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CpG oligodeoxynucleotide and double-stranded RNA synergize to enhance nitric oxide production and mRNA expression of inducible nitric oxide synthase, pro-inflammatory cytokines and chemokines in chicken monocytes

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Toll-like receptors (TLRs) recognize microbial components and initiate the innate immune responses that control microbial infections. The interaction between ligands of TLR3 and TLR9, poly I:C (an analog of viral double-stranded RNA) and CpG-ODN (a CpG-motif containing oligodeoxydinucleotide) on the inflammatory immune responses, including the production of nitric oxide (NO) and the expression of inducible NO synthase (iNOS), pro-inflammatory cytokines interleukin (IL)-1β and IL-6, and chemokines IL-8 and macrophage inflammatory protein (MIP)-1β, were investigated in chicken monocytes. The NO production was significantly higher when stimulated with a combination of CpG-ODN and poly I:C than with either CpG-ODN or poly I:C alone. Similarly, a significant synergistic effect by CpG-ODN and poly I:C was observed in the up-regulation of iNOS and IL-8 mRNA after 2 h and persisted up to 24 h. Although the combinatory treatment of CpG-ODN and poly I:C enhanced the expression of IL-1β, IL-6, and MIP-1β after 2 h stimulation, the synergism in the up-regulation of IL-1β and IL-6 mRNA was observed after 8-h and 24-h stimulation, respectively, whereas there was no synergistic effect on MIP-1β. Our results demonstrate that CpG-ODN synergizes with poly I:C to induce pro-inflammatory immune response in chicken monocytes.

Keywords: CpG-ODN, double-stranded RNA, nitric oxide, cytokines, Toll-like receptor synergy

Introduction

Toll-like receptors (TLRs) are the most important germ-line encoded pattern recognition receptors in the innate immune system that recognize pathogen-associated molecular patterns (PAMPs). As the surveillance molecules of the innate immune system, TLRs distinguish harmful invading pathogens and play a key role in the host immune defense against microbial infections.[1,2] In general, TLRs recognize three classes of structurally distinct PAMP ligands: (i) TLR3, TLR9, and TLR7/TLR8 recognize microbial nucleic acids, such as viral double-stranded (ds) RNA, unmethylated bacterial/viral DNA, and viral single-stranded RNA, respectively; (ii) TLR2 (in concert with TLR1 or TLR6) and TLR4 interact with many lipid-based microbial structures, such as lipopeptide and lipoprotein of Gram-positive bacteria and lipopolysaccharide (LPS) from Gram-negative bacteria; and (iii) TLR5 and TLR11 are receptors for microbial protein structures, recognizing flagellin and profilin, respectively.[3]

Many chicken TLRs have been identified, including homologues to human TLR1 (type b), TLR2 (types 1 and 2), TLR3, TLR4, TLR5, and TLR7; chicken specific TLR16 and TLR15; and TLR21.[4,5] Similar to mammalian TLRs, chicken TLRs mediate innate immune responses to microbial agonist stimulations and play an important role in the control of infection.[6–20] Although analysis of the chicken genome has failed to identify a chicken orthologue to mammalian TLR9,[4,5] immune

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stimulatory activities of the TLR9 ligand, CpG oligodeoxynucleotides (CpG-ODN), has been demonstrated in chicken immune cells.[7–11,21,22] These results suggest that a novel TLR structurally divergent from the typical mammalian TLR9 may have evolved in avian species to recognize microbial CpG-DNA. A recent study demonstrated that, when ectopically expressed in human HEK-293T cells, chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG-ODN.[23]

Synthetic polyinosinic-polycytidylic acid (poly I:C) is an analog of dsRNA.[24,25] In mammals, TLR3 recognizes poly I:C and mediates immune response including production of cytokines, such as type I interferons (IFNs), interleukin (IL)-12, IL-6 and tumor necrosis factor (TNF)-α.[24,26] Similarly, chicken TLR3 has also been found to mediate the immune stimulatory activity of poly I:C in avian immune cells.[18,20,27]

