Toll-like receptor (TLR)21 signalling-mediated antiviral response against avian influenza virus infection correlates with macrophage recruitment and nitric oxide production

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Abstract
Cytosine-guanosinedeoxyxynucleotide (CpG) DNA can be used for the stimulation of the toll-like receptor (TLR)21 signalling pathway in avian species which ultimately leads to up-regulation of gene transcription for pro-inflammatory molecules including nitric oxide and recruitment of innate immune cells. The objective of this study was to determine the antiviral effect of NO, produced in response to in ovo delivery of CpG DNA, against avian influenza virus (AIV) infection. We found that when CpG DNA is delivered at embryo day (ED)18 in ovo and subsequently challenged with H4N6 AIV at ED19 pre-hatch and day 1 post-hatching, CpG DNA reduces H4N6 AIV replication associated with enhanced NO production and macrophage recruitment in lungs. In vitro, we showed that NO originating from macrophages is capable of eliciting an antiviral response against H4N6 AIV infection. This study provides insights into the mechanisms of CpG DNA-mediated antiviral response, particularly against AIV infection in avian species.

INTRODUCTION
Influenza virus belongs to the family, Orthomyxoviridae and is classified into types A, B and C. Type A influenza viral strains have an avian lineage (also called avian influenza virus or AIV) and are virulent, causing severe diseases in birds and mammals as well as in humans [1]. Although the AIVs primarily circulate in wild waterfowl, they are capable of adapting to multiple species [2] and these animal species may act as a source of virus posing a threat to human health. Although vaccination is considered a key component of AIV control, the protection is unreliable because of continuous mutation of the virus in vivo due to selective pressure imposed by vaccine-mediated immune responses [3]. Given the limitations of current control measures, it is a necessity that novel measures are developed against AIV infections based on thorough understanding of host responses elicited against this infection.

Following an infection, host responses can be mounted either very early, which are known as innate host responses, or late, which are known as adaptive immune responses. The innate immune system, which mounts potent and broadly effective host responses, is equipped with a range of immune cells including macrophages. Pulmonary macrophages provide the first line of innate host responses against respiratory pathogens including AIV [4]. The macrophages are capable of recognition of microbial molecules referred to as pathogen-associated molecular patterns [5]. This recognition is mediated by pattern recognition receptors (PRRs) expressed on macrophages as well as on other immune and non-immune cells in the host. Among the PRRs, the toll-like receptors (TLRs) [6] are well studied. TLR9 in mammals and TLR21 in avian species are the only identified receptors that detect microbial DNA containing unmethylated cytosine-guanosine deoxyxynucleotide (CpG) motifs, which are generally methylated in the genomes of vertebrates [7, 8]. CpG DNA has been investigated against many clinical conditions including cancers, autoimmune diseases, hypersensitive reactions and infectious diseases, and as an adjuvant in vaccines [9], and clinical trials have shown promising effects and applicability in disease control [9]. In addition, CpG DNA has been investigated...
widely as a potent immunomodulatory agent in chickens [10–12] that activates the innate arm of the immune system against a broad range of pathogens [13]. In ovo-delivered CpG DNA also has been shown as a stand-alone treatment against viruses such as infectious laryngotracheitis virus (ILT-V) [14] and bacterial infections such as Escherichia coli [15]. Binding of CpG DNA with TLR9 and TLR21 initiates downstream signalling [9] that ultimately leads to up-regulation of gene transcription for the production of pro-inflammatory molecules including nitric oxide [16]. NO is a free radical molecule known to play a key role in the innate host responses against numerous virus infections [17–19]. Recent studies indicated that TLR9 and TLR21 activation by CpG DNA induces a large amount of NO production in mammalian [20, 21] and avian species [22–24], respectively.

In ovo or embryo vaccination was first introduced in chickens for the control of Marek’s disease [25] and since then in ovo vaccination against this disease has become a common poultry industry practice. The in ovo route of vaccination is also extensively investigated for the control of other poultry diseases and has become a commercial practice for the control of a number of these poultry diseases [26]. Following this poultry industry practice, the induction of antiviral responses pre-hatch via in ovo administration is desirable in order to provide early innate responses against circulating pathogens at the time of placing the newly hatched day-old chickens in the barn. This protection following in ovo delivery of TLR ligands has been proven against a number of microbial infections in terms of significant reduction in the microbial burdens [27, 28].

CpG DNA which interacts with avian TLR21 has been shown to elicit antibacterial effects and protection against E. coli and Salmonella Typhimurium septicaemia in chickens [28, 29]. Further, synthetic CpG DNA when delivered in ovo was reported to inhibit the replication of ILTV [14] and infectious bronchitis virus (IBV) [10]. Similarly, post-hatch immunization using CpG DNA-adjuvanted virosomes has been shown to reduce AIV shedding [30]. In some other studies, the enhancement of immune responses has been shown following administration of post-hatch avian influenza vaccine adjuvanted with CpG DNA [31, 32]. However, there is no information available on the mechanisms of CpG DNA-mediated antiviral activity against AIV or any other avian virus infections. Therefore, we hypothesized that CpG DNA is able to elicit antiviral responses against H4N6 AIV infection via increased macrophage numbers and elevated NO production originating from macrophages. The objectives of this study were to determine whether CpG DNA could elicit antiviral responses against AIV infection in vitro and in vivo, and then to investigate the mechanisms of antiviral responses. We found that CpG DNA when administered in ovo can lead to antiviral responses against H4N6 AIV replication in the respiratory tract attributable to increased macrophage numbers and enhanced NO production in macrophages.

