Marek's disease virus challenge induced immune-related gene expression and chicken repeat 1 (CR1) methylation alterations in chickens

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ABSTRACT

Marek's disease virus (MDV) challenge induces lymphoma in susceptible chickens. Host genes, especially immune related genes, are activated by the virus. DNA methylation is an epigenetic mechanism that governs gene transcription. In the present study, we found that expression of *signal transducer and activator of transcription* 1 (*STAT*1) was upregulated at 10 days post infection (dpi) in MD susceptible chickens, whereas *interleukin* 12*A* (*IL*12*A*) was elevated in both resistant and susceptible chickens. However, we did not observe MDV-induced DNA methylation variations at the promoter CpG islands (CGIs) in *STAT*1 and *IL*12*A*. Interestingly, the methylation levels at Chicken Repeat 1 (CR1), the transposable elements (TEs) located upstream of two genes, were different between resistant and susceptible chickens. Furthermore, a mutation was identified in the CR1 element near *IL*12*A*. The impact of the point mutation in transcriptional factor binding is to be examined in the near future.

Keywords: Marek's Disease; Chicken Repeat 1; DNA Methylation; Gene Expression

1. INTRODUCTION

Marek's disease (MD) is a lymphoma in chicken caused by the infection of Marek's disease virus (MDV). The infectious disease in chickens includes three phases, from the early cytolytic stage at 5 days post infection (dpi), to latent stage around 7 - 10 dpi, and then the late cytolytic phase and transformation [1]. Recently, several studies revealed that expression profiles of immune related genes were altered post MDV infection in chickens [2-4], suggesting those genes play important roles in innate and adaptive immunity in responses to MDV infection.

As we have known, viral infection is one of the environmental factors triggering DNA methylation alterations and consequently changing gene expression profiles. DNA methylation frequently occurs at the CpG sites, and increased methylation level at the promoter regions is associated with transcriptional silencing [5]. Notably, the association between DNA methylation and gene expression was observed in Marek's disease resistant and susceptible chickens post MDV infection [6,7].

Chicken Repeat 1 (CR1), a class of non-LTR retrotransposons comprising 11 subfamilies (B-H, X, Y, C3 and C4), constitutes 80% of all the interspersed repeats in the chicken genome [8,9].

Most of CR1 elements are truncated at their 5'UTR and conserved at 3'UTR [10-12]. The distribution of CR1 elements in high GC content regions makes them the potential targets for DNA methyaltion [9]. The loss of DNA methylation on repetitive DNA was associated with cancer [13,14]. However, little is known about the effects of MDV infection on methylation status of chicken repeat 1, the predominant transposable element in chicken genome.

This study aimed to uncover the MDV challenge induced DNA methylation variations on CR1s in MD resistant and susceptible chickens, and its subsequent effects on gene expression. Chicken line 6_3 and line 7_2, two highly inbred lines of specific pathogen free (SPF) chickens, are both susceptible to MDV, but line 6_3 is resistant to MD tumors while line 7_2 is highly susceptible to MD tumors [15]. Our previous result demonstrated that MDV replication was repressed infected line 6_3 than line 7_2 [16]. Cytokines and other immune related genes were differentially expressed in chickens after MDV infection [17-19]. In this study, we found that two important immune genes, *signal transducer and activator*

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of transcription 1 (*STAT*1) and *interleukin* 12*A* (*IL*12*A*) were activated in line 6_3 and line 7_2 after MDV infection. Instead of changing methylation level at promoter CpG islands, MDV infection influenced the methylation status of two CR1s near the promoters, which may be associated with *STAT*1 and *IL*12*A* mRNA expression levels. The finding may give us new insight of the potential roles of retrotransposons in MD resistance and susceptibility in chicken.

2. MATERIALS AND METHODS

2.1. Viral Challenging Experiment and Sample Preparation

SPF chickens from highly inbred lines 6_3 and line 7_2 were sampled for this study. The chickens from each of the two lines were divided into two groups. The infected groups were challenged with 500 PFU of 648A passage 40 viruses intraabdominally, at day 5 after hatch, and were designated as treatment groups. The control groups were not challenged with the MDV. Chickens from both treatment and control groups were euthanized at 10 days post infection and fresh spleen samples were collected. The spleen samples were immediately stored in RNAlater solution (QIAGEN), and then placed at –80˚C for DNA and RNA extractions. The challenged experiments were conducted in the BSL-2 facility at the USDA-ARS, Avian Disease and Oncology Laboratory at East Lansing, Michigan, USA, following the Guidelines for Animal Care and Use (Revised April, 2005) established by ADOL's IACUC.

2.2. Purification and Quantification of mRNA Levels

RNA was extracted from four samples per group using RNeasy Mini Kit (QIAGEN) based on the standard method described by the manufacturer, including the on column DNase digestion, and quantified by Nanodrop. Reverse transcription and quantification of mRNA were performed with SuperScript™ III Reverse Transcriptase (Invitrogen) with oligo (dT)$_{12-18}$ primers (Invitrogen), and the QuantiTect SYBR Green PCR Kit. In the reverse transcription control, PCR water (Invitrogen) was used to replace miRNA or RNA samples. Briefly, 1 µg of purified miRNA or total RNA was used for reverse transcription, respectively, and 2 µl of RT products (1:5 dilutions) were used for real-time PCR quantification. Two types of controls were applied in real-time PCR, including reverse transcription control and blank using PCR water. No amplicon was observed in the controls. A final volume of 20 µl real-time PCR product was incubated in an iCycler iQ PCR System (Bio-Rad), and each was performed on four biological replicates from the treat-

ment per line in each experiment. Three independent experiments were carried out for each gene. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were used as normalization.

