and adaptive arms of the immune system. For the first time, differences in response of chicken BMDC to treatment with an inflammatory stimulus (LPS), an environmental stimulus (heat), and the combination of both (LPS + heat) were characterized using BM sourced from inbred Fayoumi (disease resistant) and Leghorn (disease susceptible) lines. A variety of *in vitro* assays characterized the innate response to these complex stimulations and included NO production, phagocytic ability, MHC II surface expression, and mRNA expression. Upon stimulation, BMDC from Fayoumis produced more NO, had higher phagocytic ability, and inherently had higher MHC II surface expression. Gene expression for the heat related genes *BAG3*, *HSP25*, *HSPA2*, and *HSPH1* was strongly induced in response to heat treatment with few differences between lines, indicating conservation of this response. Gene expression for the immune related genes *CCL4*, *CCL5*, *CD40*, *GM-CSF*, *IFN-γ*, *IL10*, *IL-12β*, *IL-1β*, *IL-6*, *IL-8*, and *iNOS* were highly induced in response to LPS and differences between lines were both gene- and time-dependent. Genes that were strongly reduced due to all treatments include *CASP9*, *IL-15*, *IL-4*, *MHC-IIβ*, *TGFβ2*, *TLR4*, and *UBB*, and were largely different between lines. This research contributes to the sparse knowledge of genetic differences in DC biology in chickens using *in vitro* methods. The results of this research may contribute to future strategies used to develop effective immunomodulators and vaccines, and to breed for more disease resistant and heat tolerant chickens.

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