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Screening of Optimal CpG-Oligodeoxynucleotide for Anti-Inflammatory Responses in the Avian Macrophage Cell Line HD11

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CpG-oligodeoxynucleotides (**CpG-ODNs**) have been shown to possess immunostimulatory features in both mammals and birds. However, compared to their proinflammatory effects, little is known about the anti-inflammatory responses triggered by CpG-ODN in avian cells. Hence, in this study, the anti-inflammatory response in the chicken macrophage cell line HD11 was characterized under stimulation with five types of CpG-ODNs: CpG-A_{1585}, CpG-A_{D35}, CpG-B_{1555}, CpG-B_{K3}, and CpG-C_{2395}. Single-stimulus of CpG-B_{1555}, CpG-B_{K3}, or CpG-C_{2395} induced interleukin (*IL*)-*10* expression without causing cell injury. The effects of pretreatment with CpG-ODNs before subsequent lipopolysaccharide stimulation were also evaluated. Interestingly, pretreatment with only CpG-C_{2395} resulted in high expression levels of *IL-10* mRNA in the presence of lipopolysaccharide. Finally, gene expression analysis of inflammation-related cytokines and receptors revealed that pretreatment with CpG-C_{2395} significantly reduced the mRNA expression of tumor necrosis factor-α, *IL-1β*, *IL-6*, and Toll-like receptor 4. Overall, these results shed light on the anti-inflammatory responses triggered by CpG-C_{2395} stimulation through a comparative analysis of five types of CpG-ODNs in chicken macrophages. These results also offer insights into the use of CpG-ODNs to suppress the expression of proinflammatory cytokines, which may be valuable in the prevention of avian infectious diseases in the poultry industry.

Key words: anti-inflammatory response, chicken macrophages, CpG-ODN, HD11 cells, IL-10

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INTRODUCTION

Macrophages contribute significantly to the host immune response via phagocytosis, antigen presentation, and cytokine secretion. Avian macrophages are dispersed throughout body fluids and tissues (Klasing, 1998) and are therefore suitable for regulating host immune responses throughout the body. Avian macrophages challenged with pathogens or immunogens express several proinflammatory cytokines, such as tumor necrosis factor (**TNF**)-α, interleukin (**IL**)-1β, and IL-6, and induce immune and inflammatory responses (Klasing, 1994). However, excessive expression of proinflammatory cytokines results in severe disease. For example, infection with the avian influenza virus inappropriately regulates the host's immune system and rapidly induces the expression of proinflammatory cytokines, such as IL-6 and serum amyloid A (Burggraaf et al., 2014). In addition, chicken enteritis is characterized by excessive expression of proinflammatory cytokines (Sun et al., 2022). Thus, regulation of inflammatory responses of avian macrophages is critical to prevent avian infectious diseases in the poultry industry.

Macrophages produce IL-10, a major anti-inflammatory cytokine. IL-10 reduces the inflammatory response triggered by the stimulation of *Borrelia burgorferi*, a tick-borne spirochete, in mammalian macrophages in an autocrine manner (Chung et al., 2013). Recently, IL-10 has been shown to function as an anti-inflammatory factor in chickens and mammals (Wu et al., 2016). Moreover, enhanced IL-10 expression correlates with the reduced expression of inflammatory cytokines in the chicken intestine and macrophages (Hu et al., 2021). Therefore, the induction of IL-10 in macrophages is a good strategy for the prevention of avian diseases responsible for excessive proinflammatory responses.

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Table 1. **Nucleotide sequences of CpG-oligodeoxynucleotides (CpG-ODNs) used in this study**

Classification	Name	Sequence (5′ to 3′)
Control	GpC_{1612}	G*C*T*A*G*A*G*C*T*T*A*G*G*C*T
Class A	CpG-A_{1585}	G*G G G T C A A C G T T G A G*G*G*G*G*G
Class A	CpG-A_{D35}	G*G T G C A T C G A T G C A G G G G*G*G
Class B	CpG-B_{1555}	G*C*T*A*G*A*C*G*T*T*A*G*C*G*T
Class B	CpG-B_{K3}	A*T*C*G*A*C*T*C*T*C*G*A*G*C*G*T*T*C*T*C
Class C	CpG-C_{2395}	T*C*G*T*C*G*T*T*T*T*C*G*G*C*G*C*G*C*G*C*C*G