![Fig. 1. CpG DNA, when delivered in ovo, decreases H4N6 AIV replication in lungs, with the potential involvement of NO and macrophages as mediators in lungs. (a) Specific-pathogen-free (SPF) ED18 eggs were injected with CpG DNA (n=5) or PBS (n=5), infected with H4N6 AIV at ED19, and lung viral replication was determined at ED20. Representative Madin–Darby canine kidney (MDCK) plates showing H4N6 AIV titres and quantitative data are presented. (b) SPF ED18 eggs were delivered with CpG DNA (n=7) or PBS (n=7), infected with H4N6 AIV at ED19, and the lungs were homogenized and NO production was measured at ED20 and presented as detailed in Methods. (c) SPF ED18 eggs were injected with CpG DNA (50 μg) in ovo (n=5) or PBS (n=7) and the lung macrophages were quantified at ED19 and presented as detailed in Methods. Student’s t-tests were performed to identify group differences and the differences were considered significant at P<0.05. The error bar is the standard error of mean (SEM).](image-url)
RESULTS

CpG DNA elicits antiviral response when delivered in ovo, with potential involvement of lung macrophages and NO as mediators of the antiviral response

In ovo delivery of CpG DNA has been shown to elicit protective innate responses against bacterial and viral infections in chickens [14, 28]. Since innate responses are known to be elicited rapidly (within 24 h) following TLR ligand delivery [33], we planned to challenge embryos within 24 h of in ovo CpG DNA delivery at embryo day (ED) 18. We delivered CpG DNA (50 µg CpG DNA diluted in 200 µl sterile PBS per egg, n=5) in ovo at ED18 in SPF chicken eggs and the control eggs received 200 µl sterile PBS per egg (n=5). Both groups were infected with H4N6 AIV at ED19 via the same route (1.3×10^4 p.f.u. per egg) 24 h post-treatment. At 24 h post-infection (ED20), the lung tissues from each embryo were collected, homogenized and plaque assays were performed to quantify virus particles. In ovo-delivered CpG DNA at ED18 significantly reduces H4N6 AIV replication 24 h post-infection in lungs when compared to controls that received PBS at ED18 (P=0.04, Fig. 1a).

To study the potential mediators of antiviral response such as macrophages and NO elicited by CpG DNA against AIV infection, we delivered CpG DNA (50 µg CpG DNA diluted with 200 µl sterile PBS per egg, n=5) in ovo at ED18 in SPF chicken eggs while the control eggs received 200 µl sterile PBS per egg (n=5). Both groups were infected with H4N6 AIV at ED19 via the same route (1.3×10^4 p.f.u. per egg) 24 h post-treatment. At 24 h post-infection (ED20), the lung tissues from each embryo were collected, homogenized and plaque assays were performed to quantify virus particles. In ovo-delivered CpG DNA at ED18 significantly reduces H4N6 AIV replication 24 h post-infection in lungs when compared to controls that received PBS at ED18 (P=0.04, Fig. 1a).

Since we observed that in ovo-delivered CpG DNA elicits an antiviral response and during this response, NO production in the lungs of embryos was higher, we questioned whether the CpG DNA-mediated antiviral response against H4N6 AIV replication is attributable to NO production. To investigate whether the antiviral response elicited by in ovo CpG DNA delivery against H4N6 AIV replication is attributable to NO production, we used S-methylisothiourea (SMT), a selective inhibitor of inducible nitric oxide synthase (iNOS) that is widely used to block NO production from L-arginine in many in vivo and in vitro studies [34–36]. In our experiments, firstly, CpG DNA (50 µg CpG DNA diluted with 200 µl sterile PBS, group 1) and CpG DNA together with SMT (50 µg CpG DNA and 1.5 mg SMT diluted in 200 µl PBS per egg, group 2) were delivered in ovo at ED18 (n=5 eggs per group) with a control group receiving PBS (200 µl sterile PBS per egg, group 3). At ED19, the eggs in all three groups were infected with 1.3×10^4 p.f.u. H4N6 AIV per egg in ovo and subsequently the lungs were sampled at ED20. The samples were homogenized and the supernatants were collected to quantify live virus particles using the standard plaque assay technique. The experiment was repeated with CpG DNA (n=7), CpG DNA plus SMT (n=6) and PBS (n=6) with similar results and the data were pooled. The NO concentration in the homogenized lung supernatants was also determined using the Griess assay.