Forward and reverse primers for quantification of *IL*12*A* and *STAT*1 are listed in **Table 1**.

2.3. DNA Preparation and Bisulfite Treatment

Genomic DNA was extracted by using Nucleo Spin kit (Macherey-Nagel) from four samples of each group, and the concentration was measured by Nanodrop. Sodium bisulfite conversion reagents were used to treat 500 ng of DNA (EZ DNA Methylation Golden Kit) using the standard protocol provided by the manufacturer.

2.4. Pyrosequencing and Bisulfite Sequencing

PCR primers (**Table 1**) were designed to amplify multiple CpG sites in specific CGIs and CR1-F at an upstream region of *IL*12*A* and *STAT*1. Pyrosequencing and bisulfite sequencing were applied to detect the methylation levels of *STAT*1 and *IL*12*A,* respectively. For pyrosequencing, we used biotin labeled universal primer in the PCR reaction. The bisulfite PCR included 1 µl of 1:5 diluted bisulfite converted DNA, primers and PCR reagents from Hotstar Taq polymerase kit (QIAGEN) with four biological replicates. The methylation level detection was carried out individually by Pyro Q-CpG system (PyroMark ID, Biotage, Sweden) using 20 µl of PCR products. For bisulfite sequencing, the equal amount of DNA from four MDV challenged or control samples from each chicken line were pooled together, serving as a template for the bisulfite conversion and the bisulfite PCR, and then PCR products were purified (QIAquick Gel Extraction Kit, QIAGEN). The purified PCR products were ligated to PCR® 2.1 Vector (The Original TA cloning® Kit, Invitrogen), transformed to DH5α competent cells (ZYMO Research), and screen the white colonies for successful insertions after incubation at 37˚C overnight. Ten white colonies from each group were cultured at 37˚C shaker overnight. The plasmid DNA was isolated using QIAprep® Miniprep Kit (QIAGEN), and then M13 reverse primer was used to sequence all the samples.

2.5. Transcription Factor Binding Sites Prediction

Transcription factor binding sites (TFBS) were predicted based on the sequence of CR1-F elements upstream of *STAT*1 and *IL*12*A* using a web based tool (http://www.cbrc.jp/research/db/TFSEARCH.html). The threshold score was set as 90 to filter out poorly conserved TFBSs [20]. This algorithm predicts TFBSs based

Table 1. Primers for bisulfite PCR and real time-PCR.

Genes		Sequences
*IL*12*A*-CR1-F-1[A]	Forward	5'-GGAGGGTAGAGAGTATAAAAACGG-3'
	Reverse	5'-CCACATCCCCATAATTCTAAACA-3'
*IL*12*A*-CR1-F-2[A]	Forward	5'-GGGGTTATTATTTTTGGAGGTGT-3'
	Reverse	5'-CCATACTTTTACCCTTACAATCAC-3'
*IL*12*A*-CpG Island[A]	Forward	5'-GTCGATGTCGTGTTTTGTTATGT-3'
	Reverse	5'-GGGACACCGCTGATCGTTTAAACTCCCCGACACATCAATAC-3'
	Sequencing	5'-TTTTGTTCGATGAAATTG-3'
*STAT*1-CR1-F[A]	Forward	5'-TGTTTAGGAAGGTGGTGGAGTTA-3'
	Reverse	5'-GGGACACCGCTGATCGTTTACCTTCCCACAAAACCAAAATTA-3'
	Sequencing	5'-GGAAGGTGGTGGAGTTA-3'
*STAT*1-CpG Island[A]	Forward	5'-TGTAAYGAAGTAAAATAGGYGAGA-3'
	Reverse	5'-GGGACACCGCTGATCGTTTATCAACCTACACTACRCAACCTAA-3'
	Sequencing	5'-TAAAATAGGCGAGATATAAG-3'
*IL*12*A*[B]	Forward	5'-GATCCGGGGCCTGGAAACTGC-3'
	Reverse	5'-TGAGGTTCCCCAGCTCTGCCT-3'
GAPDH[B]	Forward	5'-GAGGGTAGTGAAGGCTGCTG-3'
	Reverse	5'-ACCAGGAAACAAGCTTGACG-3'
STAT1[B]	Forward	5'-ATCAGAAGGGCTCCAGAACA-3'
	Reverse	5'-GCAGGGAGATTGGAGATTCA-3'

[A]: Primers for bisulfite PCR; Y stands for C/T. [B]: Primers for qRT-PCR.

on the correlation calculation with binding site profile matrices, and written by Yutaka Akiyama (Kyoto University).

