Regulation of Il6 expression by single CpG methylation in downstream of Il6 transcription initiation site
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Benedict Shi Xiang Lian,1 Takumi Kawasaki,1,* Norisuke Kano,1 Daisuke Ori,1 Moe Ikegawa,1 Ayako Isotani,2 and Taro Kawai1,3,*

SUMMARY
The innate immune system is an immediate defense against infectious pathogens by the production of inflammatory cytokines and other mediators. Deficiencies of epigenetic regulatory enzymes, such as Tet1 and Dnmt1, cause dysregulation of cytokine expression. However, it is unclear if DNA methylation at a single CpG dinucleotide in a specific gene locus can regulate gene expression. In this study, we demonstrated that CpG+286 and CpG+348 in exon 2 of the Il6 gene are similar in various primary mouse cells. In lipopolysaccharide-stimulated condition, hypomethylated CpG+286 promoted Il6 expression whereas deletion of CpG+348 led to a reduction in Il6 expression associated with enhanced CTCF binding to the Il6 locus. Moreover, hypomethylation at CpG+286 in alveolar macrophages from aged mice led to higher Il6 expression in response to LPS compared with young mice. Thus, DNA methylation at specific CpG dinucleotides plays an important regulatory role in Il6 expression.

INTRODUCTION
The innate immune system is the first defense against pathogens, protecting the host from infection and initiating the antigen-specific adaptive immune response for effective clearance of pathogens (Kawai and Akira, 2010). Macrophages and dendritic cells (DCs) play an essential role in facilitating innate immune responses to produce mediators such as proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and interferons (IFN-α and IFN-β). IL-6 is a soluble mediator that exerts various pleiotropic effects on inflammation, the immune response, and hematopoiesis. Il6 gene transcription is mainly activated by the transcription factor NF-κB via the MyD88-dependent pathway initiated by several types of Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (Tanaka et al., 2014). Dysregulation of IL-6 production leads to chronic inflammation, autoimmune diseases, and tumorigenesis (Hirano, 2021).

DNA methylation in vertebrates involves the chemical modification of DNA by adding a methyl group (-CH3) at the 5-carbon position of cytosine (5mC) in the cytosine–phosphate–guanine (CpG) dinucleotide (Robertson, 2005). DNA methylation is a reversible and heritable chemical reaction, facilitated by DNA methyltransferases and DNA methyl-dioxygenase (Wu and Zhang, 2014). Two DNA methyltransferases, DNMT3a and DNMT3b, are responsible for de novo methylation, and DNMT1 maintains DNA methylation during cell division (Brenner and Fuks, 2006). Meanwhile, three classes of DNA demethylases, TET1, TET2, and TET3, perform DNA demethylation at designated regions in the genome (Ito et al., 2011). Several findings have reported the involvement of DNMTs and TETs in the innate immune response. siRNA knockdown of Tet1 showed a decrease in global DNA demethylation and reduced TNF-α expression, indicating DNA hypomethylation by Tet1 at the Tnfα gene promoter (Sun et al., 2019). Another study in Tet1-knockout (KO) mice showed that the loss of Tet1 caused DNA hypermethylation at several CpG dinucleotides in the Irf7 locus and many other gene loci, which contributed to the interferon and aryl hydrocarbon receptor (AhR) pathways to suppress mouse lung inflammation (Burleson et al., 2019). A vital role has also been demonstrated for TET2 in suppressing inflammation during bacterial lipopolysaccharide (LPS) stimulation in innate immune cells by recruiting HDAC2 to suppress Il6 expression (Zhang et al., 2015; Cull et al., 2017). Meanwhile, several studies demonstrated that Dnmt3b and Dnmt1 are needed to regulate macrophage polarization and inflammation by inducing DNA hypermethylation at the Ppary and Klf4 gene promoters (Yang et al., 2014; Wang et al., 2016; Tang et al., 2019). Overall, these findings show that regulation of DNA methylation by epigenetic enzymes controls gene expression, contributing to innate immune responses.
Gene expression is regulated by CpG dinucleotides located around the transcription initiation site (TIS) (Saxonov et al., 2006). Loci with high frequencies of CpG dinucleotides are known as CpG islands (CGI) (Takai and Jones, 2002). CGI DNA methylation plays a regulatory role in gene expression by inducing long-term gene silencing, such as X chromosome inactivation and genomic imprinting (Hashimshony et al., 2003). In contrast, DNA methylation of single CpG dinucleotides in low CpG content regions is conserved in transcription binding motifs, and it is still unclear if this plays a regulatory role in gene expression (Yin et al., 2017).

CCCTC-binding factor (CTCF) is a zinc finger protein that is highly conserved in vertebrates, with diverse regulatory roles in mediating gene expression (Kim et al., 2015). Numerous studies have demonstrated that DNA methylation of a CpG dinucleotide located in the CTCF-binding motif prevents binding of CTCF to the motif (Bell and Felsenfeld, 2000; Filippova et al., 2001; Shukla et al., 2011). CTCF also organizes the chromatin with cohesin to form topologically associating domains (TAD) that block promoter and enhancer interactions (Kentepozidou et al., 2020). Other reported functional roles of CTCF in regulating gene transcription include the recruitment of the large subunit of RNA polymerase II, and mediating alternative splicing and RNA polymerase II transcriptional pausing (Chernukhin et al., 2007; Kang and Lieberman, 2011; Shukla et al., 2011).

DNA methylation plays an essential role in the innate immune response. However, the specific mechanism by which DNA methylation regulates cytokine expression in response to LPS stimulation remains unclear. Here, we identified a functional role for DNA methylation at single CpG dinucleotides in the Il6 gene. We found that the Il6 locus has low CpG content and that DNA methylation of single CpG dinucleotides downstream of the TIS modulates Il6 expression. CRISPR/deactivated Cas9 (dCas9) fused with eukaryotic DNA methyltransferases or methyl-cleaving enzymes has been applied to study the role of DNA methylation at the Il6 locus. We found that Il6 expression after LPS stimulation was controlled by DNA methylation of a single CpG dinucleotide downstream of the TIS in the Il6 locus. Meanwhile, the loss of DNA methylation reduced recruitment of the gene insulator CTCF. Moreover, our study also revealed that alveolar macrophages (AMs) from aged mice showed significantly lower methylation levels at the single CpG dinucleotide, leading to higher Il6 expression in response to LPS compared with young mice. Thus, DNA methylation at specific CpG dinucleotides in the Il6 locus plays an important regulatory role in gene expression.

RESULTS
DNA methylation profile of the Il6 locus
To investigate the DNA methylation status at the TIS of Il6, we identified CpG dinucleotides located in the Il6 promoter region, which is 1 kb upstream and 0.5 kb downstream of the TIS. The entire 1.5 kb of the Il6 locus has no CpG islands, although it has 19 single CpG dinucleotides (Figure 1A). We separated the Il6 promoter region into three regions, region 1 (−1000 b to −350 b), region 2 (−351 b to +100 b), and region 3 (+101 b to +500 b). Region one contained five single CpG dinucleotides at positions −409, −398, −396, −394, and −386. Region 2 contained six single CpG dinucleotides at positions −195, −185, −182, −7, +26, and +79. Region three contained eight single CpG dinucleotides at positions +163, +191, +210, +263, +286, +348, +381, and +438 (Figure 1B). DNA methylation in RAW264.7 cells was analyzed using bisulfite sequencing by amplifying each region with PCR, which revealed that CpG dinucleotides at the distal end of region 3 showed a higher DNA methylation rate compared with the CpG dinucleotides in regions 1 and 2 (Figure 1B). Among the 19 CpG dinucleotides, only three CpG dinucleotides at +286 (CpG+286), +348 (CpG+348), and +381 (CpG+381) in region 3 were highly methylated (20%–70%) (Figure 1C and Table S1A). These highly methylated CpG dinucleotides are located in exon 2 of the Il6 gene (Figures 1A and 1C). Next, we examined LPS-dependent DNA methylation of the CpG dinucleotides in the Il6 gene locus. Il6 expression in RAW264.7 cells significantly increased from 3 to 48 h after LPS stimulation and peaked at 24 h (Figure S1A), whereas the DNA methylation profile was not significantly altered at 6, 12, or 24 h after LPS stimulation (Figures S1B, S9K–S9N and Table S1G). These data indicate that DNA methylation at single CpG dinucleotides in the Il6 gene was maintained irrespective of LPS stimulation.