*Phosphorothioate bond

In this study, the anti-inflammatory features of CpG-oligodeoxynucleotides (**CpG-ODNs**) were examined as potential regulators of immune responses. CpG-ODNs directly upregulate cytokines in innate immune cells, such as macrophages (Krieg, 2002). Several studies have shown that CpG-ODNs activate innate immune responses in chickens. For example, CpG-ODNs induce inflammatory responses by increasing nitric oxide, IL-1β, IL-6, and IL-12 levels in the chicken macrophage-like cell line HD11 (He et al., 2003; He and Kogut, 2003; Xie et al., 2003; He et al., 2007; Han et al., 2010). In contrast, CpG-ODNs also upregulate the anti-inflammatory response via IL-10 in mammalian cells (Yi et al., 2002; Saraiva and O'Garra, 2010). However, little is known about the anti-inflammatory response triggered by CpG-ODNs in avian cells. Because the induction of IL-10 suppresses the expression of proinflammatory cytokines, CpG-ODNs may be valuable for disease prevention in the poultry industry.

CpG-ODNs are divided into three classes, A, B, and C, based on their structural and biological characteristics (Krieg, 2006). Most studies assessing the immunostimulatory features of CpG-ODNs against chicken macrophages have been conducted using several types of Class B CpG-ODNs (He et al., 2003; He and Kogut, 2003; Xie et al., 2003; He et al., 2007; Han et al., 2010). More recently, the immunostimulatory features of Class A and C CpG-ODNs have also been assessed using CpG-A_{1585}, CpG-A_{2216}, and CpG-C_{2395} (Ciraci and Lamont, 2011; Barjesteh et al., 2014). Nevertheless, the anti-inflammatory response of each class of CpG-ODNs in chicken macrophages has not been characterized. Although He et al. (2012) have demonstrated that co-stimulation with a Class B CpG-ODN and polyinosinic-polycytidylic acid (**poly I:C**), a double-stranded RNA generally used as a viral mimic, strongly induces IL-10 in chicken monocytes, anti-inflammatory effects triggered by prophylactic use of CpG-ODNs against subsequent inflammatory responses have not yet been characterized. In the context of avian disease prevention, basic knowledge of the anti-inflammatory response induced by pretreatment with CpG-ODNs is very important.

Hence, in the present study, the effects of five different CpG-ODNs, belonging to each of the three classes of CpG-ODNs, on cell injury and *IL-10*-inducibility in HD11 cells, which are used to evaluate anti-inflammatory effects (Hu et al., 2021), were compared. These effects were also investigated in HD11 cells pretreated with CpG-ODN and then stimulated with lipopolysaccharide (**LPS**). Furthermore, the anti-inflammatory responses triggered by CpG-C_{2395} and the effects of CpG-C_{2395} pretreatment before LPS stimulation on the expression of inflammation-related genes were evaluated.

MATERIALS AND METHODS

Cell culture

HD11 cells were propagated and maintained in complete Dulbecco's modified Eagle's medium (**DMEM**) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum at 37 °C under 5% CO_2 atmosphere. The cells were subcultured every 3–4 days. HD11 cells were seeded into a 24-well cell culture plate (Corning Inc., New York, NY, USA) at a density of 1×10^5 cells/well in complete DMEM and cultured for 24 h at 37 °C in a humidified incubator supplied with 5% CO_2. The cells were harvested, the culture supernatant was removed by aspiration, and then the cells were washed once with phosphate-buffered saline (PBS) and used for further experiments.