3. RESULTS

3.1. *IL*12*A* and *STAT*1 mRNA Expression

In this study, we first detected the mRNA levels of *STAT*1 and *IL*12*A* at the latent stage of MDV infection. As shown in **Figure 1(a)**, the transcription of *STAT*1 was significantly activated in infected line 7_2, showing an approximate five-fold increases than noninfected chickens ($p < 0.01$), whereas in line 6_3, *STAT*1 was slightly upregulated after MDV infection but not significantly different from its control group ($p > 0.05$). Moreover, the *STAT*1 transcriptional levels did not show statistical significance between infected line 6_3 and line 7_2 (**Figure 1(a)**). However, the expression of *IL*12*A* was dramatically elevated in MDV challenged line 6_3 and line 7_2 groups compared to their control groups ($p < 0.01$). Meanwhile, in infected chickens, the *IL*12*A* transcripts were significantly higher in line 6_3 chickens than in line 7_2 chickens (**Figure 1(b)**). The expression levels of the two genes were not significantly different between the line 6_3 and line 7_2 control groups (**Figures 1(a)** and **(b)**).

3.2. DNA Methylation at CGIs Upstream of *STAT*1 and *IL*12*A*

To understand how MDV infection induces differential gene expression, DNA methylation at the CpG islands (CGIs) located around transcriptional start sites (TSS) of *STAT*1 and *IL*12*A* were investigated (we named them as *STAT*1_CGI and *IL*12*A*_CGI, respectively) by the pyrosequencing method. The *STAT*1_CGI overlaps with the 5'UTR and potential promoter region, and contains 6 CpG sites which are 345 bp away from the TSS. We found that all the CpG sites remained hypomethylated (less than 10%) in the control and infection groups of both lines. The methylation changes were less than 1% between the control and MDV infection groups in both lines (**Figure 2(a)**). The *IL*12*A*_CGI, containing 78 CpG sites, covers the promoter region and entire the first exon. The methylation levels of 5 CpG sites, 10 bp upstream of TSS, were measured. The methylation status of the *IL*12*A*_CGI was not different between the challenged and control groups of both lines (**Figure 2(b)**). On average, the *IL*12*A*_CGI was significantly less methylated in line 6_3 than that in line 7_2 (**Figure 3**), and the methylation difference reached 10% in the first CpG site.

3.3. DNA Methylation of CR1-F Elements Upstream of *STAT*1 and *IL*12*A*

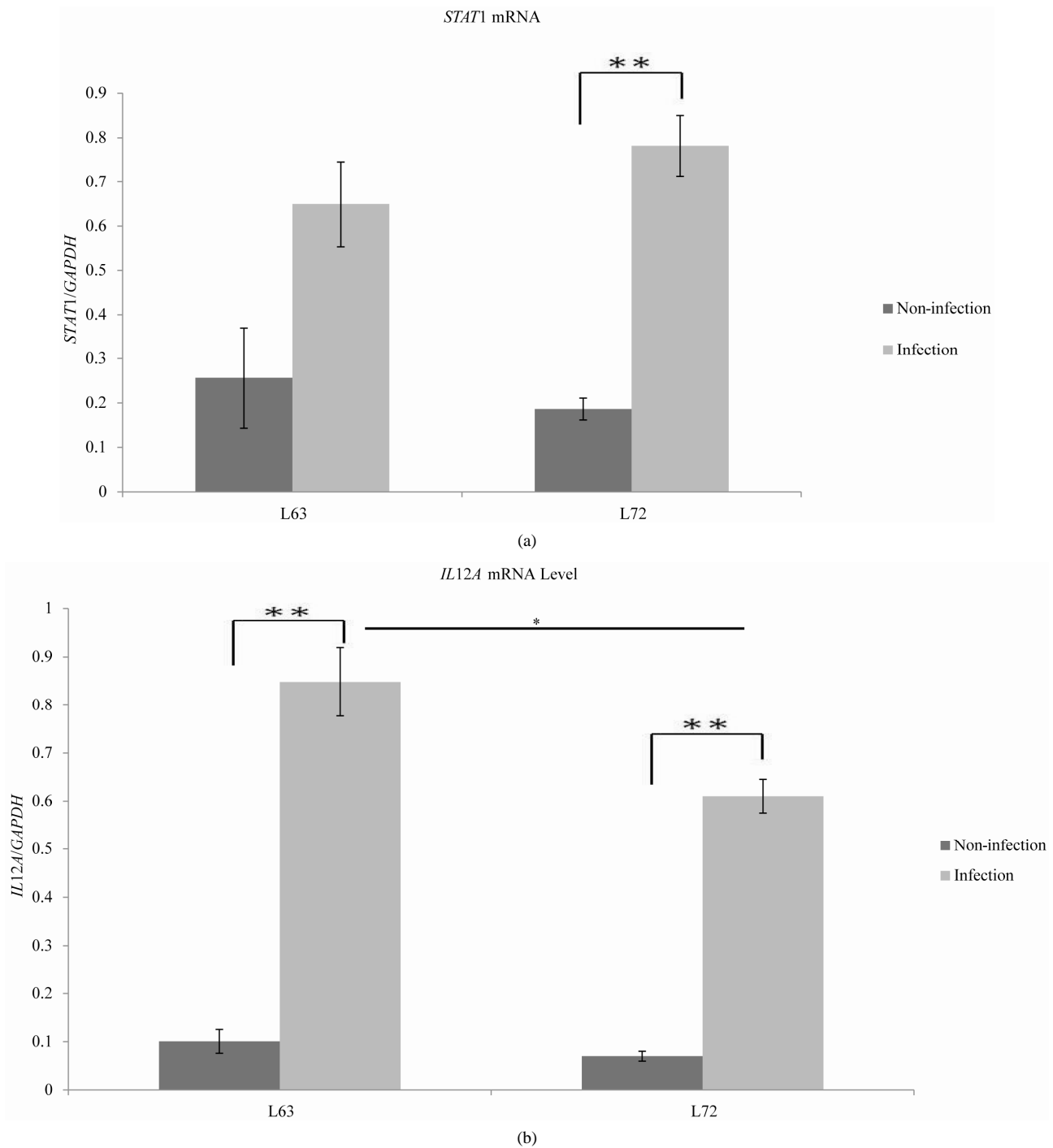Since MDV infection induced DNA methylation altera-

