Vaccine 36 (2018) 2139–2146

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Chicken immune response following *in ovo* delivery of bacterial flagellin

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ARTICLE INFO

Article history:
Received 5 December 2017
Received in revised form 17 February 2018
Accepted 26 February 2018
Available online 9 March 2018

Keywords:
Chicken
Toll-like receptor
Vaccine
Flagellin
In ovo immunization
Humoral Response

ABSTRACT

In ovo immunization of chicken embryos with live vaccines is an effective strategy to protect chickens against several viral pathogens. We investigated the immune response of chicken embryos to purified recombinant protein. *In ovo* delivery of *Salmonella* flagellin to 18-day old embryonated eggs resulted in elevated pro-inflammatory chIL-6 and chIL-8 (CXCL8-CXCLi2) cytokine transcript levels in the intestine but not in the spleen at 24 h post-injection. Analysis of the chicken Toll-like receptor (TLR) repertoire in 19-day old embryos revealed gene transcripts in intestinal and spleen tissue for most chicken TLRs, including TLR5 which recognizes *Salmonella* flagellin (FliC). The *in ovo* administration of FliC did not alter TLR transcript levels, except for an increase in intestinal chTLR15 expression. Measurement of the antibody response in sera collected at day 11 and day 21 post-hatch demonstrated high titers of FliC-specific antibodies for the animals immunized at the late-embryonic stage in contrast to the mock-treated controls. The successful *in ovo* immunization with purified bacterial antigen indicates that the immune system of the chicken embryo is sufficiently mature to yield a strong humoral immune response after single exposure to purified protein. This finding strengthens the basis for the development of *in ovo* protein-based subunit vaccines.

1. Introduction

Protection of chickens against bacterial and viral pathogens is important for animal and human health. An effective and convenient protection strategy is active immunization of embryonated eggs [1–4]. During this procedure, the vaccine is injected into the amniotic sac or intramuscularly into the chicken embryo usually at 3 days prior to hatch i.e., at day 18 of embryonic development (ED18) [5]. The immunization evokes antibodies directed against the vaccine antigens, resulting in protection early after hatching. *In ovo* vaccination is commercially widely used to protect against viral infections. Most licensed vaccines consist of live attenuated viruses that can still replicate and provoke an immune response but do not cause illness [6]. More recently, non-replicating adenovirus-vector based vaccines have been developed [7,8]. *In ovo* delivery of subunit vaccines that consist of a mixture of purified antigens is still in its infancy. Successful *in ovo* immunization has been achieved with recombinant *Eimeria* protein [9–11] but immunization with a recombinant protein of *Campylobacter jejuni* failed to induce a significant immune response [12]. The reason for the variable immune response to recombinant bacterial proteins after *in ovo* delivery is unknown.

One factor that aids the generation of a potent immune response upon immunization is the use of vaccine adjuvants or other immunomodulatory agents such as cytokines. These compounds promote the immunogenicity of vaccine antigens and influence the quality of the adaptive immune response [13–15]. The repertoire of potential adjuvants for use in chickens was boosted by the discovery of functional chicken Toll-like receptors (TLR) [16,17]. Members of the TLR family of pathogen recognition receptors sense microbial ligands and translate these signals into pro-inflammatory signals that promote amongst others antigen presentation by dendritic cells, and T- and B-cell responses [18–20]. TLR agonists are beginning to be applied as vaccine adjuvants in humans [21,22], but also in the chicken [23]. The effect of TLR stimulation on the immune response seems most effective when the antigen of interest has intrinsic TLR-stimulating activity or is conjugated to an effective TLR agonist.

TLR ligands that are investigated as adjuvants in chickens include flagellin [24,25] and CpG oligodeoxynucleotides [10,26,27] .These compounds target chTLR5 and chTLR21 receptors, respectively [28–31]. A prerequisite for the use of TLR agonists

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https://doi.org/10.1016/j.vaccine.2018.02.116
0264-410X/

as an adjuvant in combination with *in ovo* vaccination is the expression and function of the relevant TLR receptors at the late embryonic stage. Transcriptional profiling of immune genes during chicken embryo development indicates early but variable presence of TLR transcripts throughout the embryonic development [32–34].

In the present study we investigated the expression of TLR genes at the day of *in ovo* immunization and the effect of *in ovo* delivery of a recombinant bacterial antigen with intrinsic TLR5 stimulating activity on the generation and duration of an antigen-specific humoral immune response. We provide evidence that a single-dose injection of recombinant *Salmonella* flagellin into the amniotic sac of chicken embryos results in an intestinal cytokine response and the induction of specific IgY antibodies that can easily be detected up to 21 days post-hatch.