Il6 expression and DNA methylation of CpG dinucleotides in the Il6 locus were altered by Tet deficiency
To investigate the regulatory role of DNA methylation in the Il6 locus, we generated Tet1-KO RAW264.7 cells using the CRISPR/Cas9 genome editing system with sgRNA targeting exon 4 of Tet1 (Figure 2A). Cells with a frame-shift mutation of 19 base pairs (bps) and 1 bp deletion in each allele were selected (Figure 2A), and Tet1 deficiency was confirmed with immunoprecipitation-Western blot (IP-WB) (Figure 2B) and qRT-PCR (Figure 2C). TET1 expression was not detected in IP-WB; therefore, Tet1-mutant cells could be defined as Tet1-KO cells (Figure 2B), and Tet1 mRNA expression was significantly decreased in Tet1-KO...
Subsequently, both WT and Tet1-KO cells were stimulated with LPS and cytokines, and antiviral/antibacterial genes, including \textit{Il6}, \textit{Tnfa}, \textit{Ifnb1}, \textit{Cxcl10}, \textit{Mx1}, \textit{Isg15}, \textit{Ccl2}, and \textit{Ccl7}, were measured by RT-qPCR (Figure 2D). \textit{Il6} and \textit{Tnfa} gene expression increased significantly after LPS stimulation in Tet1-KO cells compared with WT cells. The \textit{Cxcl10} gene showed comparable expression to WT cells, whereas the other cytokine genes (\textit{Ifnb1}, \textit{Mx1}, \textit{Isg15}, \textit{Ccl2}, and \textit{Ccl7}) showed significantly reduced gene expression in Tet1-KO cells after LPS stimulation.

We next investigated downstream signaling pathways including NF-κB and IRF-3 activation during LPS stimulation, and found that the phosphorylation levels of NF-κB and IRF3 in WT and Tet1-KO cells were comparable (Figure 2E). These results suggest that Tet1 deficiency did not alter signal transduction during LPS stimulation. We therefore proceeded to investigate the DNA methylation profile of the CpG dinucleotides at the \textit{Il6} locus in Tet1-KO cells. The DNA methylation profile of the CpG dinucleotides at \textit{CpG}+286 and \textit{CpG}+381 showed significant hypomethylation in Tet1-KO cells compared with WT cells (Figure 2F). In contrast, \textit{CpG}+348 showed hypermethylation in Tet1-KO cells (Figures 2F, S9A and Table S1B).

Hence, these data suggest that modulation of the DNA methylation profile at single CpG dinucleotides at \textit{CpG}+286, \textit{CpG}+348, and \textit{CpG}+381 by Tet1 deficiency individually or cumulatively regulates \textit{Il6} expression.
We also investigated the functional roles of Tet2 and Tet3 because both Tet2 and Tet3 have higher gene expression than Tet1 in RAW264.7 cells (Figure S1C). Tet2-KO and Tet3-KO RAW264.7 cells were generated using the same strategy as Tet1-KO cells by targeting exon 3 for both Tet2 and Tet3 (Figures S2A and S3A). Tet2-KO and Tet3-KO cells demonstrated significant decreases in gene expression (Figure S2B and S3B). Tet2-KO cells exhibited higher Il6 expression after LPS treatment. In contrast,
LPS-treated Tet3-deficient cells showed a significant decrease in Il6 expression compared with WT cells (Figures S2C and S3C). The signal transductions during LPS stimulation in both Tet2-KO and Tet3-KO cells were also comparable to WT cells (Figures S2D and S3D). Tet2-KO cells displayed significant hypermethylation at CpG+348 and CpG+381 compared with WT cells (Figure S2E, S9O and Table S1H). Hypermethylation was also seen at CpG+286 and CpG+381 in Tet3-KO cells, but CpG+348 exhibited hypomethylation in Tet3-KO cells compared with WT cells (Figure S3E, S9P and Table S1I). Therefore, these findings suggest that DNA methylation of CpG+286, CpG+348, and CpG+381 in the downstream region of the TIS is regulated by TET family enzymes and controls Il6 expression during LPS stimulation.

Hypermethylation of CpG+286 in the downstream region of the TIS reduced Il6 expression

Although TET modulates DNA methylation in various regions of the genome, it is unclear whether DNA methylation of single CpG dinucleotides in the Il6 locus directly regulates its gene expression. We then manipulated DNA methylation using a fusion protein of inactive Cas9 (dCas9) with DNA methyltransferase MQ1, which is a methyltransferase from the prokaryote Mollicutes spiroplasma (M. Sss1), strain MQ1 (dCas9-MQ1) (Lei et al., 2017). dCas9-MQ1(WT) targets CpG dinucleotides for methylation that surround a sgRNA-targeted DNA by about 300 bases upstream and downstream (Figures S4A and S4B). We first tested two sgRNAs, sgRNA1(+251) and sgRNA2(+263), which are located close to CpG+286, CpG+348, and CpG+381 (Figure S4B). Tet1-KO cells showed a low rate of CpG+286 and CpG+381; therefore, dCas9-MQ1(WT) with sgRNA1(+251) or sgRNA2(+263) was expressed in Tet1-KO cells, and the DNA methylation profile of the Il6 locus was analyzed by bisulfite sequencing. dCas9-MQ1(WT) + sgRNA1(+251) induced robust hypermethylation at CpG+163, +191, +210, +263, +348, +381, and +438, whereas expression of dCas9-dMQ1, a mutant MQ1 with a loss of methyltransferase activity, did not enhance DNA methylation at any of these positions (Figures S4C, S9Q and Table S1J). dCas9-MQ1(WT) + sgRNA2(+263) also induced hypermethylation at these positions, except at CpG+286, which is located in the sequence targeted by sgRNA2(+263) (Figures S4D, S9R and Table S1K). Therefore, sgRNA1(+251) was selected for the subsequent experiment. Then, dCas9-MQ1(WT) or dCas9-dMQ1 with sgRNA1(+251) was expressed in RAW264.7 cells and the DNA methylation in the Il6 locus was analyzed by bisulfite sequencing. Expression of dCas9-MQ1(WT) induced a significant increase of the DNA methylation level at CpG+7, +26, +79, +163, +191, +210, +263, and +438 (Figures S5A, S9S, S9T and Table S1L), whereas expression of dCas9-dMQ1 did not. Next, we measured Il6 expression after LPS stimulation and found that Il6 expression in dCas9-MQ1(WT) + sgRNA1(+251) was comparable with that in dCas9-dMQ1 + sgRNA1(+251) (Figure S5B).