(a)

(b)

Figure 1. The relative mRNA levels of *STAT*1 (a) and *IL12A* (b) in non-infected and infected Line 6_3 and Line 7_2 was quantified by qRT-PCR and normalized using *GAPDH* (n = 4, mean ± SD). The one or two asterisks indicate the mRNA level was significantly different with $p < 0.05$ or $p < 0.01$. L7_2: line 7_2; L6_3: line 6_3.

tions were not observed at promoter CGIs, we traced further upstream sequences of *STAT*1 and *IL12A*. Interestingly, two CR1-F elements (referred as *STAT*1_CR1_F and *IL12A*_CR1_F, respectively) are located close to CGIs containing several CpG sites. The *STAT*1_CR1_F with 636 bp in length resides around 1.2 kb upstream of

*STAT*1 TSS. Using the pyrosequencing method, we found that the methylation levels of 4 CpG sites in this element were 24% and 25% in the control groups of line 6_3 and line 7_2, respectively, which decreased to 20% and 17% in the corresponding challenged groups, showing nearly a 5% - 10% reduction after MDV infection (**Figure 4(a)**).
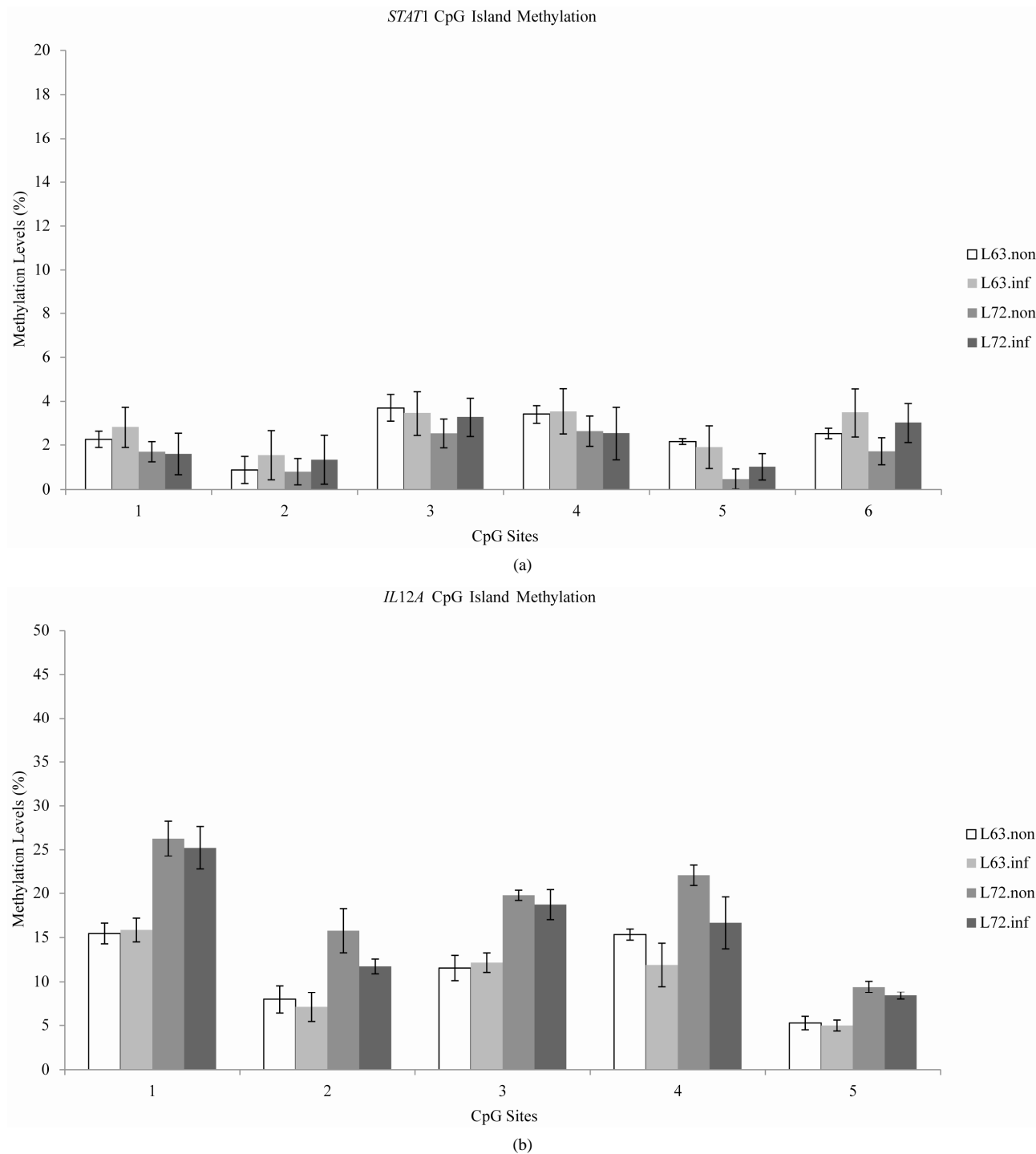
STAT1 CpG Island Methylation

(a)

IL12A CpG Island Methylation

(b)

Figure 2. DNA methylation level at CpG islands (CGIs) upstreams of *STAT*1 (a) and *IL12A* (b) were detected by pyrosequencing (n = 4, mean ± SD). L7$_2$.inf: infected line 7$_2$; L7$_2$.non: non-infected line 7$_2$; L6$_3$.non: non-infected line 6$_3$; L6$_3$.inf: infected line 6$_3$.