Toll-like receptors have been shown to mediate overlapping and specific immune responses to microbial agonists depending on TLR-associated adaptor proteins and factors that mediate the downstream signaling cascades.[2] A complex immune responses can arise upon microbial infection, in which different TLRs of the host immune cells may simultaneously engage multiple PAMPs. Emerging evidence indicates that TLRs co-operate and cross-talk when engaging multiple agonists and these interactions can result in either suppressing or synergizing a particular immune response. For example, co-stimulation of mouse peritoneal macrophages (PECs) with the TLR2 and TLR4 ligands, MALP-2 and LPS, markedly increases TNF-α production.[28] Treatment of murine macrophages with a combination of TLR9 and TLR3 agonists synergizes to produce nitric oxide (NO), IL-12, TNF-α, and IL-6.[29] Human TLR3 and TLR4 are also known to act in synergy with TLR7, TLR8, and TLR9 in the induction of IL-12p40 and IL-12p35 genes in dendritic cells (DCs).[30] Co-stimulation with CpG-ODN and flagellin synergistically enhances the secretion of IL-10 and IFN-γ, but conversely inhibits IFN-α production in human PBMCs, monocytes, and monocyte-derived DCs.[31] Toll-like receptors 2 and 3 act in concert to induce inflammatory cytokines TNF-α, IL-6, and IL-12p40 in mouse DCs, but down-regulate TLR3-induced expression of IL-12p35.[32] In human monocyte-derived macrophages and DCs, combinatory stimulation of the TLR8 ligand together with the TLR3 or TLR4 ligand lead to synergistic expression of IL-6, IL-10, IL-12, and TNF-α mRNA.[33] These observations clearly underscore the importance of TLR synergy in both the magnitude and the direction of the immune response.

Thus far, little is known of the interaction between TLR agonists on immune responses in avian immune cells, with the exception of our previous study in which we reported that CpG-ODN and poly I:C synergize in the production of NO in chicken peripheral blood monocytes.[10] In the present study, we conducted experiments to examine whether the interaction between CpG-ODN and poly I:C would also influence other pro-inflammatory immune response in chicken monocytes, including the expression of inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines and chemokines.

Materials and Methods

Reagents

Synthetic ODNs were purchased from TriLink BioTechnologies (San Diego, CA, USA). The sequences of synthetic CpG-ODNs used in the present study were: GTCGTTGTCGTTGTCGTT.[21] The synthetic dsRNA analog, poly I:C, was obtained from InvivoGen (San Diego, CA, USA).

Cell isolation

Chicken peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected from 2- or 3-day-old chickens as previously described.[10] Briefly, peripheral blood from approximately 50 chickens was pooled, mixed with 1% methylcellulose (1 : 1, v/v), and centrifuged at 25 *g* for 15 min. The supernatant was collected and diluted with Ca^{2+} and Mg^{2+}free Hanks balanced salt solution, carefully layered onto a discontinuous Histopaque gradient (specific gravity 1.077/1.119) in 50-ml conical centrifuge tubes, and centrifuged at 250 *g* for 60 min. The PBMC layer at the 1.077/1.119 interface was collected, washed, and resuspended in RPMI-1640 containing gentamicin (50 μg/ml). Cell counts were performed and cells were diluted to appropriate concentrations for each assay.

Monocyte culture and stimulation

Aliquots of 2 ml of PBMCs (1×10^7 cells/ml) were dispensed into a 12-well plate and incubated at room temperature (~22°C) for 2 h. After incubation, non-adherent cells were removed by washing three times with RPMI-1640. The adherent-enriched monocytes were cultured for 18 h in a complete DMEM medium (Dulbecco's modified Eagle's medium containing 10% chicken serum, antibiotics [100 U penicillin/ml and 100 μg streptomycin/ml], and 1.5 mM L-glutamine). Prior to stimulation, cells were washed once more with fresh media. Cells were then stimulated with CpG-ODN,

poly I:C, or a combination of two for the indicated period (2, 4, 8, and 24 h) at 41°C in a 5% CO_2 and 95% humidity incubator. Thrombocytes were the significant contaminating cell population in the isolated monocytes after initial washings; however, the number of adherent thrombocytes was drastically reduced (to less than 10%) after overnight (18 h) culture and subsequent washing with fresh media prior to stimulation. The reduction of the thrombocyte population in monocyte cultures might be due to the loss of adherence during culturing as reported in a previous study.[34]