At ED18 when in ovo delivery of CpG DNA is combined with SMT which inhibits NO production, we found an increasing trend of H4N6 AIV replication in the lungs similar to the PBS-treated group (P=0.081, Fig. 2a) coinciding with a significant decrease in NO production when compared to the group that received in ovo CpG DNA alone (P=0.0019, Fig. 2b). The difference in H4N6 AIV replication between CpG DNA plus SMT and PBS groups was not significantly different (P>0.05, Fig. 2a).

In ovo-delivered CpG DNA-mediated antiviral response post-hatch correlates with macrophage response in the respiratory tract

Since in ovo-delivered CpG DNA elicits antiviral responses against H4N6 AIV within 24 h, we wanted to see whether this antiviral response persists post-hatch. Since the poultry industry practice is placing the day-old chickens immediately following hatch in the contaminated poultry barn environment, we simulated this field condition by infecting the day-old chickens following in ovo delivery of CpG DNA. For this, we delivered CpG DNA (50 µg CpG DNA diluted with 200 µl sterile PBS per egg, n=6) in ovo at ED18 in SPF eggs and the control eggs received 200 µl sterile PBS per egg (n=6). Both groups were infected with H4N6 AIV intranasally under gas anaesthesia (isoflurane) at day 1 post-hatch (2.7×10^4 p.f.u. per chick). At 3 days post-infection, the chicks were euthanized, tracheas were collected and an immunofluorescence assay was performed to quantify AIV nuclear antigen.

At ED18, in ovo-delivered CpG DNA significantly reduces H4N6 AIV replication in post-hatch chickens 3 days post-infection in the trachea when compared to controls that received PBS (P=0.03, Fig. 3a). As hypothesized, we further found that in ovo-delivered CpG DNA at ED18 showed an increasing trend in the number of macrophages in the trachea at day 1 post-hatch (at the time of infection) compared to the controls (P=0.07, Fig. 3b).
**H4N6 AIV infection does not increase macrophage numbers in in ovo CpG DNA-, CpG DNA plus SMT- or PBS-treated lungs**

Due to the observation that the CpG DNA-mediated antiviral response against H4N6 AIV infection correlates with macrophage recruitment, we then investigated to see whether this pattern of macrophage recruitment is due to the H4N6 AIV infection. When we injected CpG DNA ($n=11$) or CpG DNA plus SMT ($n=10$), with controls receiving PBS ($n=12$), at ED18 in ovo and subsequently infected subsets of eggs of all three groups with H4N6 AIV in ovo at ED19 balanced with uninfected controls of PBS ($n=6$), CpG ($n=4$) and CpG plus SMT ($n=4$), we found that there was no significant difference between the infected CpG DNA-treated and uninfected CpG DNA-treated groups ($P>0.05$, Fig. 4). However, in the H4N6 AIV infected and uninfected groups, higher macrophage numbers were observed in in ovo CpG DNA-treated groups when compared to in ovo PBS-treated groups ($P=0.045$ and 0.0082 for H4N6 AIV uninfected and infected groups, respectively).

**Macrophages are efficient in the uptake of CpG DNA in vitro**

Since we observed that in ovo-delivered CpG DNA elicits antiviral responses against H4N6 AIV replication correlating with macrophage recruitment in the lungs, we hypothesized that avian macrophages may be involved in the uptake of CpG DNA, potentially initiating CpG DNA–TLR21 interaction leading to the antiviral responses against H4N6 AIV infection. CpG DNA conjugated with fluorescein isothiocyanate (FITC) was used to track the uptake of CpG DNA in macrophages and we found that more than half of the macrophages were efficient in the uptake of CpG DNA within 1 h post-treatment ($P=0.004$). As shown in Fig. 5(a, b), it is also evident that the rate of uptake of CpG DNA was not influenced by transfection ($P>0.05$).

**Macrophages serve as a source of NO production following CpG DNA treatment, leading to the antiviral response against H4N6 AIV**

As we observed that macrophages are able to uptake CpG DNA efficiently and an exogenous source of NO could mediate the antiviral response against H4N6 AIV, we evaluated whether macrophages were able to produce NO leading to the antiviral response against H4N6 AIV infection. Briefly, the chicken macrophage cells, Muquarrab Qureshi-North Carolina State University (MQ-NCSU), were cultured in six-well plates with $1.5 \times 10^5$ viable cells per well for 24 h and stimulated with CpG DNA ($10 \mu M$) or Roswell Park Memorial Institute (RPMI) medium as a control (five replicates per treatment). The resultant MQ-NCSU cell culture supernatants were collected at 24 h post-treatment and
a portion (500 µl) was transferred to Madin–Darby canine kidney (MDCK) cell monolayers before inoculation with H4N6 AIV (70 p.f.u. per well). RPMI medium and PBS added in separate MDCK six-well plates acted as negative controls for macrophage culture supernatants and these controls were also inoculated similarly with H4N6 AIV. The resulting plaques were counted after staining with crystal violet at 40–48 h post-infection. The NO concentration in the remaining culture supernatants was determined using the Griess assay with the view of quantifying NO production by macrophages. Culture supernatants derived following stimulation of macrophages with CpG DNA were able to elicit antiviral responses (Fig. 6a, P<0.0001). The same culture supernatants originating from CpG DNA-treated macrophages had significantly higher NO production when compared to the controls (Fig. 6b, P<0.0001) potentially attributing the antiviral response against H4N6 AIV replication to NO originating from macrophages. The negative controls (cell culture growth medium and PBS) had the highest viral replication of H4N6 AIV when compared to macrophage culture supernatants (Fig. 6a, P<0.0001).