Moreover, the methylation variations showed different trends in the 4 CpG sites. In line 7$_2$, the methylation levels in the first three CpG sites were significantly decreased after MDV infection (p < 0.05 or p < 0.01), and methylation level of the last CpG site was not significantly decreased (p > 0.05). In contrast, in line 6$_3$, the methylation levels of the first two CpG sites were significantly declined by more than 10%, which was similar to the changes in line 7$_2$. At the third CpG site, the methylation level was dramatically increased after MDV infection (p < 0.01), and the slightly increased methylation level was also observed at the fourth CpG site (p >
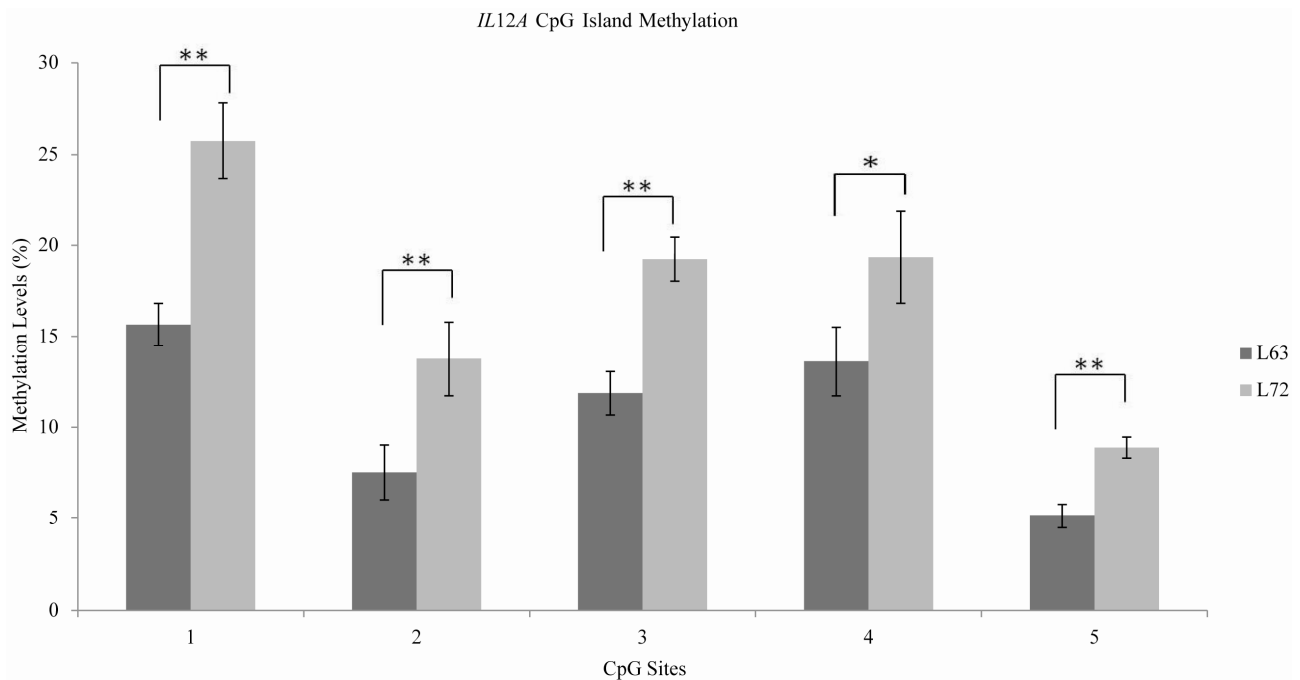
IL12A CpG Island Methylation

Figure 3. DNA methylation level at CpG islands (CGIs) upstream of *IL12A* were detected by pyrosequencing (n = 8 from each chicken line, including 4 control birds and 4 MDV infected birds, mean ± SD). The one or two asterisks denote the methylation level at the CpG sites was significantly different with $p < 0.05$ or $p < 0.01$. $L7_2$: line 7_2; $L6_3$: line 6_3.

0.05).

As for the *IL12A*_CR1_F element, it locates at 732 bp upstream of TSS. Four CpG sites were examined by bi-sulfite sequencing. As demonstrated in **Figure 4(b)**, the methylation levels were enhanced in this region in infected chickens, from 36.7% to 56.7% in line 6_3 and 50% to 75% line 7_2, respectively. However, the increased average methylation in infected groups was largely due to the occurrence of hypermethylation at the second and third CpG sites in infected line 6_3, and first and fourth CpG sites in infected line 7_2. After further sequencing analysis, we found that 80% of the last CpG site in this element was mutated to AT in line 6_3 birds regardless of infection (**Figure 4(c)**). The mutation at the CpG sites in line 6_3 resulted in decreasing methylation level of the entire region in line 6_3 compared to line 7_2.

