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# Epigenetic regulation of pro-inflammatory cytokine genes in lipopolysaccharide -stimulated peripheral blood mononuclear cells from broilers

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ABSTRACT

Inflammatory response which can be mediated by inflammatory genes, can be induced by pathogenic microorganisms, be associated with enteric diseases and the loss of growth performance in broilers. The understanding of epigenetic regulation of inflammatory genes could help explain the response to infection of microorganisms and inhibit the reaction of inflammation in broilers. This study investigated the effect of histone acetylation by histone deacetylases (HDAC) inhibitors trichostain A (TSA) and DNA methylation by demethylation agent 5-Aza-2′-deoxycytidine (AZA) and methyl donor methionine (Met) and folic acid (FA) on the expression of pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) from healthy broilers. The results showed that the mRNA expression of *IL-1β*, *IL-6* and *TNF-α* can be down-regulated by pre-treatment of TSA in LPS-stimulated broiler PBMC. The expression of pro-inflammatory cytokines related with the expression of *HDAC7* and *HDAC10* which can influence histone acetylation, and may also be affected by increasing the acetylation of non-histone proteins. The demethylation by AZA increased the expression of *IL-6* and *TNF-α* in LPS-stimulated broiler PBMC. The addition of FA and Met decreased the expression of DNA methyltransferases *(DNMT)1* and *DNMT3a*, while the Met also down-regulated the expression of *IL-6* and *TNF-α* in LPS-stimulated cells. In addition, the Met administration increased the methylation of −191 CpG site (up-stream from transcription start site) of *IL-6* and −419 CpG site of *TNF-α*. This study indicated that the expression of pro-inflammatory cytokines is regulated by protein acetylation. Demethylation also increased the expression of *IL-6* and *TNF-α*, which can be regulated by Met through increasing the promoter methylation. These results may have implications for controlling inflammation by epigenetic regulation in broilers.



## 1. Introduction

Inflammation, a complex response to pathogenic microorganisms infection, injured tissue and other environmental factors involved in feeding and rearing, could contribute enteric diseases and the loss of growth performance and economic income to the animal industry. The inflammatory response is orchestrated by pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Takeuchi and Akira, 2010), whose expression can be induced by lipopolysaccharide (LPS) *in vivo* (Wang et al., 2014; Liu et al., 2015) and *in vitro* (Wang et al., 2013a,b). The inflammatory genes of LPS signaling can be regulated by TLR4 signaling pathway (Kawai and Akira, 2006; Newton and Dixit, 2012), and also can be regulated by epigenetic mechanisms (Hübner et al., 2013; Poplutz et al., 2014).

Several studies demonstrated that the expression of *IL-1β*, *IL-6*, and *TNF-α* is related to their promoter chromatin structure (Poplutz et al., 2014; Wessels et al., 2013) and promoter DNA methylation (Tekpli et al., 2013; Wilson 2008).

Histone acetylation which plays key roles in remolding chromatin conformation is highly dynamic and regulated by the opposing action of two families of enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC) (Bannister and Kouzarides, 2011). HDACs remove acetyl groups from histones resulting a condensed chromatin structure reducing access by

*Abbreviations:* HDAC, histone deacetylase; TSA, trichostain A; AZA, 5-Aza-2′-deoxycytidine; Met, methionine; FA, folic acid; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; HAT, histone acetylase; SAM, S-adenosylmethionine; DNMT, DNA methyltransferase; SAH, S-adenosylhomocysteine.

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transcription factors, finally preventing gene transcription. It is recognised that HDACs can also deacetylate non histone proteins (Glozak et al., 2005). Today trichostatin A (TSA) is used mainly as a reference substance to examine the effects of deacetylation inhibition on genes expression (Halili et al., 2010; Munro et al., 2013).

DNA methylation influences the expression of some genes and depends upon the availability of methyl group from S-adenosylmethionine (SAM) and DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) that transfer methyl group from SAM to cytosines within CpG dinucleotides (Niculescu and Zeisel, 2002), by convert SAM to S-adenosylhomocysteine (SAH). Many reports analyzed the effect of methylation on gene expression by AZA which can inactivate DNMTs (Wessels et al., 2010; Poplutz et al., 2014). Methionine (Met), an essential amino acid, plays a unique role in DNA methylation processes by serving as the penultimate methyl donor for methylation reactions (Waterland, 2006). Folic acid (FA) plays an essential role in one-carbon transfer involving remethylation of Hcy to Met (Kim, 2005). Supplementation with high-dose folic acid attenuated LPS-induced *IL-1β*, *IL-6* and *TNF-α* in pregnant mice (Zhao et al., 2014). Maternal FA supplementation could elevate *DNMT1* expression in intrauterine growth retardation piglets (Liu et al., 2011).

Several studies have indicated that pro-inflammatory cytokines expression can be regulated by histone acetylation and DNA methylation, there is less study about the epigenetic regulation of inflammatory genes in broiler PBMC. In the present study, we investigated the effect of histone acetylation by TSA, the effect of DNA methylation by AZA, Met and FA on inflammatory genes expression in LPS-stimulated broiler PBMC.