**CpG DNA-mediated inhibition of H4N6 AIV replication is attributable to NO production originating from macrophages**

Since we observed that CpG DNA-mediated inhibition of H4N6 AIV replication is associated with enhanced NO production originating from avian macrophages, we investigated whether this antiviral response is attributable to NO production originating from macrophages. Briefly, MQ-NCSU cells were cultured in six-well plates with 1.5×10^6 viable cells per well for 24 h and stimulated with CpG DNA.
(10 µM), CpG DNA (10 µM) together with SMT (1 mM), SMT (1 mM) or RPMI medium as a control (five replicates per treatment). The resultant MQ-NCSU cell culture supernatants were collected at 24 h post-treatment and a portion was transferred to MDCK cells before inoculation with H4N6 AIV (50 p.f.u. per well). RPMI medium and PBS added in separate MDCK six-well plates acted as negative controls for macrophage culture supernatants and these controls were also inoculated similarly with H4N6 AIV. The resulting plaques were counted after staining with 1% crystal violet at 40–48 h post-inoculation. The NO concentration in the remaining culture supernatants was quantified using the Griess assay. The experiment was repeated with the same number of replicates. Here, we found that SMT-mediated inhibition of CpG DNA-stimulated NO production and basal NO in macrophages significantly increases H4N6 AIV replication when compared to the group that received only CpG DNA (P<0.0001, Fig. 7a) coinciding with the significant decrease in NO production by macrophages (P<0.0001, Fig. 7b). The increase in H4N6 AIV replication we observed in the CpG DNA plus SMT group was not as high as that observed in untreated control groups (P<0.0001, Fig. 7a).

**DISCUSSION**

The findings of the current study are fourfold. First, CpG DNA can elicit antiviral responses against H4N6 AIV replication as demonstrated in vivo and in vitro. Second, the CpG DNA-mediated antiviral response was associated with NO production and macrophage recruitment in the...
lungs. Third, the CpG DNA-mediated antiviral response is attributable to NO production in vitro. Finally, we discovered that NO originating from macrophages elicits the antiviral response against H4N6 AIV replication.

It has been recorded previously that CpG DNA is able to induce protective host responses against many acute viral infections such as ILTV, Ebola virus, vaccinia virus, AIV, herpes simplex virus (HSV), murine cytomegalovirus and adenovirus infections [14, 30, 37–39]. The antiviral effects of CpG DNA have also been demonstrated against many chronic viral infections such as hepatitis C virus [40], hepatitis B virus [41] and retrovirus infections. Although CpG DNA was found to be effective against many viral infections, there is a paucity of literature on the antiviral effect of CpG DNA against AIV infection in vivo and in vitro. Recently, it has been shown that CpG DNA can act as an adjuvant when combined with the H4N6 AIV virosomes vaccine. In the study of Mallick et al., the CpG-adjuvanted vaccine has been shown to reduce virus shedding of a homologous challenge at day 6 post-challenge compared to control chickens, correlating with enhanced antibody-mediated immune responses [30]. In an in vitro study, it has also been recorded that stimulation of chicken

![Fig. 5. Macrophages uptake CpG DNA efficiently in vitro. Avian macrophages were cultured on coverslips for 24 h and stimulated with either CpG DNA conjugated with FITC alone, CpG DNA conjugated with FITC plus lipofectamine transfection reagent, lipofectamine alone or RPMI growth medium (mock) (four replicates per treatment). One hour post-treatment, the coverslips were stained using a live cell-wall staining kit, fixed, nuclear stained, mounted on glass slides and observed under an epifluorescence microscope. The CpG DNA uptake by macrophages was estimated as described in Methods. ANOVA tests were used to identify group differences and the differences were considered significant at P<0.05. Error bar, SEM. (a) Quantitative data for CpG DNA uptake by macrophages. (b) Representative CpG DNA uptake images for each treatment group.](image)
macrophages, MQ-NCSU cells, with different types of CpG DNA at the time of infection or prior to infection significantly decreases H4N6 AIV replication [42]. Our study was directed to investigate whether CpG DNA is able to elicit antiviral responses against H4N6 AIV infection when CpG DNA is delivered in ovo. In agreement with previous studies using other viruses and also with the in vitro study using AIV [42], we found that in ovo-delivered CpG DNA is capable of eliciting an antiviral response against H4N6 AIV infection encountered pre- and post-hatch. We investigated the factors that may have led to the antiviral response against H4N6 AIV infection in the lungs by evaluating NO production and macrophage numbers in the lungs following in ovo CpG DNA delivery. Indeed, we found NO and macrophages as potential mediators of the CpG DNA-mediated antiviral response against H4N6 AIV infection in the lungs. This observation of the involvement of NO and macrophages in the antiviral response of CpG DNA against H4N6 AIV replication is in agreement with the findings of previous studies conducted by our group using another avian virus, ILTV [14].