Treatments of HD11 cells with CpG-ODN and LPS

Endotoxin-free desalted phosphorothioate-ODN (**PS-ODN**) was synthesized by GeneDesign, Inc. (Osaka, Japan). PS-ODN was reconstituted in PBS and passed through a 0.22 μm pore microfilter (Nihon Millipore K.K., Tokyo, Japan). The ODN sequences, namely CpG-A_{1585}, CpG-A_{D35}, CpG-B_{1555}, CpG-B_{K3}, CpG-C_{2395}, and GpC_{1612} are presented in Table 1. CpG-A_{1585} and CpG-B_{1555} have been used in mice and mouse cells (Shimosato et al., 2010; Yamamoto et al., 2017); CpG-A_{D35} and CpG-B_{K3} have been used in human cells (Aoshi et al., 2015); and CpG-C_{2395} have been used in mice and human cells (Roda et al., 2005; Yamamoto et al., 2016). GpC_{1612}, which does not include unmethylated CpG dinucleotides that are important for CpG-ODN activity, was used as a control (Yamamoto et al., 2017). LPS from *Escherichia coli* O127:B8 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Seeded HD11 cells were incubated with 3 μM CpG-ODNs for 24 h and subjected to cell viability assays and quantitative reverse transcription-polymerase chain reaction (**qRT-PCR**) analysis. In addition, HD11 cells incubated with 3 μM CpG-ODNs for 24 h followed by stimulation with 500 ng/mL LPS for 2 h were also assayed using the above methods.

Table 2. **Nucleotide sequences of primers used in quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Primer	Sequence (5′ to 3′)	Reference	Annealing temperature (°C)
β-actin F	ACCCCAAAGCCAACAGAGAG	Ichikawa et al., 2021	60
β-actin R	AACACCATCACCAGAGTCCATC		
IL-10 F	CGCTGTCACCGCTTCTTCA	Lammers et al., 2010	66
IL-10 R	TCCCGTTCTCATCCATCTTCTC		
TNFα F	CGCTCAGAACGACGTCAA	Rohde et al., 2018	60
TNFα R	GTCGTCCACACCAACGAG		
IL-6 F	AAATCCCTCCTCGCCAATCT	Zhao et al., 2018	60
IL-6 R	CCCTCACGGTCTTCTCCATAAA		
IL-1β F	TGGGCATCAAGGGCTACA	Lee et al., 2010	64
IL-1β R	TCGGGTTGGTTGGTGATG		
TLR4 F	TCTTTCAAGGTGCCACATCCA	Tang et al., 2021	64
TLR4 R	AGCGACGTTAAGCCATGGAA		

Cell viability assay

The proportion of viable CpG-ODNs and LPS-treated cells was observed using an inverted microscope IX71 (Olympus, Tokyo, Japan) and photographed using a DP70 camera (Olympus). Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (**MTT**) assay with some modifications (Ito et al., 2013; Hattori et al., 2021). Briefly, 300 µL complete DMEM containing 0.5 mg/mL MTT was added to cells after aspiration of medium, and the cells were incubated for 4 h at 37 °C. The cells were then washed with PBS to remove the non-internalized MTT, and 300 µL of 40 mM hydrochloric acid-isopropanol solution was added to lyse the cells to release the MTT internalized by the viable cells. Next, 100 µL MTT solution was transferred to a 96-well plate (Thermo Fisher Scientific), and the MTT concentration was measured colorimetrically. The cell viability was estimated as the viability of CpG-ODN- or CpG-ODN plus LPS-treated cells relative to that of PBS-treated (control) cells by measuring the optical density at 570 nm. The effect of actinomycin D, a cytotoxic reagent (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), was also examined to confirm the proportion and viability of HD11 cells used in this study.

qRT-PCR analysis

Total RNA from cells treated with CpG-ODN and LPS was purified using the FastGene RNA Premium Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). cDNA was prepared by reverse transcription of 100 µg total RNA per sample using SuperScript IV reverse transcriptase (Thermo Fisher Scientific). Equal volumes of cDNA were used to quantify various immune response-related cytokine factors by qRT-PCR using the StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA) and KOD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) with specific primers. The nucleotide sequences of the primers that were used are listed in Table 2. qRT-PCR was performed in duplicate. The relative expression levels of each target gene were calculated using the ΔΔCt method (Livak and Schmittgen, 2001) and normalized against β-actin expression.

Statistical analysis

Statistical analyses were performed using GraphPad Prism8 (GraphPad Software, Inc., San Diego, CA, USA). Data obtained from the cell viability assay were analyzed using the Dunnett's test. Data obtained from qRT-PCR were analyzed using one-way analysis of variance with the Tukey–Kramer post-hoc test.