dCas9-MQ1(WT) with a sgRNA methylates a wide range of CpG dinucleotides around the targeted DNA, whereas the Q147L mutation of MQ1 (dCas9-MQ1Q147L) methylates a specific CpG dinucleotide located 20–30 bases downstream of the sgRNA binding site (Figure 3A) (Lei et al., 2017). RAW264.7 cells were expressed with dCas9-dMQ1 + sgRNA2(+263) or dCas9-MQ1Q147L + sgRNA2(+263) and the methylation profile of the Il6, Mx1, and Ifnb locus was measured by bisulfite sequencing analysis. We found that the methylation level of CpG+286 at region 3 in the Il6 locus was enhanced by the expression of dCas9-MQ1Q147L + sgRNA2(+263), whereas CpG dinucleotides at other positions did not show enhanced levels (Figure 3B). We also measured DNA methylation in region 3’ (+42 b to +403 b) which contained CpG+79, +163, +191, +210, +263, +286, +348, and +381 and obtained similar results with the bisulfite sequence of region 3 (Figures S6A, S9U, S9V, and Table S1M). In addition, methylation level of Mx1 and Ifnb locus was not altered by the expression of dCas9-MQ1Q147L + sgRNA2(+263) (Figures S6B, S6C, S9W–S9Z, Table S1N, and S1O). Il6 expression after LPS stimulation was reduced by expression of dCas9-MQ1Q147L + sgRNA2(+263) compared with dCas9-dMQ1 + sgRNA2(+263) (Figure 3C). On the contrary, dCas9-MQ1Q147L expression did not alter Mx1, Ifnb1, or Ig51 expression (Figures 4D–4F). These results demonstrate that DNA methylation at +286 inhibits Il6 expression. Conversely, the expression of dCas9-MQ1Q147L + sgRNA2(+263) in Tet1-KO did not reduce the Il6 expression after the LPS stimulation, despite the methylation level was significantly enhanced in CpG+286 (Figures S6D, S6E, S9AA, AB and Table S1P).

Hypermethylation of CpG+286 increased Il6 expression

To further study the regulatory role of DNA methylation at CpG+286 in the Il6 locus on Il6 expression, we expressed a fusion protein of inactive Cas9 (dCas9) with the TET1 catalytic domain (CD) tethered by bacteriophage MS2-coating protein (dCas9-TET1-CD) (Xu et al., 2016). dCas9-TET1-CD demethylates CpG dinucleotides at a specific locus 100–300 bases downstream of the targeted sgRNA (Figure 4A)
dCas9-TET1-CD + sgRNA1(+251) was expressed in RAW264.7 cells and CpG methylation in the Il6, Mx1, and Ifnb locus was analyzed by bisulfite sequencing. We found that the methylation level of CpG+286 was reduced by the expression of dCas9-TET1-CD + sgRNA1(+251), but methylation at CpG+348 and CpG+381 was not altered (Figures 4B, S9D, S9E, and Table S1D). In addition, methylation level of Mx1 and Ifnb locus was not changed by the expression of dCas9-MQ1(Q147L) + sgRNA2(+263) were stimulated with LPS, and the expression of Il6 (C), Mx1 (D), Ifnb1 (E), and Isg15 (F) were measured by qRT-PCR (mean ± s.e.m; n = 3). Multiple unpaired t-test (B) or two-way ANOVA with Tukey’s multiple comparison test (C, D, E, F).

Deletion of CpG+348 reduced Il6 gene expression but recruited higher CTCF binding

The highest DNA methylation level in the Il6 locus was at CpG+348; however, it could not be altered by the dCas9-MQ1 or dCas9-TET1-CD systems (Figures 3B and 4B). To reveal the functional role of CpG+348 in
the regulation of Il6 expression, we deleted CpG+348 in RAW264.7 cells using the CRISPR/Cas9 genome editing system. Deletion of CpG+348 was confirmed by sequencing analysis, and cells were selected that had a non-frame-shifted mutation in at least one allele to minimize the possibility of mRNA degradation (Figure 5A). Then, we treated both WT cells and CpG+348-deletion cells with actinomycin D after LPS stimulation and the Il6 mRNA stability was measured. The half-life (t1/2) of Il6 mRNA in WT and CpG+348-deletion cells was 1.40 and 1.46 h, respectively, indicating that the stability of Il6 mRNA was not changed by CpG+348 deletion (Figure 5B). Then, both WT and CpG+348-deletion cells were stimulated with LPS and Il6 expression was measured by qRT-PCR. Il6 expression in CpG+348-deletion cells was suppressed after LPS stimulation compared with WT cells, whereas the expression of Mx1, Ifnb1, and Isg15 did not change (Figures 5C–5F). Therefore, these results suggest that DNA methylation at CpG+348 promotes Il6 expression after LPS stimulation.

Figure 4. DNA demethylation at CpG+286 induced Il6 expression

(A) Schematic diagram of the mechanism by which dCas9-TET1-CD with bacteriophage MS2 coating protein facilities demethylation of the CpG dinucleotide.

(B) The methylation profile of CpG dinucleotides in RAW264.7 cells after expression of dCas9-TET1-CD + sgRNA1(+251) was measured by bisulfite sequencing. Bar graph shows percentage of methylated CpG dinucleotides (mean ± s.e.m).

(C–F) RAW264.7 cells expressing dCas9-TET1-CD + sgRNA1(+251) were stimulated with LPS, and the expression of Il6 (C), Mx1 (D), Ifnb1 (E), and Isg15 (F) was measured by qRT-PCR (mean ± s.e.m; n ≥ 3). Multiple unpaired t-test (B) or two-way ANOVA with Tukey’s multiple comparison test (C–F).
Figure 5. DNA methylation loss at CpG+348 reduced Il6 expression and increased the binding of CTCF to the CTCF-2 binding site

(A) Upper: schematic diagram of the Il6 locus. The sgRNA targeting exon 2 is highlighted in the red band. Lower: sequence of CpG+348 deletion cells. The sgRNA is indicated in gray and the PAM sequence is indicated in yellow.

(B) WT and CpG+348-deleted cells were treated with 5 mg/mL of actinomycin D according to the indicated time periods after LPS stimulation for 2 h. Il6 expression was measured by qRT-PCR and fitted by exponential decay (mean ± s.e.m; n = 3).

(C–F) Expression of Il6 (C), Mx1 (D), Ifnb1 (E), and Isg15 (F) in WT and CpG+348-deleted cells was measured by qRT-PCR (mean ± s.e.m; n = 3).
CTCF is a transcription regulator with versatile functions that binds specific sites in the mouse genome and plays many important roles in transcriptional regulation and chromatin modeling (de Almeida et al., 2011). The Il6 locus contains two CTCF-binding motifs (CTCF-1, -160 to +240; CTCF-2, +1439 to +1839) and CTCF recruitment has been reported (Figure 5A) (Howe et al., 2021). To further understand whether DNA methylation of CpG+348 regulates CTCF binding to the Il6 locus, we performed a chromatin immunoprecipitation (ChIP) assay with anti-CTCF antibody. LPS-treated or untreated WT and CpG+348-deletion cells were fixed and sonicated, and then isolated DNA fragments from these cells were precipitated by anti-CTCF antibody. We selected three regions in the Il6 locus, CTCF-1, CTCF-2, non-CTCF as a negative control and the Mx1 locus, and the precipitated DNA was quantified by qRT-PCR (Figures 5G–5J). LPS stimulation did not alter CTCF binding to any region in either WT or CpG+348-deletion cells. We found that CpG+348 deletion increased CTCF binding to the CTCF-2 region compared with WT, but it did not alter CTCF binding to CTCF-1 region, the non-CTCF region in the Il6 locus (Figures 5H and 5I), and CTCF region in the Mx1 locus (Figure 5J). These findings suggest that CTCF binding to the CTCF-2 region in the Il6 locus is regulated by DNA methylation of CpG+348.