NO-mediated inhibition has been demonstrated in vitro against several viruses, including influenza virus [43, 44], dengue virus, HSV [18], vesicular stomatitis virus (VSV)

Fig. 6. Avian macrophages serve as a source of NO production following CpG DNA treatment, potentially leading to the antiviral response against H4N6 AIV replication in vitro. Avian macrophages were cultured in six-well plates for 24 h and stimulated with either CpG DNA or RPMI medium as a control (five replicates per group). Twenty-four hours post-treatment, culture supernatants were collected, a portion was transferred to MDCK cell monolayers grown in six-well plates, and cells were infected with H4N6 AIV employing appropriate controls. Two days post-infection, the six-well plates were stained with 1 % crystal violet to enumerate plaques. The remaining culture supernatants were used to quantify NO production. ANOVA and Student’s t-tests were used to identify group differences and the differences were considered significant at P<0.05. Error bar, SEM. (a) H4N6 AIV replication in different groups. (b) NO production in different groups.
[17], Japanese encephalitis virus, ILTV [45], Marek’s disease virus (MDV) [36], coxsackievirus [46, 47], vaccinia virus, porcine respiratory coronavirus, rhinovirus, flavivirus [48], and hantavirus [49]. Similarly, based on in vivo studies, NO-mediated antiviral responses against influenza virus, dengue virus, HSV, mouse hepatitis virus, Friend murine
leukaemia virus, hepatitis B virus, respiratory syncytial virus, infectious bursal disease virus, murine cytomegalovirus, MDV [36], coxsackievirus [46], adenovirus, VSV and hantavirus [49] have been shown. The antiviral responses recorded in the aforementioned studies were based on exogenous NO supplied via NO donors. We demonstrated that \textit{in ovo} CpG DNA delivery induced significant NO production in the lungs as well as in macrophages. Furthermore, our findings confirmed that the CpG DNA-mediated antiviral response against H4N6 AIV replication is attributable to NO production \textit{in vitro}. \textit{In vivo}, there was a trend of involvement of NO in the antiviral response against H4N6 AIV replication. The difference in observations between \textit{in vitro} and \textit{in vivo} experiments may be related to the complexity of the \textit{in vivo} system involving many factors as opposed to simple \textit{in vitro} systems. It was also interesting that avian macrophages used in our study were capable of eliciting basal NO production in the absence of any stimulation. However, this observation agrees with previous observations made using avian macrophage cell lines [14, 50]. When stimulated with CpG DNA, macrophages were capable of producing 30–40 µM nitrite during 24 h following the treatment in our experiments and this observation agrees with previous observations [14, 50]. Although, we did not investigate the mechanisms involved in the NO-mediated inhibition of AIV replication, the importance of NO in AIV infection could be many-fold. First, due to its hydrophobicity, NO diffuses freely across cell membranes (without receptors or carrier proteins) [51, 52]. An unpaired electron makes NO highly chemically reactive, and its antiviral mechanism depends on diffusion through the cell membrane and reducing the synthesis of the viral genome and viral proteins in host cells by inactivating enzymes involved in viral replication such as viral protease [19, 47]. Second, NO is a molecule capable of directly damaging the viral genome [53, 54]. However, one possible explanation why the host cell DNA is not damaged by excessive production of NO is the presence of superoxide dismutase, a rapid degradation system for reactive nitrogen free radicals [55]. Third, NO may be able to modify the molecules necessary for viral replication, such as transcriptional factors, through nitrosylation thus inhibiting virus replication and subsequent spread of viruses in the host [52, 56].

Although we observed that SMT is capable of inhibiting NO production in macrophages to a very low concentration, the abrogation of the antiviral response against H4N6 AIV was not comparable to the reduction in NO production. When compared to culture supernatants of untreated macrophages, the macrophages treated with SMT should have resulted in higher H4N6 AIV titres due to abrogation of the antiviral response induced by NO. This discrepancy in our observation is very difficult to explain but it is possible that SMT could have had direct or indirect antiviral responses in the absence of NO. We could not find literature to support our view but it has been shown that CpG DNA can elicit antiviral mechanisms such as activation of reactive oxygen species in immune cells [57] and further investigations are required to elucidate the potential reasons for this unexpected observation.