RESULTS

Cell viability of HD11 cells treated with CpG-ODN under normal and inflammatory conditions

Whether the viability of HD11 cells was affected by CpG-ODN was examined using an IX71 inverted microscope and an MTT assay. Microscopic observations revealed that CpG-ODNs treatment did not affect the proportion of viable HD11 cells under normal conditions. However, actinomycin D treatment reduced the proportion of viable cells under the same conditions (Figure 1A). The MTT assay revealed that treatment with CpG-A_{1585}, CpG-B_{1555}, CpG-B_{K3}, and CpG-C_{2395} significantly increased the cell proliferation index compared to that obtained with treatment with PBS alone (Figure 1B), thereby indicating their enhanced mitogenic activity. Thus, it is likely that CpG-ODNs, except for CpG-A_{D35}, enhance mitogenic activity in HD11 cells under normal conditions.

The same analyses were conducted using HD11 cells under LPS-induced inflammatory conditions. To induce an inflammatory response, HD11 cells were incubated with LPS for 2 h, as described previously (Lian et al., 2012; Xu et al., 2013; Qi et al., 2017). The proportion of viable cells did not change under these conditions (Figure 1C). However, contrary to the results of the MTT assay under normal conditions, pretreatment with CpG-ODN did not change the cell proliferation index compared to that obtained with treatment with LPS alone (Figure 1D). Therefore, all CpG-ODNs used in this study were non-toxic to HD11 cells under both normal and inflammatory conditions.

Comparison of the effect of CpG-ODN on the induction of IL-10 mRNA expression in HD11 cells