Nitrite assay

Nitrite, a stable metabolite of nitric oxide, produced by activated monocytes was measured by the Griess assay.[35] Briefly, an aliquot of 100 µl culture supernatant from each well was transferred to the wells of a new 96-well flat-bottom plate and combined with 50 µl of 1% sulfanilamide and 50 µl of 0.1% naphthylenediamine (both were prepared in 2.5% phosphoric acid solution). After 10 min incubation at room temperature, the nitrite concentration was determined by measuring optical density (OD_{550}) of each well using a SPECTRA MAX microplate reader (Molecular Devices; Sunnyvale, CA, USA). Sodium nitrite (Sigma) was used as a standard to determine nitrite concentrations in the cell-free medium.

Quantitative real-time reverse transcription PCR (QRT-PCR) analysis of cytokine and iNOS gene expression

Total RNA devoid of gDNA contamination was isolated from chicken monocytes using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Total RNA samples (1 µg each reaction) were reverse transcribed to cDNA using the ThermoScript RT-PCR System (Invitrogen; Carlsbad, CA, USA). Expression levels of chicken iNOS, pro-inflammatory cytokines IL-1β and IL-6, chemokines IL-8 and MIP-1β, and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined by QRT-PCR using MX3000P® (Stratagene; La Jolla, CA, USA). Primers and probes (Table 1) were obtained from Applied Biosystem (Austin, TX, USA). Primer amplification efficiency was verified for each gene using 2-fold serial dilutions of cDNA. Analysis of QRT-PCR was performed for each sample in duplicate in a total volume of 25 µl, consisting of 12.5 µl Brilliant® II QPCR Master Mix (Stratagene), 0.5 µl ROX reference dye diluted 1 : 500, 1.25 µl primer/probe mix (900 nM/250 nM final concentrations, respectively), 5.75 µl RNase/DNase-free water, and 5 µl diluted cDNA (25 ng RNA). All reaction plates were run under identical cycle conditions, 95°C for 10 min, and 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. The fluorescence threshold was set at 0.2 and the resulting cycle threshold values (Ct), normalized to the reference gene, were used for analysis.

Table 1. QRT-PCR primer and probe sequences

	Name	Sequence (5′ → 3′)
ch-GAPDH	Probe	CTTGGCTGGTTTCTCC-(FAM)
	F	CCCCAATGTCTCTGTTGTTGAC
	R	CAGCCTTCACTACCCTCTTGAT
ch_iNOS	Probe	CCCAATAGCCACCTTCAG-(FAM)
	F	CCCTCCAGCTGATCAGACTATC
	R	GTGTGCAAGCCGGAATCTTTT
ch-IL-1β*	Probe	CCACACTGCAGCTGGAGGAAGCC-(FAM)
	F	GCTCTACATGTCGTGTGTGATGAG
	R	TGTCGATGTCCCGCATGA
ch-IL-6	Probe	CTTGCACATCTCGTCCTGC-(FAM)
	F	ACCGCGCCGTCCAG
	R	TTCTCGCACACGGTGAACTT
ch-IL-8*	Probe	TCTTTACCAGCGTCCTACCTTGCGACA-(FAM)
	F	GCCCTCCTCCTGGTTTCAG
	R	TGGCACCGCAGCTCATT
ch-MIP-1β	Probe	ACCGGTGGGTTCTGAC-(FAM)
	F	CCTCATTGCCATCTGCTACCA
	R	CGGGAGATGTAGGTGAAGCA

F, forward; R, reverse.
*Primer and probe sequences were kindly provided by Dr Peter Kaiser, Institute for Animal Health, Compton, UK.

Data analysis

At least two independent experiments were conducted at different times. Within each experiment, nitrite levels from 3–5 replicate wells of cell culture were measured for each treatment. For each gene, the threshold cycle (Ct) values of different treatments at each time point post-treatment were normalized to the respective endogenous control, GAPDH, to obtain the ΔCt (dCt) values. To assess differences between treatments at each time point post-treatment, ddCt values (the difference of dCt value between stimulated and unstimulated control) were analyzed.[36] For presentation purposes, results are expressed as fold-changes (2^{-ddCt}) in gene expression.