3.4. Transcriptional Factor Binding Site Prediction

To uncover the potential function of the CR1-F element methylation or mutation on gene expression, the tran-scriptional factor binding sites (TFBS) were predicted using sequences of CR1-F elements upstream of *STAT*1 and *IL12A*, respectively. By computational prediction, as shown in **Table 2**, we found that CR1-F upstream of *STAT*1 contained two *GATA*-1 recognition sites, at 360 - 370 bp and 578 - 593 bp regions. The fourth CpG site was resided in the second putative *GATA*-1 binding site

(**Table 2**). Comparatively, two putative binding sites were predicted in *IL12A*. *E*47 and *MZF*1 were located at the 16 - 20 bp and 117 - 124 bp of CR1-F upstream of *IL12A*, respectively. The putative *E*47 binding site con-tains at the first CpG site of the CR1-F element. The mutation, 12 bp away from the predicted *MZF*1 binding site, did not change the TFBS prediction results (**Table 2**).

4. DISCUSSION

Latency is a crucial step to establish permanent immu-nosuppression in MDV infection [17]. It has been proven that the differences in viral load were detectable at 10 dpi in the spleen of infected line 6_3 and 7_2 chickens [2]. Therefore, all the spleen samples were collected at 10 dpi to elucidate the influence of MDV infection on host gene

Table 2. Transcription factor predicted in CR1-F elements.

| Transcription Factors[A]	Gene[B]	Position	Score[C]	Core Sequence[D]
GATA-1	*STAT*1	360 - 370	93.1	GCTGATAGGA
GATA-1	*STAT*1	578 - 593	93.9	CACGATGGTGATAAG
*E*47	*IL12A*	16 - 29	90.4	ACAGCTGCCCGGGG
*MZF*1	*IL12A*	117 - 124	91.3	GTTGGGGA

[A]: Names of transcription factors; [B]: Genes located downstream of the CR1-F elements; [C]: Calculated based on matrix similarity; [D]: Sequences of predicted TFBS.

expression and DNA methylation. In our previous study, we proved the MDV was successfully challenged in both line 6_3 and 7_2, and replicated faster in infected line 7_2 than line 6_3 [16].

STAT1, a member of STAT protein family, acts as a transcriptional activator and mediates gene expression in response to pathogens [21]. STAT1 transcription was induced by MDV challenge in the early cytolytic phase at 5 dpi [17]. Here, we found that its expression was also significantly activated at latency in infected line 7_2 (p < 0.05), but not in infected line 6_3 (p < 0.05). It was reported that IL12A interacts with IL12B to form a cytokine IL12, and stimulates downstream signaling pathways for innate immunity [22]. After MDV infection,

IL12A was upregulated in the lytic infection (5 dpi) [23]. In our study, we found the IL12A mRNA level was continuously elevated at latent stage, further illustrating the importance of IL12A in MD pathogenesis.

DNA methylation fluctuation triggered by virus infection has been well documented [24,25]. Recently, distinct DNA methylation patterns were identified in MD susceptible chickens after exposure to MDV [6]. In this study, we found that promoter CGI methylation remains either very low or stable after MDV challenge, whereas the methylation status of two CR1-F elements, further upstream of promoter CGIs, was altered upon MDV challenge, agreeing with previous research [26]. It has been found that true differential methylation regions were not
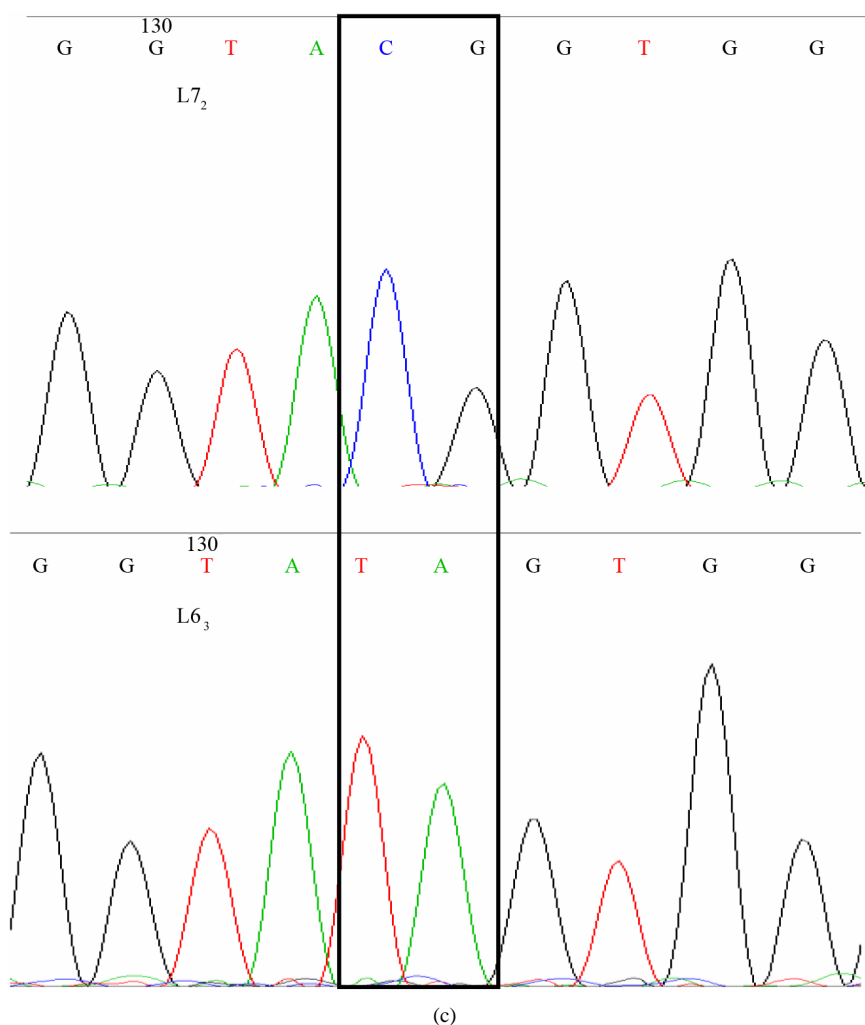