Age-related DNA methylation of a single CpG dinucleotide controls Il6 expression

To further understand the functional role of CpG methylation, the DNA methylation profile of the Il6 locus was analyzed in primary murine cells: bone-marrow-derived DCs (BMDCs), bone-marrow-derived macrophages (BMDMs), peritoneal macrophages (PECs), and AMs from mice aged from 8–12 weeks, and mouse embryonic fibroblast (MEFs) cells. The DNA methylation levels at CpG+348 were broadly similar in BMDMs, PECs, AMs, and MEFs, but not in BMDCs, while the DNA methylation levels at CpG+286 were high in AMs and MEFs, but not in the other primary cells (Figures 6A, S9F–S9I, and Table S1E). However, BMDCs showed a hypomethylated profile at the Il6 locus compared with RAW264.7 cells. These results suggest that hypermethylation in CpG+286 and CpG+348 are distinctive features of macrophages, but not other cell lines.

Aging is highly associated with DNA methylation. The DNA methylation pattern in the gene promoter, the region near the TIS, the 5‘-UTR, exons, and exon–intron boundaries all change significantly with age (Sziráki et al., 2018). To further investigate whether aging is associated with DNA methylation in the Il6 locus, we isolated AMs from the lungs of young mice (8–12 weeks old) and aged mice (48–52 weeks). AMs of young mice showed higher DNA methylation levels at CpG+286 than AMs of aged mice (Figures 6B, S9I, S9J and Table S1F), whereas Il6 expression in AMs from young mice showed lower expression levels than AMs from aged mice after LPS stimulation (Figure 6C). Then, we differentiated BMDMs from the bone marrow of young and aged mice. In contrast, BMDMs from young mice showed higher DNA methylation at CpG+348 than BMDMs from aged mice (Figures 58A, S9A, S9AJ, and Table S1T), and BMDMs of young mice showed higher Il6 expression than BMDMs of aged mice (Figure 58B). Therefore, these findings suggest that regulation of DNA methylation at a single CpG dinucleotide in the Il6 locus controls Il6 expression.

DISCUSSION

We found that the regions downstream and upstream of the Il6 TIS contained 19 single CpG dinucleotides, with no CGI. Of these, only 3–5 CpG dinucleotides are methylated in RAW264.7 cells and other primary cells, which are located in exon 2 of Il6, downstream of the TIS. The hypermethylation or hypomethylation of CpG+286 by a CRISPR/dCas9-based system showed that hypermethylation of CpG+286 specifically suppresses Il6 expression. Deletion of CpG+348, which shows the highest DNA methylation level in the Il6 locus in RAW264.7 cells, showed suppression of Il6 expression and recruitment of CTCF. These results suggest that DNA methylation of CpG+348 inhibits recruitment of CTCF, which follows suppression of Il6 expression. In addition, we compared DNA methylation of AMs in aged and young mice and found that DNA methylation at CpG+286 was suppressed in AMs of aged mice and Il6 expression was consequently increased. Aged mice showed higher IL-6 protein expression in the alveolar lining fluid (ALF) than young mice (Moliva et al., 2014), and in agreement with this, our results showed that the DNA methylation pattern of
AMs in aged mice showed hypomethylation at CpG+286 compared with young mice. We found that DNA methylation of a single CpG dinucleotide downstream of the TIS controlled gene expression, and an age-related decrease of DNA methylation levels led to changes in gene expression.

Our results indicated that a single CpG dinucleotide has a distinct regulatory role in gene expression. Expression of dCas9-MQ1(Q147L) + sgRNA2(+263) in RAW264.7 cells hypermethylated the single CpG dinucleotide at CpG+286 and decreased Il6 expression. However, expression of dCas9-MQ1(WT) + sgRNA2(+263) in RAW264.7 cells, which induces DNA methylation at a wide range of single CpG dinucleotides in the Il6 locus, failed to suppress Il6 expression. Our results also showed that hypomethylation of CpG+286 increased Il6 expression, whereas deletion of CpG+348 to mimic hypomethylation decreased Il6 expression. Therefore, DNA methylation at a wide range of single CpG dinucleotides by dCas9-MQ1(WT) may compensate regulatory function at each CpG dinucleotide and thus did not cause gene expression changes. Our results indicated that each single CpG dinucleotide has a distinct regulatory function for gene expression.

Tet2 and Tet3 have higher gene expression levels compared with Tet1 in RAW264.7 cells. Deficiency of Tet family genes showed a distinct distribution of altered DNA methylation in the Il6 locus and had a regulatory role in Il6 expression. These results indicated that DNA methylation at each CpG dinucleotide is maintained by the combination of Tet family enzymatic function. Tet1 deficiency induced hypomethylation at CpG+286 and CpG+381 and hypermethylation at CpG+348, and increased Il6 expression. As expression of dCas9-MQ1(Q147L) + sgRNA2(+263) hypermethylated CpG+286 in RAW264.7 cells,
dCas9-MQ1(Q147L) + sgRNA2(+263) restored DNA methylation at CpG+286 in Tet1-KO cells. Expression of dCas9-MQ1(Q147L) + sgRNA2(+263) in Tet1-KO cells restored DNA methylation levels at CpG+286 without affecting DNA methylation at other CpG dinucleotides; however, Il6 expression was not restored. These results suggest that DNA methylation of CpG+348 and CpG+381 contributes to the regulation of Il6 expression, or that DNA methylation at other CpG dinucleotide loci indirectly controls Il6 expression.

DNA methylation modification by dCas9-MQ1(Q147L) or dCas9-TET1 only modified methylation at CpG+286. We have tested several gRNAs to modify the DNA methylation levels at CpG+348 and CpG+381, but none of these were successful. dCas9-MQ1 and dCas9-TET1 can directly modulate DNA methylation at CpG dinucleotides located in open chromatin regions, whereas CpG+348 and CpG+381 may be protected by chromatin-remodeling complexes or methyl-CpG binding proteins that selectively bind to methylated DNA. We deleted CpG+348 by the CRISPR/Cas9 system to mimic hypomethylation of CpG+348 and found that DNA methylation of CpG+348 inhibits recruitment of CTCF, which will induce suppression of Il6 expression. It is unclear whether CTCF enrichment at the CTCF-2 binding site is associated with the establishment of a robust TAD boundary that leads to a stronger insulation effect on Il6 gene transcription by blocking the interaction between enhancers and the Il6 promoter. However, it is also possible that the CTCF enrichment might lead to histone mark modifications that silence Il6 expression. Further study is necessary to fully determine the regulation of gene expression by DNA methylation at CpG+348, and it is important to develop tools to modulate DNA methylation of CpG dinucleotides protected by other proteins or chromatin structure. The CTCF-1 binding site spans the TIS region that contains several transcription factor binding sites, which possibly play vital roles in Il6 transcriptional regulation. As proposed by Chernukhin et al. (2007), CTCF may recruit RNA polymerase II to the gene promoter of the Il6 gene locus, which is also a CTCF-binding site, to initiate gene transcription in response to LPS stimulation. Hypomethylation at CpG+286 favors the recruitment of CTCF to the CTCF-1-binding site, which further promotes Il6 expression. Overall, we propose that CTCF enrichment at the CTCF-1 and CTCF-2-binding sites might exert different functional properties in modulating Il6 gene transcription. The correlation between CTCF recruitment and the DNA methylation level at CpG+286 and CpG+348 should be clarified in future.