## 2. Materials and methods

### 2.1. Cell isolation and cultivation

Blood (10 mL) was collected in a heparinized centrifuge tube from the wing vein from each of six healthy male Cobb broilers who were two to three months old. The experiment was approved by the Animal Care and Use Committee of Northwest A&F University. PBMC were isolated using lymphocyte separation medium (TED science, Tianjin, China) by centrifuging for 25 min at 2000 × *g*. The cells were washed twice with RPMI1640 culture and then resuspended in RPMI1640 (normal, no folic acid or no methionine; Gibco, Carlsbad, CA) complete culture medium and then seeded at a concentration of $5 \times 10^6$ cells/mL in 6-well plates. Complete culture medium used was RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). The cells were cultured for a total 48 h at 37 °C in a humid atmosphere of 5% $CO_2$.

### 2.2. Experimental design

#### 2.2.1. Trial 1

As shown in Fig. 1, broiler PBMC were cultured 48 h, PBMC cells were incubated with or without TSA (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) (5 μM) for 4 h, and subsequently were stimulated with or without LPS (*E. coli* 055: B5; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) (10 mg/L) for 3 h prior to harvest cells, six replicates for each treatment. The cells were collected and lysed in RNAiso Plus (Takara, Dalian, China) and stored at −80 °C prior to RNA extraction.

#### 2.2.2. Trial 2

As shown in Fig. 2, broiler PBMC were cultured 48 h, PBMC cells were incubated with or without AZA (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) (1 mg/L) for 4 h, and subsequently were stimulated with or without LPS (10 mg/L) for 3 h prior to harvest cells, six replicates for each treatment. The cells were lysed in RNAiso Plus and stored at −80 °C prior to RNA extraction.

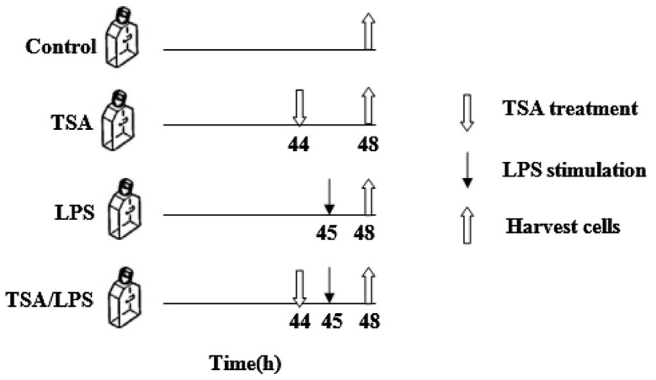


**Fig. 1.** Experimental design for trial 1. *In vitro* treatments of peripheral blood mononuclear cells (PBMC) with Trichostatin A (TSA, 5 μM) and lipopolysaccharide (LPS, 10 mg/L).

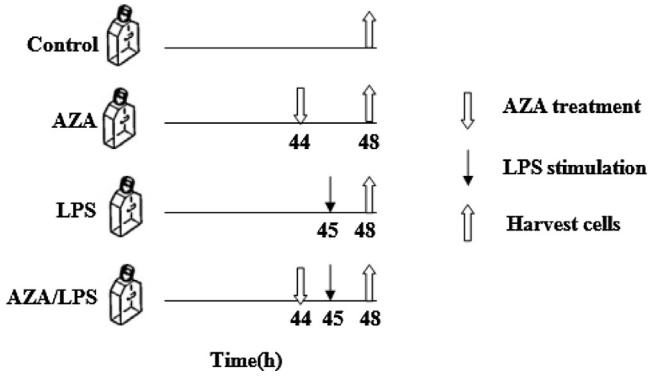


**Fig. 2.** Experimental design for trial 2. *In vitro* treatment of PBMC with 5-aza-2′-deoxycytidine (AZA, 1 mg/L) and LPS (10 mg/L).

#### 2.2.3. Trial 3

This trial used a 3 × 3 factorial design. The main factors were L-Met (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) concentrations (7.5, 15, 30 mg/L, marked Low-Met, Medium-Met, High-Met) and FA (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) concentrations (0.5, 1, 2 mg/L, marked Low-FA, Medium-FA, High-FA). The L-Met concentrations of 15 mg/L and FA concentrations of 1 mg/L were the normal concentration in RPMI1640 culture medium. After culture for 45 h, LPS (10 mg/L) was added into for an additional 3 h. Six replications were used for each treatment. The cells were collected and stored at −80 °C until RNA, DNA extraction.

### 2.3. RNA extraction and cDNA synthesis

Total RNA from cells was extracted using RNAiso Plus reagent according to the manufacturer's instruction. The NanoDrop ND-1000 spectrophotometer (Nano-drop Technologies, DE, USA) was used to detect the concentration and purity of extracted total RNA. All RNA samples were diluted to a concentration of 100 ng/μL. Total RNA (500 ng) was reverse transcribed to cDNA using PrimeScript RT reagent Kit (Takara, Dalian, China) according to manufacturer's instruction. The cDNA was stored at −20 °C subsequently until use.

### 2.4. qRT-PCR

The mRNA expression of pro-inflammatory cytokines (*IL-1β*, *IL-6*, and *TNF-α*), HDACs and DNMTs was quantified using real time-PCR and performed with SYBR Premix Ex Taq Kit. The real time PCR