To screen for the CpG-ODN that strongly induces the expres-

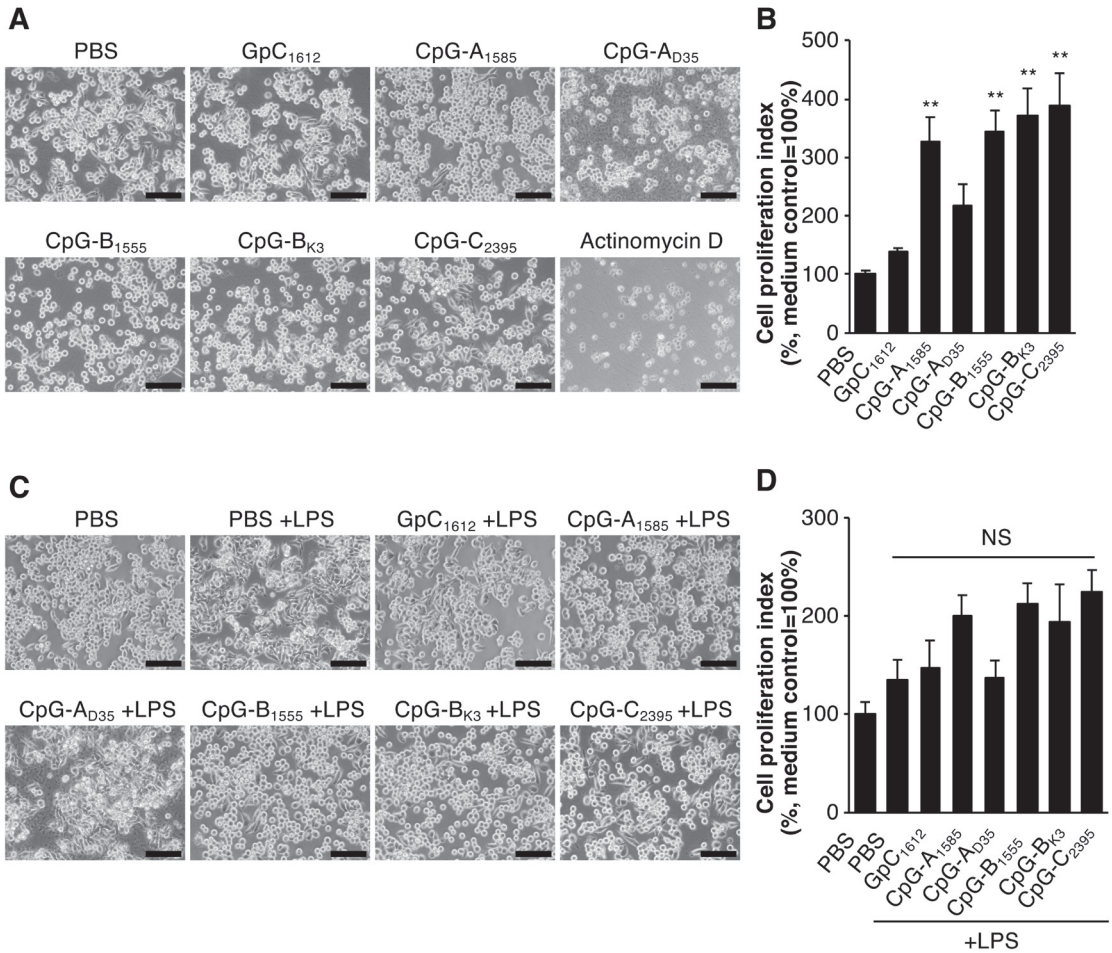

Fig. 1. **Evaluation of cell viability.** (A, C) Cellular morphology of HD11 cells treated with oligodeoxynucleotides (ODN) (A) or stimulated by lipopolysaccharide (LPS) after pretreatment with each ODN (C). Scale bars = 100 μm. (B, D) Results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay using HD11 cells treated with ODN (B) or stimulated by LPS after pretreatment with each ODN (D). Error bars indicate the standard error of the mean (SEM) of the cell proliferation index calculated from three independent experiments (n = 3). Statistical significance was evaluated using the Dunnett's test comparing HD11 cells treated with phosphate-buffered saline (PBS) (B) or stimulated by LPS after pretreatment with PBS (D) (**$P < 0.01$). NS = not significant.

sion of anti-inflammatory cytokines in HD11 cells, the effects of CpG-A_{1585}, CpG-A_{D35}, CpG-B_{1555}, CpG-B_{K3}, and CpG-C_{2395} on *IL-10* mRNA expression were examined in HD11 cells under normal and inflammatory conditions. Treatment with CpG-B_{1555}, CpG-B_{K3}, and CpG-C_{2395} significantly induced *IL-10* mRNA expression compared to the induction achieved upon treatment with other ODNs and the PBS control under normal conditions, with the highest induction obtained with CpG-C_{2395} (Figure 2A). In addition, pretreatment with CpG-C_{2395} resulted in the highest induction of *IL-10* mRNA expression under LPS-induced inflammation condition. However, similar results were not observed in HD11 cells pre-treated with CpG-B_{1555} or CpG-B_{K3} plus LPS (Figure 2B). Thus, CpG-C_{2395} strongly induced *IL-10* mRNA expression in HD11 cells under both conditions. The role of CpG-C_{2395} as an effective inducer of IL-10 was further evaluated in subsequent functional analyses, focusing on the regulation of inflammatory responses.

Effects of CpG-C_{2395} treatment on LPS-induced inflammatory responses in HD11 cells

Finally, the effects of CpG-C_{2395} pretreatment on LPS-induced inflammatory responses were evaluated, focusing on its effects on the proinflammatory cytokines TNF-α, IL-1β, and IL-6. The mRNA levels of *TNF-α*, *IL-1β*, and *IL-6* remarkably increased in cells treated with LPS, but were significantly inhibited by CpG-C_{2395} pretreatment. Additionally, the mRNA levels of LPS-induced Toll-like receptor (***TLR***) *4*, a pattern recognition receptor that specifically recognizes LPS (Brownlie and Allan, 2011), were significantly inhibited by CpG-C_{2395} (Figure 3). These