Data were analyzed by one-way ANOVA, followed by multiple comparisons (Tukey test) using SigmaStat® software (Jandel Scientific, San Rafael, CA, USA). *P*-values of <0.05 were considered to be significant.

RESULTS

Synergistic interaction of CpG-ODN and poly I:C on iNOS gene expression and NO production in chicken monocytes

The synthetic CpG-ODN itself was a potent immune stimulant and induced significant production of NO in chicken monocytes, while poly I:C did not induce NO production (Fig. 1A). However, combined stimulation with both CpG-ODN and poly I:C strongly synergized in the production of NO in chicken monocytes. Similarly, synergism between CpG-ODN and poly I:C was observed in respect to iNOS gene expression in stimulated chicken monocytes (Fig. 1B). During the 24-h stimulation period, transient increase in iNOS mRNA (between 2–8 h) was observed in the monocytes stimulated with poly I:C alone, but this increase in iNOS mRNA did not give rise to NO production. On the other hand, CpG-ODN stimulation yielded both elevated iNOS mRNA levels and a significant increase in NO production. Furthermore, simultaneous stimulation with CpG-ODN and poly I:C not only strongly up-regulated, but also prolonged, iNOS gene expression, resulting in a significantly higher iNOS mRNA level and NO production than stimulation with either agonist alone. These results suggest 'cross-talk' between the TLR signaling pathways.

Differential and synergistic effect of CpG-ODN and poly I:C on pro-inflammatory cytokines IL-1β and IL-6 gene expressions in chicken monocytes

Pro-inflammatory cytokines, such as IL-1β and IL-6, regulate the host immune response to infection and play

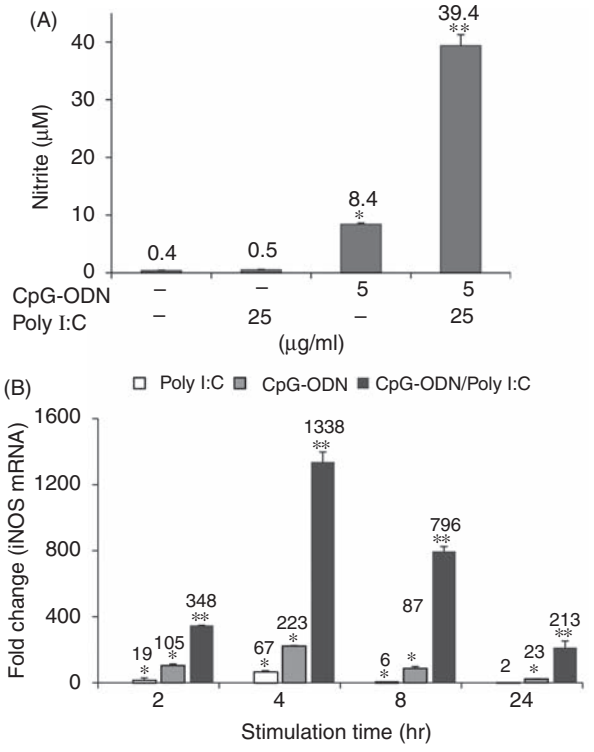

Fig. 1. NO induction and iNOS mRNA expression in chicken monocytes stimulated by CpG-ODN, poly I:C, or a combination of the two. (A) NO production after 72 h stimulation and (B) iNOS mRNA expression after 2, 4, 8, and 24 h stimulation. Data are mean values and SD from three (NO production) or two (iNOS mRNA expression) independent experiments. *Differences between treatments and the control are statistically significant ($P = 0.05$). **Significant synergistic effect by a combinatory treatment with CpG-ODN and poly I:C ($P = 0.05$).

an important role in controlling and eliminating invading pathogens. Recognition of mammalian TLR9 agonist CpG-ODN by chicken blood monocytes has led to a strong up-regulation of the expression of both IL-1β (Fig. 2A) and IL-6 (Fig. 2B). In contrast, exposure to poly I:C resulted in only a slight rise in IL-1β and IL-6 mRNA after 2 h and 4 h of stimulation and these mRNA levels returned to control levels by 8 h. However, a combinatory stimulation with CpG-ODN and poly I:C brought enhanced expressions of IL-1β after 4 h and IL-6 after 8 h of stimulation. A strong synergistic effect on mRNA expression was observed after 8 h of stimulation for IL-1β and after 24 h of stimulation for IL-6.