Since we observed that the CpG DNA-mediated antiviral response against H4N6 AIV infection was associated with macrophage recruitment, we then investigated to see whether this pattern of macrophage recruitment is due to H4N6 AIV infection. We found an increasing trend of macrophage populations in H4N6 AIV-infected CpG DNA-treated lungs compared to uninfected CpG DNA-treated lungs (Fig. 4). This may potentially be due to the stimulation of macrophages by CpG DNA prior to infection and that may have increased the mobilization of macrophages further following H4N6 AIV infection. However, the difference between the infected CpG DNA-treated and CpG DNA-treated uninfected groups is not significantly different \( (P>0.05) \). Interestingly, we also did not observe an increase in macrophage numbers in the lungs following H4N6 AIV infection in the \textit{in ovo} PBS-treated group \( (P>0.05) \) and in the CpG DNA plus SMT-delivered group \( (P>0.05) \). However, in this experiment, we found that the macrophage populations were increased following \textit{in ovo} CpG DNA and CpG DNA plus SMT delivery in the lungs and this could be attributable to a CpG DNA-mediated antiviral response \textit{in vivo}. These observations confirmed our previous observation that \textit{in ovo} CpG DNA delivery increases macrophage numbers in the lungs [14].

Although we did not clarify the role of lung macrophages in the innate host responses against H4N6 AIV infection to a great extent, the importance of lung macrophages in AIV antiviral responses mediated by CpG DNA could be several-fold. First, it is possible that macrophages may have played a role as phagocytic cells, reducing H4N6 AIV burden in the lungs of the \textit{in ovo} CpG DNA-delivered group [58]. Second, avian macrophages could have been a source of a number of antiviral cytokines such as IL-1β [59] and IFNγ [60], and these cytokines may have played a role in reducing H4N6 AIV replication in the lungs following \textit{in ovo} CpG DNA delivery. Third, avian macrophages can be a source of reactive nitrogen species such as NO [61], and NO has been shown to elicit the antiviral response against a number of avian viruses [36, 45, 62]. Consistent with these reports, we have observed here that inhibition of H4N6 AIV replication is attributable to NO produced from macrophages. The latter finding may be a plausible reason for a significant reduction in H4N6 AIV replication in lungs observed following \textit{in ovo} CpG DNA delivery in the current study.

The implications of our observations described in this manuscript are many-fold. First, we found that the \textit{in ovo} administration of CpG DNA stimulates the innate arm of the immune system as indicated by the expansion of macrophage numbers and the increased production of an innate antiviral molecule, NO, in the lung. Consequently, this induction of innate host responses resulted in the reduction of H4N6 low-pathogenic avian influenza virus (LPAIV) replication \textit{in vivo}. In agreement with the practice of \textit{in ovo} vaccination by the poultry industry [26], we delivered CpG
DNA in ovo and this is the first study to our knowledge that has demonstrated the in ovo-delivered CpG DNA-mediated antiviral response against AIV infection. Although these findings are preliminary and need further investigations, in ovo CpG delivery may provide a basis for developing novel control measures against LPAIV infections in chickens. Second, although we studied the mechanistic aspects of in ovo CpG DNA delivery against a viral infection (i.e., LPAIV infection), our finding of the mechanisms of induction of innate immunity following in ovo delivery of CpG DNA may be applicable to various pathogens including bacteria. Previously, in ovo-delivered CpG DNA has been shown to be effective against avian pathogens including ILTV [14], IBV [10], E. coli and S. Typhimurium [28, 29]. Due to the non-specific nature of the functions of macrophages and NO, it is plausible, but remains to be seen, that these two innate immune mediators may be playing potential roles in the antimicrobial responses against the aforementioned avian pathogens as has been observed in the current study against H4N6 LPAIV infection. Finally, although the antiviral effect of NO has been previously shown against AIV infection in vitro using an exogenous source of NO [43, 44], our studies demonstrated that H4N6 LPAIV infection can be significantly reduced by activating the TLR21 signalling pathway leading to endogenous NO production both in vitro and in vivo.

Although we demonstrated that the antiviral effect of CpG DNA delivered in ovo was associated with the expansion of the macrophage population in the lungs and enhanced production of NO pre-hatch and day 1 post-hatch, the existence of the same associations of macrophages and NO was not investigated beyond day 1 post-hatch. However, it has been shown previously that the effect of subcutaneous administration of CpG DNA in the adult chickens persists up to 6 days post-treatment against E. coli infection and is absent 9 days post-infection onwards [15]. We previously observed that in ovo-delivered CpG DNA protects chickens for at least 12 days post-infection when infected with ILTV on day 1 post-hatch [14]. However, it is also important to evaluate the efficacy of in ovo-delivered CpG DNA against AIV encountered beyond day 1 post-hatch since the birds can be exposed to AIV in the barn environment not only on the day of placing the birds on day 1, but also at any age. Furthermore, we investigated the effect of in ovo-delivered CpG DNA against an LPAIV strain of AIV and whether in ovo-delivered CpG DNA is effective against highly-pathogenic AIV needs further investigation. Furthermore, in the current study we observed the innate host responses in the respiratory tract and unlike LPAIV, highly-pathogenic AIV induces systemic infection.

In conclusion, CpG DNA when administered in ovo can lead to antiviral responses against H4N6 AIV replication attributable to macrophage recruitment and enhanced NO production in lungs. Inhibition of H4N6 LPAIV replication following CpG DNA treatment is dependent on the production of NO originating from avian macrophages. Our results provide insights into the mechanisms of induction of antiviral responses against AIV replication by CpG DNA. Although the findings have implications in antiviral host responses against AIV infection in chickens, further investigations are necessary to elucidate the role of other innate immune cells and molecules in the CpG DNA-mediated antiviral responses against AIV infection.