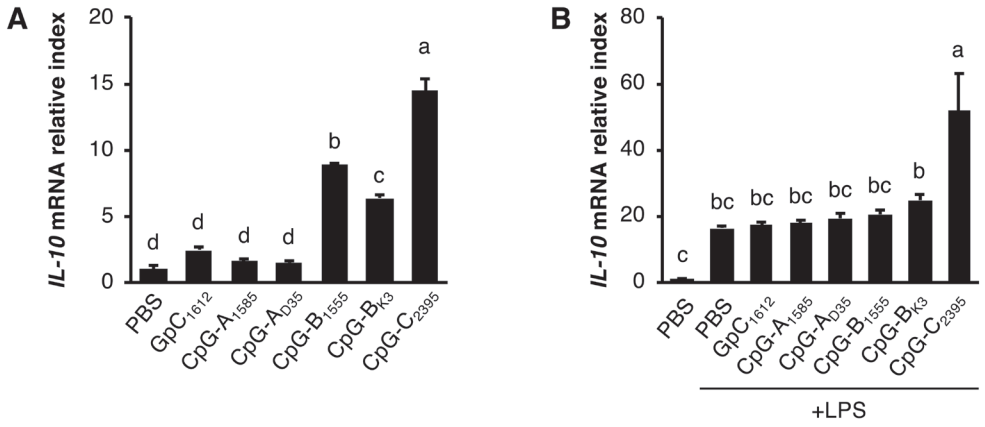

Fig. 2. **Expression analysis of interleukin (IL)-10 using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).** (A) Expression analysis of HD11 under single-stimulus conditions. (B) Expression analysis of HD11 stimulated by lipopolysaccharide (LPS) after pretreatment with each oligodeoxynucleotide (ODN). The ΔΔCt method was used to calculate relative expression levels. Levels of *IL-10* mRNA were normalized to β-actin mRNA expression. Error bars indicate the standard error of the mean (SEM) of relative expression levels for three independent experiments (n = 3). Statistical significance was evaluated using Tukey's test. Different letters represent significant differences ($P < 0.05$). PBS, phosphate-buffered saline.

results suggested that CpG-C_{2395} inhibited the LPS-induced inflammatory response in HD11 cells.

DISCUSSION

The main goal of this study was to characterize the anti-inflammatory features of CpG-ODNs in avian cells. Five CpG-ODNs generally used in mammals were screened and it was observed that prophylactic use of CpG-C_{2395} strongly induced an anti-inflammatory response in HD11 cells upon subsequent LPS stimulation. To the best of our knowledge, this is the first study to elucidate the anti-inflammatory responses of chicken macrophages using a comparative analysis of CpG-ODN.

A single stimulus of each ODN to HD11 cells showed that CpG-B_{1555}, CpG-B_{K3}, and CpG-C_{2395}, but not CpG-A_{1585} and CpG-A_{D35}, induced *IL-10* expression (Figure 2A) without cell injury (Figures 1B and 1D). Class B CpG-ODNs containing a full PS backbone with one or more CpG dinucleotides, called the CpG motif, directly stimulate mammalian macrophages and induce IL-10 expression (Yi et al., 2002; Boonstra et al., 2006). Overall, it was shown that Class B CpG-ODNs induced IL-10 expression in both mammalian and avian macrophages. Additionally, Class C CpG-ODNs possess intermediate structural features between Class A CpG-ODNs, which have a CpG-containing palindromic motif, and Class B CpG-ODNs (Hanagata, 2012). Therefore, it was suggested that CpG-C_{2395}, belonging to Class C CpG-ODN, may strongly induce *IL-10* expression in HD11 cells because its structural features are similar to those of Class B CpG-ODN. CpG-A_{1585}, a Class A CpG-ODN, also induces anti-inflammatory responses in mice (Yamamoto et al., 2017). However, in the current study, a single stimulus of CpG-A_{1585} upregulated cell viability, but did not induce *IL-10* expression in HD11 cells. Moreover, a single stimulus of CpG-A_{D35} did not affect the cell viability or *IL-10* expression in HD11 cells. As the functions of Class A CpG-ODNs in chickens are poorly understood, further studies are needed.

Comparative analysis using each ODN showed that only pretreatment with CpG-C_{2395} promoted the expression of *IL-10* under LPS-induced inflammatory conditions (Figure 2B). Additionally, pretreatment with CpG-C_{2395} downregulated several inflammation-related genes without causing cell injury (Figures 1B, 1D, and 3). Thus, CpG-C_{2395} induced anti-inflammatory responses following LPS stimulation. Previous comparative studies using different classes of CpG-ODNs have shown the efficacy of Class B CpG-ODNs against antiviral or bacterial responses in chickens (Dar et al., 2009; Barjesteh et al., 2014). Additionally, Class B CpG-ODNs have been identified upon administering CpG-ODNs as vaccine adjuvants to chickens (Wang et al., 2009). Thus, previous studies have mainly focused on the immunostimulatory features of Class B CpG-ODNs in poultry. In contrast, the present findings showed that pretreatment with Class C CpG-ODNs was effective for anti-inflammatory responses in chicken macrophages.