Differential and synergistic effect of CpG-ODN and poly I:C on chemokines IL-8 and MIP-1β gene expressions in chicken monocytes

Chemokines mediate trafficking of leukocytes to the site of infection by inducing chemotaxis and activation of different types of inflammatory cells. Chemokines IL-8 and MIP-1β are members of the CXC and CC

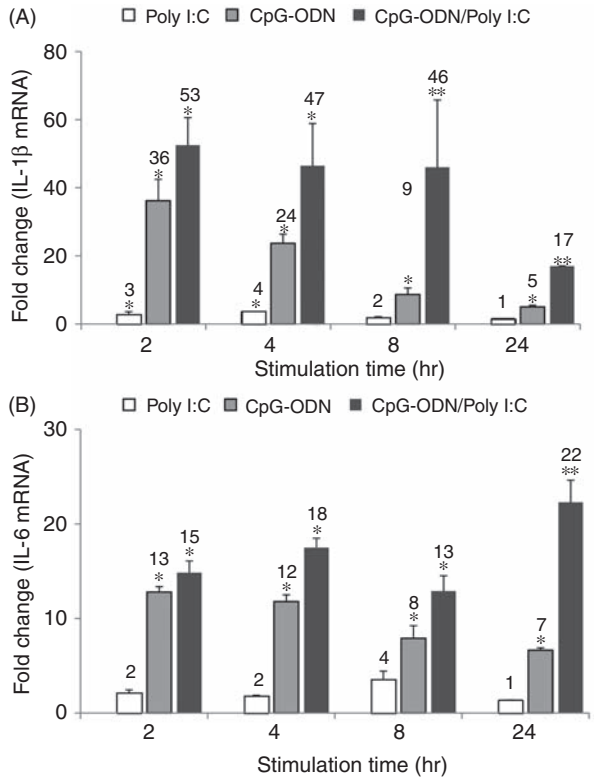

Fig. 2. The expression of IL-1β and IL-6 mRNA in chicken monocytes stimulated by CpG-ODN (5 μg/ml), poly I:C (25 μg/ml) or a combination of the two after 2, 4, 8, and 24 h stimulation. (A) IL-1β and (B) IL-6. Data are mean values and SD of two independent experiments. *Differences between treatments and the control are statistically significant ($P = 0.05$). **Significant synergistic effect by a combinatory treatment with CpG-ODN and poly I:C ($P = 0.05$).

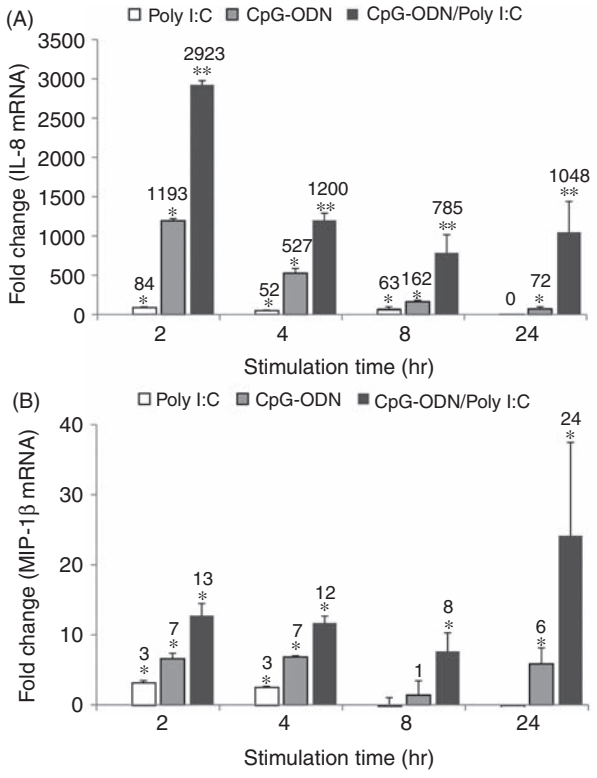

Fig. 3. The expression of IL-8 and MIP-1β mRNA in chicken monocytes stimulated by CpG-ODN (5 μg/ml), poly I:C (25 μg/ml) or a combination of the two after 2, 4, 8, and 24 h stimulation. (A) IL-8 and (B) MIP-1β. Data are mean values and SD of two independent experiments. *Differences between treatments and the control are statistically significant ($P = 0.05$). **Significant synergistic effect by a combinatory treatment with CpG-ODN and poly I:C ($P = 0.05$).