(a)

(b)

(c)
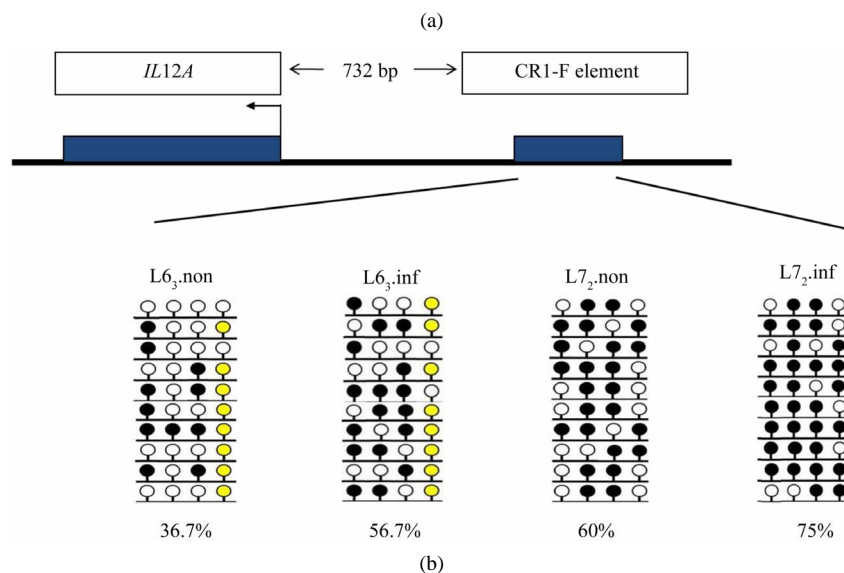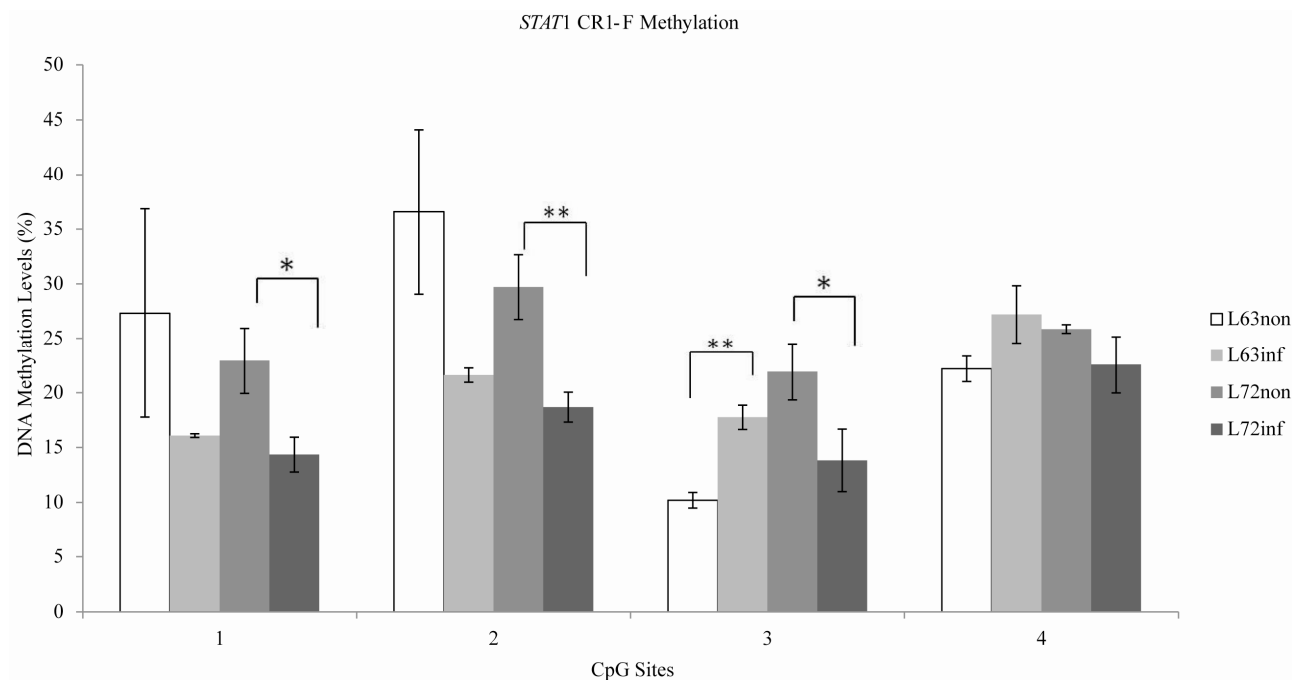
Figure 4. (a) DNA methylation level at CR1-F upstream of *STAT*1 was measured by pyrosequencing (n = 4, mean ± SD). The one or two asterisks indicate the methylation level was significantly different with $p < 0.05$ or $p < 0.01$. $L7_2$.inf: infected line 7_2; $L7_2$.non: non-infected line 7_2; $L6_3$.non: non-infected line 6_3; $L6_3$.inf: infected line 6_3; (b) DNA methylation level at CR1-F upstream of *IL12A* was measured by bisulfite sequencing. From each group, 10 clones were sequenced. The top panel indicates the position of *IL12A* gene and CR1-F elements, and the arrow denotes the direction of transcription start sites. In bottom panel, the open circle denotes ummethylated CpG, the black circle denotes the methylated CpG, and yellow circle denotes the mutation. $L7_2$.inf: infected line 7_2; $L7_2$.non: non-infected line 7_2; $L6_3$.non: non-infected line 6_3; $L6_3$.inf: infected line 6_3; (c) The mutations in *IL12A*_CR1_F were identified in line 6_3, pointed out by the square, and the top is the sequences in line 7_2, and bottom is sequence from line 6_3. $L7_2$: line 7_2; $L6_3$: line 6_3.