Mammals and birds have different mechanisms to recognize CpG-ODNs. In mammals, CpG-ODNs are recognized by TLR9 (Hemmi et al., 2000). However, birds have evolutionarily lost TLR9 and TLR21 acts as its functional homolog (Brownlie et al., 2009; Keestra et al., 2010). Interestingly, although mammalian TLR9 and avian TLR21 are functionally identical in that they recognize CpG-ODNs, they have minimal sequence similarity and different CpG-ODN sequence recognition profiles (Xie et al., 2003; Keestra et al., 2010; Chuang et al., 2020). TLR15, a Toll-like receptor unique to birds, also contributes to CpG-ODN

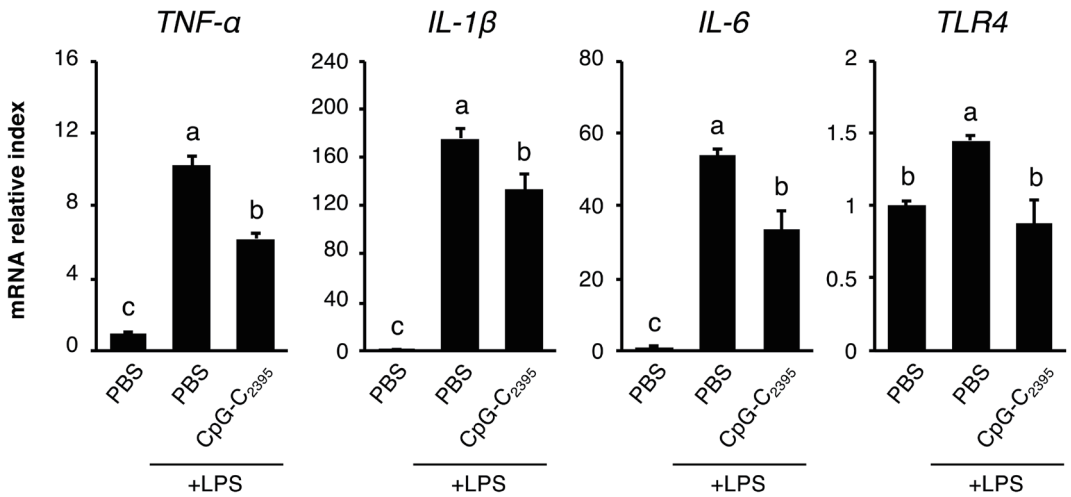

Fig. 3. **Expression analysis of inflammation-related genes using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The ΔΔCt method was used to calculate relative expression levels.** The mRNA levels of each gene were normalized to β-actin mRNA expression. Error bars indicate the standard error of the mean (SEM) of relative expression levels for three independent experiments (n = 3). Statistical significance was evaluated using Tukey's test. Different letters represent significant differences ($P < 0.05$). PBS, phosphate-buffered saline; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α, IL-1β, interleukin-1β; TLR4, Toll-like receptor 4.

recognition (Ciraci and Lamont, 2011). Although the detailed mechanisms and differences in selectivity of CpG-ODN remain unclear, basic knowledge of the immunostimulatory features of CpG-ODNs against avian cells, but not mammalian cells, is required to use CpG-ODN as an anti-infective agent in the poultry industry. These results provide a new perspective on the superiority of CpG-C_{2395} in the suppression of diseases caused by excessive expression of proinflammatory cytokines in chickens.

In conclusion, CpG-C_{2395} directly upregulated *IL-10* and reduced the expression of LPS-induced inflammatory cytokines in HD11 cells. These findings provide a new perspective on the superiority of CpG-C_{2395} in preventing diseases responsible for excessive expression of proinflammatory cytokines, such as avian influenza and enteritis. However, this study is limited in that cell–cell interactions and preventive effects against infection and intestinal inflammatory disease in birds following pretreatment with CpG-C_{2395} were not evaluated. Hence, further studies are needed to evaluate the effects of CpG-C_{2395} *in vivo* and to apply the findings of this study in the poultry industry.

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AUTHOR CONTRIBUTIONS

Kennosuke Ichikawa conducted the experiments and wrote the manuscript. Mei Matsuzaki, Ryo Ezaki, and Hiroyuki Horiuchi discussed the data. Yoshinari Yamamoto designed the experiments and supervised the study. All authors have read and agreed to the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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