chemokine families, respectively, and their primary function is to recruit granulocytes, such as heterophils in chickens. Expression of the IL-8 gene in chicken monocytes was highly inducible by either CpG-ODN or poly I:C (Fig. 3A). The IL-8 mRNA responded rapidly, and was the highest increase in each treatment after 2 h of treatment. In the monocytes treated with a combination of CpG-ODN and poly I:C, a significantly larger increase (2923-fold) in IL-8 mRNA expression was observed than in cells treated with either CpG-ODN (1193-fold) or poly I:C (84-fold) alone after 2 h of stimulation. Furthermore, when stimulated with either CpG-ODN alone or a combination of CpG-ODN and poly I:C, the cells exhibited a prolonged strong up-regulation of IL-8 gene expression, whereas the IL-8 mRNA in poly I:C treated monocytes leveled off after 8 h of stimulation. Treatments with CpG-ODN or a combination of CpG-ODN and poly I:C also induced increased MIP-1β mRNA expression in chicken monocytes, while poly I:C alone showed only a modest effect on MIP-1β gene expression after 4 h of stimulation (Fig. 3B).

Discussion

Monocytes and macrophages are mononuclear phagocytes that play a critical role in the innate immune response and as antigen processing and presenting cells in the acquired immune response. As a component of the first-line immunological defense, monocytes and macrophages recognize infectious agents through the PAMP recognition-receptors TLRs.[37] Recognition of microbial components by TLRs initiates signal cascades which trigger expression of genes that produce reactive oxygen and nitrogen intermediates (ROI and RNI), pro-inflammatory cytokines, and co-stimulatory molecules.[38,39] Nitric oxide is an important pro-inflammatory mediator, involved in macrophage antimicrobial and tumoricidal activities.[40] Nitric oxide mediates host defense against intracellular pathogenic micro-organisms such as *Salmonella* spp.[40,41] and against virus proliferation.[41,42] Interleukin-1β and IL-6 are two major pro-inflammatory cytokines, regulating host immune response to infection and play an important role in controlling and eliminating invading pathogens.

Higher IL-1β and IL-6 mRNA expression levels in blood leukocytes have been associated with better resistance to *Salmonella* infection in broiler chicken lines.[43] Chemokines IL-8 and MIP-1β, representatives of the CXC and CC chemokine families respectively, are produced mainly by macrophages to induce chemotaxis and cell activation of different types of inflammatory cells and inflammation at the site of infection. However, information concerning TLR-mediated inflammatory response in chicken monocytes is limited.

Many TLRs share largely common signaling cascades which eventually converge at the NF-κB pathway.[44] However, adaptor proteins that are recruited to, and interact with, TLRs upon activation by engaging microbial agonists can modulate the direction of the signaling cascade to confer the specificity of TLR-mediated immune response.[45,46] Under physiological conditions, host immune cells that encounter microbes may be exposed to multiple PAMPs. Increasing evidence indicates that, when engaging multiple agonists, TLRs cross-talk through their downstream signaling components, and such interaction can greatly influence the outcome of the host immune response.[29–33] In this study, we demonstrated that this co-operative interaction between CpG-ODN and poly I:C synergistically amplified the pro-inflammatory immune response in chicken blood monocytes. Agonists CpG-ODN and poly I:C demonstrated vast differences in their immune stimulatory effects on chicken monocytes. In general, CpG-ODN exhibited much greater immune stimulatory capacity for inducing pro-inflammatory responses, including NO production and expression of iNOS, pro-inflammatory cytokines IL-1β and IL-6, and chemokines IL-8 and MIP-1β mRNA; conversely, poly I:C showed minimal effects on most of the immune response measured in this study, except for a moderate, brief up-regulation of iNOS and IL-8 mRNA expression after 8 h of stimulation. Compared to CpG-ODN which induced both high level of iNOS mRNA and NO production, poly I:C stimulation was limited to a shorter period of up-regulation of iNOS mRNA expression. This transient induction of iNOS mRNA, however, failed to yield enough iNOS activity to produce measurable amounts of NO. However, a combinatory stimulation with CpG-ODN and poly I:C brought a strong synergistic effect, dramatically increasing not only NO production, but also the mRNA expression of iNOS in chicken monocytes. Clearly, the synergistic increase of NO production was a result of a strong and prolonged up-regulation of iNOS gene expression in chicken monocytes. In addition to iNOS, a combinatory stimulation with CpG-ODN and poly I:C also synergistically enhanced the expressions of IL-1β, IL-6, IL8, and MIP-1β mRNA.