In summary, we found that DNA methylation of a single CpG dinucleotide in the downstream region of the TIS regulates gene expression by modulating DNA-binding proteins. The DNA methylation level is affected by various factors, such as age, smoking, inflammation, and diet, and alterations to the DNA methylation cause changes of gene expression. It is still unclear whether this change in DNA methylation is beneficial. However, some recent reports have proposed that several CpG dinucleotides can be used as biomarkers to predict treatment outcomes and illness severity for many diseases, including cancer, pediatric diseases, autoimmune diseases, and even the recent COVID-19 (Nile et al., 2008; Claus et al., 2012; Shanthikumar et al., 2020; de Moura et al., 2021). Furthermore, a single CpG dinucleotide has a greater advantage in targeted therapy for suppressing gene expression in cells, as it has high specificity and creates fewer off-target effects. Further study is needed to determine the regulatory role of DNA methylation in gene expression and the regulation of physiological outputs by changes in DNA methylation.

Limitations of the study
In this study, we modulated DNA methylation of single CpG dinucleotide by CRISPR/Cas9-based system. CpG+286 in the Il6 locus was modified by the method, however, CpG+348 and CpG+381 were not. CRISPR/Cas9-based DNA methylation system has the limitation of modification in the specific CpG site; therefore, further development of the technology for DNA methylation is necessary. We proposed that Il6 gene expression is controlled by a single DNA methylation which regulates binding of CTCF to the gene locus. Further studies are needed to clarify whether the other genes are also controlled by the DNA methylation of single CpG nucleotide in the downstream of TIS.

STAR+METHODS
Detailed methods are provided in the online version of this paper and include the following:

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104118.

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AUTHOR CONTRIBUTIONS
BSXL and TKawasaki performed experiments, analyzed the data, and wrote the manuscript. NK assisted with the chromatin immunoprecipitation (ChIP) protocols. AI contributed the dCas9-MQ1 plasmid system. MI and DO supported the experiment. TKawai wrote the manuscript and supervised the research.

DECLARATION OF INTERESTS
The authors declare no conflicts of interest.

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REFERENCES

Baccam, M., Woo, S.-Y., Vinson, C., and Bishop, G.A. (2003). CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-kB, AP-1, and C/EBP. J. Immunol. 170, 3099–3108. https://doi.org/10.4049/jimmunol.170.6.3099.

Bell, A.C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature 405, 482–488. https://doi.org/10.1038/35013100.

Brenner, C., and Fuku, F. (2006). DNA methyltransferases: facts, clues, mysteries. DNA Methylation: basic Mechanisms. Curr. Top. Microbiol. Immunol. 301, 45–66. https://doi.org/10.1007/3-540-31990-7_3.

Burleson, J., Sniard, D., Yadagiri, V.K., Chen, X., Werrauch, M.T., Ruff, B.P., Brandt, E.B., Hershey, G.K.K., and Ji, H. (2019). TET1 contributes to allergic airway inflammation and regulates interferon and aryl hydrocarbon receptor signaling pathways in bronchial epithelial cells. Sci. Rep. 9, 1–18. https://doi.org/10.1038/s41598-019-43767-6.

Chernukhin, I., Shamsuddin, S., Kang, S.Y., Bergstrom, R., Kwon, Y.-W., Yu, W., Whitehead, J., Mukhopadhyay, R., Dossouer, F., and Farrar, D. (2007). CTCF interacts with and recruits the largest subunit of RNA polymerase II to CTCF target sites genome-wide. Mol. Cell. Biol. 27, 1631–1648. https://doi.org/10.1128/MCB.01993-06.

Claus, R., Lucas, D.M., Stilgenbauer, S., Ruppert, A.S., Yu, L., Zucknick, M., Mertens, D., Buhler, A., Oakes, C.C., and Larson, R.A. (2012). Quantitative DNA methylation analysis identifies a single CpG dinucleotide important for ZAP-70 expression and predictive of prognosis in chronic lymphocytic leukemia. J. Clin. Oncol. 30, 2483. https://doi.org/10.1200/JCO.2011.39.3090.

Cull, A.H., Nentsinger, B., Buckstein, R., Wells, R.A., and Rauh, M.J. (2017). Tet2 restrains...
inflammatory gene expression in macrophages. Exp. Hematol. 55, 56–70.e13. https://doi.org/10.1016/j.exphem.2017.08.001.

de Almeida, C.R., Stadhouders, R., de Bruijn, M.J., Bergen, I.M., Thongue, S., Lenhard, B., Van Ijcken, W., Grosveld, F., Galjart, N., and Soler, E. (2011). The DNA-binding protein CTCF limits proximal V recombination and restricts enhancer interactions to the immunoglobulin κ light chain locus. Immunity 35, 501–513. https://doi.org/10.1016/j.immuni.2011.07.014.

de Moura, M.C., Davalos, V., Planas-Serra, L., Alvarez-Errodo, D., Arribas, C., Ruiz, M., Aguilar-Albesa, S., Troya, J., Valencia-Ramos, J., and Vélez-Santamaría, V. (2021). Epigenome-wide association study of COVID-19 severity with respiratory failure. EBioMedicine 66, 103339. https://doi.org/10.1016/j.ebiom.2021.08.001.

Filippova, G.N., Thienes, C.P., Penn, B.H., Cho, D.H., Hu, Y.J., Moore, J.M., Klesert, T.R., Lobanenkov, V.V., and Tapsott, S.J. (2001). CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DMI locus. Nat. Gene 28, 333–343. https://doi.org/10.1038/ng570.

Hashimshony, T., Zhang, J., Keshet, I., Bustin, M., and Cedar, H. (2003). The role of DNA methylation in setting up chromatin structure during development. Nat. Commun. 8, 1–10. https://doi.org/10.1038/ncomms16026.

Li, L.C., and Dahia, R. (2002). MethPrimer: designing primers for methylation PCRs. Bioinformatics 18, 1427–1431. https://doi.org/10.1093/bioinformatics/18.11.1427.

Molvía, J.I., Rajaram, M.V., Sickli, S., Sisindran, S.J., Guirado, E., Pan, X.J., Wang, S.-H., Ross, P., Lasure, W.P., and Schlesinger, L.S. (2014). Molecular composition of the alveolar lining fluid in the aging lung. Age 36, 1187–1199. https://doi.org/10.1007/s11357-014-9633-4.

Nile, C.J., Read, R.C., Akił, M., Duff, G.W., and Wilson, A.G. (2008). Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. Arthritis Rheum. 58, 2686–2693. https://doi.org/10.1002/art.23758.

Robertson, K.D. (2005). DNA methylation and human disease. Nat. Rev. Gene. 6, 597–610. https://doi.org/10.1038/nrg1655.

Saxov, S., Berg, P., and Brutlag, D.L. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc. Nat. Acad. Sci. U S A 103, 1412–1417. https://doi.org/10.1073/pnas.0510310103.

Shanthikumar, S., Neeland, M.R., Maksimovic, J., Ranganathan, S.C., and Saffery, R. (2020). DNA methylation biomarkers of future health outcomes in children. Mol. Cell. Pediatr. 7, 1–11. https://doi.org/10.1016/j.mcpedi.2020.09.002.