in the CpG island but in the low CpG density regions near the traditional CpG islands [26]. These results suggest that, instead of the CGIs at promoter regions, the methylation level of CR1-F elements might be influenced by MDV infection.

As we have known, about 25% of human gene promoter regions harbor sequences derived from TEs, indicating the potential contribution of TEs to regulatory elements [27]. Interestingly, the CR1 also contains putative TFBSs. Two *GATA*-1 binding sites were predicted in the CR1-F element upstream of *STAT*1, and their sequences were same as the chicken *GATA* binding consensus sequence WGATAR, in which W and R refer to

A/T and A/G, respectively [28]. The regulation of *GATA*-1 on *STAT*1 transcription has been proven in mice [29], we thus speculated that *GATA*-1 might control chicken *STAT*1 expression with the same manner. Moreover, DNA methylation regulates gene expression by blocking transcription factor binding [30,31]. Therefore, we hypothesized that DNA demethylation induced by MDV infection in CR1-F might mediate the *GATA*-1 binding to the upstream of *STAT*1 and thereby enhance its expression. The methylation level of the last two CpG sites, where the putative *GATA*-1 binding site is located, was enhanced in line 6_3 after MDV infection, but declined in line 7_2. This difference may help us explain the smaller

upregulation of *STAT*1 transcription in line 6_3 than in line 7_2. The hypothesis will be confirmed in the future.

In contrast, MDV infection was associated with not only the increased methylation level of CR1-F element near *IL*12*A,* but also increased *IL*12*A* mRNA levels. We reasoned that the increased methylation level may reduce the binding affinity of *myeloid zinc finger protein* 1 (*MZF*1) and *E*47, two transcriptional repressors [32,33], hereby explaining the correlation of increased methylation levels at *IL*12*A*_CR1-F element and the activation of *IL*12*A* transcription after MDV infection in line 6_3 and line 7_2. We also observed that *IL*12*A* was also more actively transcribed in line 6_3 than line 7_2 regardless of MDV infection. In control groups, *IL*12*A* mRNA was about 30% higher in line 6_3 than in line 7_2 although not statistically significant (p = 0.2); in infected groups, line 6_3 had significantly higher *IL*12*A* mRNA level than line 7_2. Correspondingly, the promoter CGI and *IL*12*A*_CR1-F element were greatly methylated in line 7_2 compared to line 6_3 despite MDV infection (**Figures 3** and **4(b)**), implicating that the repression of *IL*12*A* transcription was probably mediated by the methylation at both the promoter and CR1-F region. Therefore, the methylation at promoter CGI and upstream CR1-F elements may contribute to transcriptional differences in *IL*12*A* in line 6_3 and line 7_2. Because the methylation on promoter region was stable, we think the MDV infection triggered the methylation alterations at *IL*12*A*_CR1-F element, which may be involved in the transcription stimulation of *IL*12*A* in MDV infection. Moreover, it has been reported that the increased number of methylated CpG sites at the distal region inhibited the activity of adjacent gene promoter [34]. Therefore, we assume that the mutation at CR1 region in line 6_3 may affect the activity of *IL*12*A* in line 6_3. Taken together, the methylation change on *IL*12*A*_CR1-F element was most likely involved in the transcription stimulation of *IL*12*A* in response to MDV infection in two lines. The differences in genetic and epigenetic aspects, in terms of SNPs and DNA methylation patterns, may comprehensively account for the difference of *IL*12*A* mRNA levels between two lines.

Collectively, MDV challenge activated *STAT*1 and *IL*12*A* transcription in the MD resistant line 6_3 and susceptible line 7_2 chickens at the latency. The methylation status at the promoter CpG islands of *STAT*1 and *IL*12*A* were stable after MDV infection. The enhanced expression levels of the two immune-related genes might be mediated by DNA methylation variations at the upstream CR1-F elements, the chicken repetitive DNA sequences. These results indicated that these two CR1-F elements were presumably *cis*-regulatory sequences and their methylation alterations thereby might be involved in responses to MDV infection. Further work is required to demonstrate the biological functions of the CR1-F ele-

ments and the influences of DNA methylation on transcription factor binding affinity and gene expression.

5. ACKNOWLEDGEMENTS

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