The mechanism for the synergy observed between CpG-ODN and poly I:C on NO production and pro-inflammatory cytokine and chemokine expression cannot be readily explained. In mammalian cells, the proximate intracellular location of both TLR3 and TLR9 in the endosomal compartment[47,48] may result in a closer interaction between these two pathways. Although their signaling pathways are divergent, with TLR9 being MyD88-dependent while TLR3 transduces signaling through TRIF,[46] their downstream signaling cascades eventually converge at the NF-κB pathway. Blocking NF-κB activation in macrophages attenuates both TLR3- and TLR9-mediated inflammatory cytokine gene expressions.[49] Thus, the combination of CpG-ODN and poly I:C may result in the activation of NF-κB by two simultaneously activated signaling cascades of chicken TLR3 and chicken TLR21, a functional homolog to mammalian TLR9.[23]

The innate immune response of producing inflammatory cytokines and other pro-inflammatory mediators such as NO is critical for controlling pathogenic infections. It has been demonstrated that chickens with increased innate immune responses, such as heterophil oxidative burst, degranulation, and pro-inflammatory cytokine expression have a greater resistance to infectious pathogens.[50] More specifically, higher IL-1β and IL-6 mRNA expression levels in blood leukocytes upon stimulation have been associated with better resistance to *Salmonella* infection in broiler chicken lines.[43] Previous studies have demonstrated that treatment with immune stimulatory CpG-ODN enhances resistance to bacterial infection[9,51–54] in neonatal chickens and suppresses infectious bronchitis virus replication in chicken embryos.[55] The results of this study show that a combination of bacterial DNA and dsRNA induces a markedly enhanced inflammatory immune response that has both antiviral and antibacterial activity in primary chicken monocytes. Whether the synergistic immune stimulatory property of CpG-ODN and poly I:C in combination can be further explored as an immunological strategy for controlling viral and bacterial infections in chickens remains to be investigated. In humans, concomitant viral and bacterial co-infection causes a 'lethal synergism'.[56] The murine model reveals that viral and bacterial co-infections induce excessive inflammatory responses, which contribute to lethal immunopathology and sepsis.[56] Thus far, there is no indication that viral and bacterial co-infection causes a 'lethal synergism' in avian species. However, the synergistic interaction between CpG-ODN and poly I:C, mimicking bacterial DNA and viral ds-RNA respectively, on the pro-inflammatory immune response described in the present study is similar to that found in mammalian cells.[29]

Conclusions

Ligands of mammalian TLR9 and TLR3, CpG-ODN and poly I:C, have been shown to exhibit vast differences in their immune stimulatory properties on chicken monocytes. Clearly, CpG-ODN exhibited a much greater capacity to induce pro-inflammatory immune responses in chicken monocytes, including NO production and expression of iNOS, pro-inflammatory cytokines IL-1β and IL-6, and chemokines IL-8 and MIP-1β mRNA, than poly I:C. However, in combination, CpG-ODN and poly I:C greatly synergized to stimulate NO production and the expression of iNOS, pro-inflammatory cytokines IL-1β and IL-6, and chemokines IL-8 and MIP-1β. The synergistic properties of CpG-ODN and poly I:C in combination may be important for the future development of an immunological strategy for controlling food-borne pathogens in poultry.

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Mention of commercial or proprietary products in this paper does not constitute an endorsement of these products by the USDA, nor does it imply the recommendation of products by the USDA to the exclusion of similar products.

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