Shukla, S., Kavak, E., Gregory, M., Imashimizu, M., Shutinoksi, B., Kashiev, M., Oberdoerffer, P., Sandberg, R., and Oberdoerffer, S. (2011). CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. Nature 479, 74–79. https://doi.org/10.1038/nature10442.

Sun, F., Abreu-Rodriguez, I., Ye, S., Gay, S., Distler, O., Neidhart, M., and Karouzakis, E. (2019). TET1 is an important transcriptional activator of TNFα expression in macrophages. PLoS One 14, e0218551. https://doi.org/10.1371/journal.pone.0218551.

Suzuki, A., Kawano, S., Mitsuyama, T., Suyama, M., Kansai, Y., Shiraide, K., Sasaki, H., Tokunaga, K., Tsuchihara, K., Sugano, S., and Nakai, K. (2018). DBTSS/DBKERO for integrated analysis of transcriptional regulation. Nucleic Acids Res. 46, D229–D238. https://doi.org/10.1093/nar/gkx1001.

Sziráki, A., Tyskovskiy, A., and Gladyshev, V.N. (2018). Global remodeling of the mouse DNA methylome during aging and in response to calorie restriction. Aging cell 17, e12738. https://doi.org/10.1111/acel.12738.

Takai, D., and Jones, P.A. (2002). Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc. Nat. Acad. Sci. U S A 99, 3740–3745. https://doi.org/10.1073/pnas.052410099.

Tanaka, T., Narazaki, M., and Kishimoto, T. (2014). IL-6 in inflammation, immunity, and disease. Cold Spring Harb. Perspect. Biol. 6, a016295. https://doi.org/10.1101/cshperspect.a016295.

Tang, R.-Z., Zhu, J.-J., Yang, F.-F., Zhang, Y.-P., Xie, S.-A., Liu, Y.-F., Yao, W.-J., Pang, W., Han, L.-L., and Kong, W. (2019). DNA methyltransferase 1 and Krüppel-like factor 4 axis regulates macrophage inflammation and atherosclerosis. J. Mol. Cell. Cardiol. 128, 11–24. https://doi.org/10.1016/j.yjmcc.2019.01.009.

Wang, X., Cao, Q., Yu, L., Shi, H., Xue, B., and Shi, H. (2016). Epigenetic regulation of macrophage polarization and inflammation by DNA methylation in obesity. JCI insight 1. https://doi.org/10.1172/jci.insight.87748.

Wu, H., and Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell 156, 45–68. https://doi.org/10.1016/j.cell.2013.12.019.

Xu, X., Tao, Y., Gao, X., Zhang, L., Li, X., Zou, W., Ruan, K., Wang, F., Xu, G.-L., and Hu, R. (2016). A CRISPR-based approach for targeted DNA demethylation. Cell Discov. 2, 1–12. https://doi.org/10.1038/celldisc.2016.9.

Yang, X., Wang, X., Liu, D., Yu, L., Xue, B., and Shi, H. (2014). Epigenetic regulation of macrophage polarization by DNA methyltransferase 3b. Mol. Endocrinol. 28, 565–574. https://doi.org/10.1210/mend.2013-1293.

Yin, Y., Morgunova, E., Joilma, A., Kaasinen, E., Sahu, B., Khund-Sayeed, S., Das, P.K., Kivioja, T., Dave, K., and Zhong, F. (2017). Impact of cytosine methylation on DNA binding specificities of human transcription factors. Science 356. https://doi.org/10.1126/science.aas2239.

Zhang, Q., Zhao, K., Shen, Q., Han, Y., Gu, Y., Li, X., Zhao, D., Liu, Y., Wang, C., Zhang, X., et al. (2015). Tet2 is required to resolve inflammation by recruiting Hdac2 to specifically repress IL6. Nature 525, 389–393. https://doi.org/10.1038/nature15252.
KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-phospho-IRF3 (Ser396) | Cell Signaling Technology | 4D4G; RRID: AB_823547 |
| Mouse anti-IRF3     | Cell Signaling Technology | D83B9; RRID: AB_1904036 |
| Mouse anti-phosphor-p65 (Ser536) | Cell Signaling Technology | 93H1; RRID: AB_10827881 |
| Mouse anti-p65      | Cell Signaling Technology | D14E12; RRID: AB_10859369 |
| Rabbit anti-TET1    | Gene Tex | GTX124207; RRID: AB_11176491 |
| Rat anti-TET1       | EMD Millipore | MABE1144; RRID: AB_2910103 |
| Mouse anti-actin    | Santa Cruz | sc-47778; RRID: AB_2714189 |
| Rabbit anti-CTCF    | EMD Millipore | 07-729; RRID: AB_441965 |
| HRP-conjugated anti-rabbit | Sigma | A0545; RRID: AB_257896 |
| HRP-conjugated anti-mouse | Sigma | A4416; RRID: AB_258167 |
| APC anti mouse CD11c | BD Biosciences | 550261; RRID: AB_398460 |
| PE anti mouse Siglec-F | BD Biosciences | 552126; RRID: AB_394341 |
| Bacterial and virus strains |        |            |
| Competent Quick DH5-alpha | Toyobo | DNA-913F |
| One Shot™ TOP10 Chemically Competent E. coli | ThermoFisher Scientific | C404010 |
| Chemicals, peptides, and recombinant proteins |        |            |
| TaKαRa ExTaq HS     | TaKαRa | RR006A |
| pCR2.1-TOPO-TA cloning kit | Invitrogen | 45-0641 |
| KOD FX Polymerase   | TaKαRa | KFX-101 |
| ReverTra Ace Synthesis | Toyobo | TRT-101 |
| T4 polynucleotidopoly nucleotide Kinase | New England Biolabs | M02015 |
| BbsI               | Thermo Scientific | ER1011 |
| Ligation Convenience Kit | Nippon Gene | 319-05961 |
| Puromycin          | Invivogen | Anti-pr-1 |
| Hygromycin B Gold  | Invivogen | Anti-hg-1 |
| Actinomycin D      | Sigma | A9415 |
| Protein A Sepharose 4 Fast flow | Cytiva | 17528001 |
| GM-CSF             | Pepro Tech | 315-03 |
| M-CSF              | Pepro Tech | 315-02 |
| Collagenase        | Fujifilm | 032-22364 |
| DNase              | Promega | M610A |
| Brewer thioglycollate medium | Sigma | B2551-500G |
| Deposited data     |        |            |
| Supplementary Data | This paper | N/A |
| Critical commercial assays |        |            |
| EZ DNA Methylation Kit | Zymo Research | D5002 |
| RNA Extraction Kit | Zymo Research | R1035 |
| Fast Gene Plasmid Mini Kit | Nippon Genetics | FG-90502 |
| Nucleo Bond Xtra Midi Kit | TaKαRa | U0410C |
| Neon Transfection System | Invitrogen | MPK10096 |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

### Experimental models: Cell lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RAW264.7 cells      | ATCC   | TIB-71     |
| HEK293T cells       | ATCC   | CRL-3216   |

### Experimental models: Organisms/strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse C57BL/6       | CLEA Japan | N/A        |
| Bone marrow derived macrophage from C57BL/6 mice | This paper | N/A |
| Bone marrow derived dendritic cells from C57BL/6 mice | This paper | N/A |
| Peritoneal macrophage from C57BL/6 mice | This paper | N/A |
| Alveolar macrophage from C57BL/6 mice | This paper | N/A |

### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Tet1-KO Genotyping Primer Forward | This paper | N/A |
| 5'-GTCAAGTCAAAGGAAGCTGTGATGC-3' |
| Tet1-KO Genotyping Primer Reverse | This paper | N/A |
| 5'-AACTGTGAAAGACAGACTACGGGTCTTG-3' |
| Tet2-KO Genotyping Primer Forward | This paper | N/A |
| 5'-ATTCCAGGATCACACAGGAAGC-3' |
| Tet2-KO Genotyping Primer Reverse | This paper | N/A |
| 5'-TGACACTCCTGACTGCTCTGGG-3' |
| Tet3-KO Genotyping Primer Forward | This paper | N/A |
| 5'-AAGGGGCTCTGAAACCCACCC-3' |
| Tet3-KO Genotyping Primer Reverse | This paper | N/A |
| 5'-TCAGGGTCTCTGAAATGGGGCAC-3' |
| CpG+348 Genotyping Primer Forward | This paper | N/A |
| 5'-AAGTGAAAGGCAGTTCTGAG-3' |
| CpG+348 Genotyping Primer Reverse | This paper | N/A |
| 5'-TCACATTCTGTATCTCCAGACG-3' |

### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pX330-U6-Chimeric_BB-CBhhSpCas9 | Addgene | #42230 |
| pcDNA3.1-dCas9-MQ1(WT)-EGFP | Addgene | #89633 |
| pcDNA3.1-dCas9-dMQ1-EGFP | Addgene | #89637 |
| pcDNA3.1-dCas9-MQ1(Q147L)-EGFP | Addgene | #89634 |
| pdCas9-TET1-CD | Addgene | #83340 |
| pcDNA3.1-MS2-Tet1-CD | Addgene | #83341 |

### Software and algorithms

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Il6 gene TIS Start Sites (DBTSS) | Database of Transcription | https://dbtss.hgc.jp/ |
| Transcription factor-binding site at upstream of TIS | Baccam et al. (2003) | N/A |
| CGI prediction and location of single CpG dinucleotide MethPrimer 2.0 online database program | MethPrimer2 | http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi |
| CTCF binding sites Ensembl | Ensembl | https://asia.ensembl.org/Mus_musculus/Info/Index |
| CTCF binding sites UCSC | UCSC | https://genome.ucsc.edu/ |
| CRISPR single guide RNA CHOPCHOP | CHOPCHOP | https://chopchop.cbu.uib.no/ |
| Graph drawing and statistical analysis GraphPad Prism 9 | GraphPad Prism | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Taro Kawai (tarokawai@bs.naist.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
All data reported in this paper will be shared by corresponding authors upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from corresponding authors upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
C57BL/6 mice (CLEA Japan) were bred and maintained in the specific pathogen-free animal facility. Young male mice (8–12 weeks old) and aged male mice (48–52 weeks) were used for AMs isolation. The animal maintenance and experiments performed for this study were approved by the Committee of Animal Research at Nara Institute of Science and Technology. All methods were performed based on the Policy on the Care and Use of Laboratory Animals at Nara Institute of Science and Technology.

Cells and reagents
RAW264.7 cells, a macrophage-like cell line from Mus musculus, were cultured in DMEM (Nacalai Tesque) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies). Murine bone marrow cells were treated with 10 ng/mL M-CSF (Proteo Tech) (for BMDMs) or 10 ng/mL GM-CSF (Proteo Tech) (for BMDCs), and incubated for differentiation for 7 to 8 days at 37°C and 5% CO2 with RPMI 1640 (Nacalai Tesque) containing 100 units/mL penicillin, 100 μg/mL streptomycin (Nacalai Tesque), 2-mercaptoethanol and 10% FBS. Brewer thioglycollate medium at 3% (w/v) was pre-injected into the peritoneal cavity of mice for 3–5 days, and 5 mL of PBS containing 3% FBS was injected to collect the peritoneal fluids. The cells obtained from the peritoneal fluids were used as primary PECs. Lungs from mice were isolated and treated with collagenase (Fujifilm032-22364) and DNase (Promega M610A) for 30 min in RPMI 1640 medium. CD11c and Siglec F positive cells were isolated by FACS Aria II (BD Bioscience) and were used as primary AMs.

KO cells
To generate gene KO or CpG dinucleotide deletion using RAW264.7 cells, guide sequences located in exon 4 of Tet1 and exon 3 of Tet2 and Tet3 were subcloned into pX330-U6-Chimeric_BB-CBhhSpCas9 (42230, Addgene) expressing Cas9 and gRNA: Tet1, sense 5’-CCACCGCATGAGTGTCACCCTC-3’; Tet2, antisense 5’-CACCAGGTTGTTGTTCTCGGTG-3’; Tet3, sense 5’- CACCATTTGCACCTAGTC CCTCCG-3’; CpG+348, sense 5’- CCACATACCCTCCAAAGTGCCG-3’. The gRNAs targeting exon 3 of both Tet2 and Tet3 have no potential off-target sites (Table S2). The gRNA for Tet1 and CpG+348 deletion have a few potentials off-target sites, but no mutation was found at the off-target sites in Tet1-KO and CpG+348-deletion cells (data not shown). Genomic regions containing guide sequences were amplified from the genome of RAW264.7 cells and inserted into the pCAG EGxxFP plasmid, acting as a reporter for genome editing. Then these plasmids at a concentration of 500 ng/μL were electroporated into RAW264.7 cells by NEON (Invitrogen) at 1680 V, 20 ms, and 1 pulse. GFP-positive cells were sorted by a BD FACS Aria (BD Bioscience). Cells were cultured for 2 weeks until cellular density reached 70%. Then, cells were transferred to 24-well plates and DNA was isolated for sequence analysis.

METHOD DETAILS

RNA isolation and qRT-PCR
Total RNA from samples was isolated with TRIzol reagent (Invitrogen) and reverse transcribed with ReverTra Ace (Toyobo) according to the manufacturer’s instructions. qRT-PCR was performed with the following primers: mTet1, forward 5’-GGACTTACATTAGCAAGCCTG-3’, reverse 5’-GGGCGCTTTCTTTTCTTTT
TTGTGTACC-3', mTel2, forward 5'-CTCCTGGTGAACAACTGTCAATGG-3', reverse 5'-CTAATAGCTGCCACATCAGACC-3', mTel3, forward 5'-GTATGGGAAAGGGAAGAGGC-3', reverse 5'-AGGATCAGAATAACATCACTGCGG-3', mii6, forward 5'-GTAGCTATAGTACTCCAGAAG-3', reverse 5'-ACGATGATGACCATTTGGGAA-3', mTnf, forward 5'-CTGTAGCCCGCTGCAGTCA-3', reverse 5'-TTCGATGATGTGCAGCAGAGAT-3', mTnf, forward 5'-CTGTAGCCCGCTGCAGTCA-3', reverse 5'-TTCGATGATGTGCAGCAGAGAT-3'.