METHODS

Animals

SPF eggs purchased from the Canadian Food Inspection Agency (CFIA), Ottawa, Canada were incubated at the Health Research Innovation Centre (HRIC), University of Calgary in digital incubators (Rcom Pro 20 and 50, Kingsuromax 20 and Rcom MARU Deluxe max; Autoelex Co.). During the incubation, the eggs were candled to select fertile eggs at ED11 for the experiments.

Virus and TLR ligand

A low-pathogenic AIV, A/Duck/Czech/56 (H4N6), which was kindly provided by Dr Eva Nagy (University of Guelph, Canada), was used in the studies. Initially the virus was propagated in the embryonated chicken eggs at ED9–11 by infecting them through the allantoic cavity route. H4N6 AIV titre in the harvested allantoic fluid was determined by plaque assay using MDCK cells. The ligand for TLR21, Class B CpG DNA 2007 (5’-TCGTCGGTGTTCGTTGTCGTT-3’), FITC-conjugated CpG DNA 2006 (5’-TCGTCG-TCGTTGTCGTTGTCGTT-3’) and non-CpG DNA (5’-TCGTCGTTGTCGTTGTCGTT-3’) (Invivo-gen) were purchased from Cedarlane. In all experiments, CpG DNA 2007 was used except in the CpG DNA uptake experiment, which required FITC-conjugated CpG DNA 2006. These CpG DNA sequences, which consist of a complete phosphorothioate backbone, have been shown to induce strong NO production in vitro in avian cells when compared to non-CpG DNA [12, 63]. As we have previously shown that non-CpG DNA and PBS elicit similar host responses in vivo [14], most of the experiments were conducted using PBS or cell culture medium as controls rather than non-CpG DNA.

Cells and cell culture

MQ-NCSU cell line [64], was kindly gifted by Dr Shayan Sharif (University of Guelph, Canada). This virally transformed avian macrophage cell line has been commonly used for in vitro NO production experiments [45, 65–67]. Previously, we have observed that MQ-NCSU cells and primary macrophages generate similar NO production in response to treatment with CpG DNA [14]. This cell line was maintained in LM-HAHN medium which was prepared from equal volumes of McCoy’s 5A medium and Leibovitz L-15 medium (both Gibco Life Technologies) supplemented with chicken serum (10 %), foetal bovine serum (8 %), 1 mM 2-mercaptoethanol, sodium pyruvate (1 %), l-glutamine (1 %), penicillin (100 U ml−1), streptomycin (100 μg ml−1), tetrerase phosphate broth (1 %) and
Fungizone (250 µg ml⁻¹), in an incubator with the conditions of 5 % carbon dioxide (CO₂) and 40 °C. The other cell line used in the studies, MDCK, was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % foetal bovine serum, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹), in an incubator with the conditions of 5 % CO₂ and 37 °C.

In ovo delivery technique

In ovo delivery at ED18 has become a standard route of vaccination in the poultry industry, inducing early immune responses leading to more optimal protection of the birds than post-hatch vaccination [68]. In agreement with this practice, we performed an in ovo delivery technique for treatment and pre-hatch H4N6 AIV infection as previously described [27, 68, 69]. Briefly, the egg shell was punctured through the air sac at the broader end of the eggs using a sterile 23-gauge needle after disinfecting the shell surface with 70 % ethanol. A 2.5 cm long, 23-gauge needle was then used for in ovo delivery of compounds and AIV into the amniotic cavity by inserting the entire length of the needle perpendicularly through the punctured hole. At the end of the procedure the holes were sealed with lacquer and eggs were placed in the incubator.

Sample collection and tissue homogenization

The embryos and chicks were euthanized and lung tissues from each embryo or animal were collected in 1 ml sterile PBS and kept on ice. On the same day of sample collection or at a later day following storage at −80 °C, the lung tissues in 1 ml PBS were homogenized using a Bio-Gen PRO200 homogenizer (PRO Scientific) at medium speed, and centrifuged at 2000 g, 4 °C for 10 min. The supernatants were collected, divided into aliquots and stored at −80 °C until use in plaque and NO assays.

Plaque assay for the determination of H4N6 AIV viral titres

MDCK cells suspended in complete DMEM medium were seeded in six-well plates at a density of 1.2×10⁶ viable cells per well. After 24 h, the confluent monolayers of MDCK cells in six-well plates were washed with sterile and warm PBS and inoculated with 500 µl of 10-fold serially diluted supernatants collected from lung homogenates. After incubation for 30 min at 37 °C, the plates were overlaid with serum-free 2× MEM medium containing an equal volume of 2.4 % Avicel (FMC BioPolymer) and 1 µg ml⁻¹ bovine l-lysylamine-2-phenylethyl chloromethyl ketone (TPCK)-trypsin (Sigma). The inoculated plates were incubated for 40–48 h at 37 °C and 5 % CO₂. Visible plaques were counted under an inverted microscope (CKX41, Olympus) after staining with 1 % crystal violet.