WB assay

Cells were stimulated with LPS and lysed with RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS). Whole-cell lysates were mixed with 2 x SDS sample buffer (1 M Tris-HCl, 10% SDS, 20% glycerol, 0.01% bromphenol blue, 0.2 M DTT) and heat-treated at 95°C for 5 min. Samples were subjected to SDS-PAGE and proteins were transferred to PVDF membrane (Bio-Rad). Transferred membranes were blocked with 5% skim milk in TBST buffer (0.5 M Tris, 1.38 M NaCl, 0.027 M KCl, 0.05% Tween 20) and then were incubated with anti-phospho-IRF3 (Ser396) mouse antibody (D14E12; Cell Signaling Technology), phosphor-p65 (Ser536) mouse antibody (93H1; Cell Signaling Technology), or anti-p65 mouse antibody (4D4G; Cell Signaling Technology), anti-IRF3 mouse antibody (D83B9; Cell Signaling Technology), anti-acid sodium, 0.01% (w/v) SDS]. The chromatin was sheared for 60 min by sonication with Covaris S220 (75 W, 3 mL of Lysis buffer 3 [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA (pH 8.0), 150 mM NaCl, 10 mM EDTA (pH 8.0), 0.5% (w/v) Deoxycholic acid sodium, 0.1% (w/v) SDS, 1% NP-40, 0.5% (w/v) Deoxycholic acid sodium, 0.1% (w/v) SDS, 1% NP-40, and once with 1 mL of high-salt buffer [10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% (w/v) Deoxycholic acid sodium, 0.1% (w/v) SDS, 1% NP-40], and once with 1 mL of LiCl buffer [10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA (pH 8.0), 0.5% (w/v) Deoxycholic acid sodium, 0.1% (w/v) SDS, 1% NP-40], and last resuspended in 400 μL of elution buffer [10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA (pH 8.0), 0.5% (w/v) SDS]. The eluted protein-DNA complex was treated with 10 mg/mL of RNaseA (Nippon Gene) at 37°C for one hour, and then reverse cross-linked with 10 mg/mL of iScience 25, 104118, April 15, 2022 17
Proteinase K (Wako) at 65°C overnight. The DNA was purified with phenol/chloroform/isoamyl alcohol. The DNA samples were then analyzed by qRT-PCR using the following primers for the Il6 locus: CTCF-1 forward, 5'-AGCCCAACAAAGAGTAC-3', reverse 5'-CAAGTGACAGATAGCAC-3'; CTCF-2, forward 5'-GCCCTTTTCTCTGTC-3', reverse 5'-GTTTTCTCGCAAGTGC-3'; non-CTCF, forward 5'-TGGAGTTAGGGACTTGG-3', reverse 5'-GGAGAAGCTTTGGAATTGGG-3', the Mx1 locus: forward 5'-TGTTCCATTCCAGCATCTC-3', reverse 5'-TCCACTCTCCTCTCTTTC-3'.

**DNA methylation analysis**

Bisulfite conversion of DNA was done using the EZ DNA methylation kit (Zymo Research) following the manufacturer's instructions. Briefly, about 250 ng to 2 μg of DNA samples in 50 mM sodium hydroxide (NaOH) (Wako) with 1M Tris-HCl at pH 8.0 (Nacalai Tesque) were taken and prepared for bisulfite conversion with 100 μL of CT conversion reagent after heating at 37°C for 15 min. Then, the samples were incubated in the dark at 50°C for 18–20 h. The bisulfite-converted DNA samples were purified according to the manufacturer's protocols. PCR was performed using Ex Taq (Takara). The PCR primers were amplified for the Il6 locus: forward 5'-AGGGAGTGTGTGTTTTTGTATG-3', reverse 5'-CTCTCAAAAACCTACCCCTTTT-3'; Region 1, forward 5'-CACAACCTAACCCTCTCT-3', reverse 5'-GTTTTCTGCAAGTGCATCATC-3'; Region 2, forward 5'-AGGGAGGAGGAGGAGG-3', reverse 5'-GTTATAGGTTATGAAGGAAG-3'; Region 3, forward 5'-GGAGAGCATTGGAAATTGGG-3', reverse 5'-TCCACTCCTCTCTCTTTC-3'. The amplified products were subjected to electrophoresis on a 2% agarose gel, and were then sub-cloned into a pCR2.1-TOPO cloning vector (Invitrogen). Bisulfite conversion reactions were performed independently more than two times and the PCR fragments in pCR2.1-TOPO were sequenced at least 10 clones in each dot spot. The rate of methylation was calculated as the number of methylated cytosines divided by the number of total samples sequenced.

**mRNA stability assay**

The cells were stimulated with 100 ng/mL LPS (Sigma) for 2 h. Then, 5 mg/mL of Actinomycin D (Sigma) was added to the culture medium. The total RNAs were prepared at the indicated periods. The total mRNA was quantified by qRT-PCR.

**Targeted DNA methylation and demethylation in CpG dinucleotides**

sgRNA1(+251) (forward 5'-CCACTGTCTCTTGGGACTGATGC-3', reverse 5'-AAACGCATCAGTCCCCAAGAAGGCA-3') was subcloned into pcDNA3.1-dCas9-MQ1(MQ1)-EGFP (Addgene #89633), pcDNA3.1-dCas9-dMQ1-EGFP (Addgene #89637), and pcCas9-TET1-CD (Addgene #83340). sgRNA2(+263) (forward 5'-CCACTGTGTGCTGACACACCA-3', reverse 5'-AAACCTGTTGTCCACGACATG-3') was subcloned into pcDNA3.1-dCas9-MQ1(Q147L)-EGFP (Addgene #89634) and pcDNA3.1-dCas9-MQ1-EGFP (Table S2). For the transfection of pcDNA3.1-dCas9-MQ1(MQ1)-EGFP, pcDNA3.1-dCas9-MQ1(Q147L)-EGFP and pcDNA3.1-dCas9-dMQ1-EGFP, 5 μL of plasmids at a concentration of 2–5 μg/μL were electroporated into RAW264.7 cells by NEON (Invitrogen) at 1680 V and 20 ms. The transfected cells were cultured in Penicillin/Streptomycin-supplemented DMEM media for 2 days and GFP-positive cells were sorted by a BD FACS Aria (BD Bioscience). Similarly, for the transfection of pcDNA3.1-M52-Tet1-CD (Addgene #83341) and dCas9-TET1-CD, plasmids at a concentration of 2–5 μg/μL, which adjusted to a fixed molar ratio of 1:2:6 as suggested by Xu et al., were electroporated into RAW264.7 cells by NEON (Invitrogen) at 1680 V and 20 ms (Xu et al., 2016). The transfected cells were cultured in Penicillin/Streptomycin-supplemented DMEM media for 2 days and were selected by 250 μg/mL of hygromycin B gold (Invivogen) for 4 days. Both the FACS-sorted or hygromycin-selected cells were collected and stimulated with 100 ng/mL of LPS (Sigma) for 6 h. Total RNA was prepared from the samples by RNA Extraction Kit (Zymo Research) according to the manufacturer’s instructions.

**Genome annotation**

The TIS of Il6 was obtained from the Database of Transcription Start Sites (DBTSS; Suzuki et al., 2018). CGI prediction and single CpG dinucleotide identification in Il6 were performed using MethPrimer 2.0 online database program (Li and Dahiya, 2002). The CTCF binding sites in the Il6 locus were obtained from genome annotation.
Ensembl genome browser and USCS genome browser. The transcription factor-binding sites in the promoter region of Il6 of Mus musculus were taken from Baccam et al. (2003) (Baccam et al., 2003).

QUANTIFICATION AND STATISTICAL ANALYSIS

The methylation rate of a CpG dinucleotide is calculated as follow: rate of methylation = number of methylated cytosines/total number of clones sequenced. A two-tailed Student’s unpaired t test was used for two-group comparisons, a one-way or two-way ANOVA with Tukey’s multiple comparison test was used when three or more groups were compared. All statistical analysis were processed by the GraphPad Prism Version 9.0 software package (GraphPad Software, San Diego, CA).