Determination of NO concentrations in tissue homogenates and cell culture supernatants

NO concentrations in homogenized lung samples and supernatants of MQ-NCSU cell cultures were assayed by measuring the end-product, nitrite, using the Griess assay (Promega) [45].

Immunofluorescence assay

Tissues preserved in optimal cutting temperature (OCT) compound were sectioned (thickness of 5 µm) and an indirect immunofluorescence assay was used to quantify macrophage numbers in lung sections and to quantify expression of H4N6 AIV nuclear antigen in tracheal sections. For macrophage staining, 5 % goat serum in TBS buffer (2.42 g Trizma base and 8 g NaCl in 11 distilled water, pH 7.6) was used for blocking and was incubated at room temperature for 30 min. Mouse monoclonal antibody specific for chicken macrophages, KUL01 (SouthernBiotech), was used at 1:200 dilution in blocking buffer and incubated for 30 min at room temperature. DyLight 550-conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories) was used at 1:500 dilution in blocking buffer as the secondary antibody and incubated for 1 h at room temperature. TBS-T buffer (TBS with 0.1 % Tween 20) was used as the washing buffer and after each step, the sections were washed 2 or 3 times with an interval of 3–5 min. Finally, the slides were mounted in Vectashield mounting medium with DAPI (Vector Laboratories), coverslipped and sealed with lacquer.

For the immunofluorescence assay to quantify the expression of H4N6 AIV nuclear antigen in tracheal sections, 2.5 % horse serum in TBS buffer was used for blocking and was incubated at room temperature for 1 h. Mouse monoclonal anti-influenza A nuclear protein antibody (SouthernBiotech) was used at 1:10 dilution in blocking buffer and incubated at room temperature for 1 h. Goat anti-mouse IgG (H+L) (ready-to-use, RTU) (Vector Laboratories) was used as amplifier antibody and incubated for 15 min. DyLight 594-conjugated horse anti-goat IgG (RTU) (Vector Laboratories) was used as the secondary antibody and incubated for 30 min at room temperature. Triton X-100 (0.2 %) (Sigma) and PBS were used as the washing buffers and after adding primary and secondary antibodies, the sections were washed twice with an interval of 20 min with 0.2 % Triton X-100 and once with an interval of 5–10 min with PBS. Finally, the slides were mounted in Vectashield mounting medium with DAPI as described above.

CpG DNA uptake assay in avian macrophages

Avian macrophages (MQ-NCSU cells) were cultured on coverslips placed in six-well plates (1.5×10⁶ viable cells per well) for 24 h and stimulated with either CpG DNA conjugated with FITC (Invivogen) alone (1 µM), CpG DNA conjugated with FITC (1 µM) plus lipofectamine transfection reagent (2 µl; Invitrogen), lipofectamine alone (2 µl) or growth medium, RPMI, with four replicates per treatment. One hour post-treatment, the coverslips were stained with cell-wall stain wheat germ agglutinin Alexa Fluor 594 conjugate [Image-IT LIVE Plasma Membrane and Nuclear Labeling kit (134406); Invitrogen], after fixation with 4 % paraformaldehyde. Then the coverslips were mounted on glass slides with Vectashield mounting medium with DAPI.
(Vector Laboratories) or with nuclear staining of Hoechst 33342 trihydrochloride [Image-iT LIVE Plasma Membrane and Nuclear Labeling kit (I34406); Invitrogen] and observed under an epifluorescence microscope (BX51, Olympus). To estimate the percentage of macrophages with CpG DNA, five fields with highest fluorescence signals were chosen and cells with and without FITC signals were counted. The number of macrophages with CpG DNA was expressed in relation to number of cells present in the field as estimated by number of nuclei.

**Data analyses**

**ImageJ analysis of fluorescence signals**

For quantification of the number of macrophages in lung tissues and H4N6 AIV nuclear protein expression, five areas with highest DyLight 550 (macrophages) and DyLight 594 (AIV nuclear protein) fluorescence signals and corresponding nuclear-stained (DAPI) areas were captured under ×20 magnification from each lung section. These images were then subjected to fluorescence intensity quantification using ImageJ software (National Institutes of Health, USA). The resultant fluorescence intensities for DyLight 594- or DyLight 550-positive signals were expressed relative to the total areas as a percentage.

**Statistical analysis**

Student’s t-test (GraphPad Prism Software 5) was used for identifying differences between two groups. When multiple groups were present in an experiment, analysis of variance (ANOVA) followed by Tukey’s test were used to compare group differences. Before each set of data was analysed, the outlier test was conducted using the Grubbs’ test (GraphPad software). Differences between groups were considered significant at $P<0.05$.

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**Conflicts of interest**

All authors declare that there are no conflicts of interest.

**Ethical statement**

The University of Calgary Animal Care Committees have approved the use of SPF eggs and chickens used in all our experimental procedures.

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