2. Materials and methods

2.1. Construction, expression, and purification of recombinant Salmonella His-tagged flagellin

Recombinant flagellin (FliC) of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) *was* produced as previously described [35] with some minor modifications. Briefly, the *fliC* gene of *S.* Enteritidis strain 90-13-706 was amplified by PCR as described [29], cloned with an N-terminal 6xHis-tag into the pT7.7 protein expression vector [36], and transformed into *E. coli* BL21 star (DE3). Protein expression was induced by adding 1 mM of IPTG to bacteria (OD_{550} of 0.4) grown (37 °C) in LB broth containing 100 μg/ml of ampicillin. Four hours after induction, bacteria were harvested by centrifugation and resuspended into urea solution (8 M urea in 100 mM Tris-HCl, 100 mM NaH_2PO_4, pH 8). After 16 h of incubation (20 °C, constant rotation), the insoluble fraction were removed by centrifugation. The supernatant containing the FliC protein was mixed (1 h) with Ni-NTA beads (Qiagen). After washing of the beads with 4 × 4 ml of 8 M urea solution with pH 6.3, bound FliC protein was eluted with 4 × 0.5 ml of 8 M urea solution with pH 5.9 and, subsequently, with 4 × 0.5 ml of 8 M urea solution with pH 4.5. The FliC containing fractions were determined by 12.5% SDS-PAGE, pooled, and stored in 4 M urea solution. Protein concentrations were measured with the Pierce BCA protein assay kit.

2.2. Animal experiments

Fertilized eggs from SPF (Ross 308) broilers (Gezondheidsdienst, Deventer, the Netherlands) were kept at 38 °C and 65–75% relative humidity in a forced air egg incubator. At embryonic day 18, the eggs were candled to check their fertilization, and then divided into three groups of 15 eggs. Group 1 received 20 μg of FliC protein diluted in 100 μl of 10 mM Tris (pH 9.0)/20% glycerol/5 mM sucrose/80 mM urea (FliC group). Embryonated eggs of group 2 were injected with 100 μl of the same solution lacking FliC protein (mock group). The eggs of group 3 were kept intact and received no treatment (non-injected group). For *in ovo* delivery we followed the procedure described by Sharma [1]. Briefly, after cleaning the eggs with 0.5% hypochlorite (bleach), a small hole was made at the air cell end of the egg using an 18G sterile needle. A 22G one-inch bevel needle (Monoject) was then used to manually deliver 20 μg of flagellin (or solvent) through the air sac membrane directly into the amniotic fluid. After 24 h of incubation (to allow transport to the embryo), the embryos of five eggs from each group were aseptically removed to isolate the gut and spleen tissue. Organ samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. The remaining eggs were kept in the incubator until hatch. After hatch, the chickens were reared in a ground stable under controlled hygienic conditions for up to 21 days. Chickens were given access to water and commercial broiler diet *ad libitum* without antibiotics or coccidiostats. At day 11 post-hatch, blood samples were taken from the wing vein for antibody analysis. At day 21 post-hatch, all chickens were sacrificed by electrocution and blood was collected by exsanguination. After blood clotting and centrifugation (2000×*g*, 5 min, 4 °C), sera were collected and stored at -20 °C until analyzed. The entire experiment was repeated in the same setup with eggs from a commercial (non-SPF) flock (Lagerweij, Lunteren, the Netherlands). The *in ovo* immunization procedure did not influence the hatchability and chicken survival. All experiments were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

2.3. RNA isolation and RT-PCR analysis

Total RNA was isolated from 50 (±5) mg of the collected embryonic tissue specimens. Samples were homogenized (6500×*g* for 50 s at 4 °C) in a MagNA Lyser instrument (Roche) using Lysing Matrix D tubes (MPbio) filled with 1 ml of RNA-Bee (Bio-connect USA). Total RNA was extracted using the RNA-Bee isolation kit according to the instructions of the manufacturer. The quantity and purity of the extracted RNA was measured at 260/280 nm in a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). After treatment with DNAse (1 U/μg of RNA, Fermentas), one microgram of RNA was reverse transcribed to cDNA using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Transcripts of chicken *TLR* genes and (as control) the chicken *GAPDH* gene (*chGAPDH*), were determined by PCR using the primers listed in Table 1 [37]. In all cases, RT-negative control samples were run to verify the absence of contaminating DNA. PCR amplification was performed using 1 μl of cDNA, 200 nM of each primer, 1 mM of dNTPs, and 1 Unit of Taq DNA polymerase (Fermentas) in a total reaction volume of 20 μl. The following cycle conditions were used: one initialization step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 35 s, followed by one cycle at 72 °C (10 min). RT-PCR products were resolved by electrophoresis using 2% TBE agarose gels stained with ethidium bromide and imaged under UV illumination (Pharmacia Biotech). Results shown

Table 1
Chicken RT primers used in this study [37].

RNA target		Primer sequence (5′-3′)
chTLR1b	Forward	AGAAAAGGCTCCAGGCTACGA
	Reverse	TACGACGTTCCCCAGTTGTGT
chTLR2t1	Forward	TGAAGCTGATGTGGAAGCA
	Reverse	ACACCGTGATTTTGCCTGTGA
cTLR2t2	Forward	GATGCCAGCGCCAATAACTTTA
	Reverse	TCCATCAGTGACAGCTGCACA
chTLR3	Forward	GCACCTGTGAAAGCATTGCTT
	Reverse	AATGGAGCACTGTCCTTGCA
chTLR4	Forward	CCTGAAATGGGTCAAGGAAAAG
	Reverse	CTGTGGTTGGGTTGGGATG
chTLR5	Forward	GAAATTGGAACACAACGCGTC
	Reverse	CGGAGTATGGTCAAACGTTGC
chTLR7	Forward	GGCTGTGAATGAATGAATGGGTGA
	Reverse	GCTGAATGCTCTGGGAAAGG
chTLR15	Forward	GAAAATAAGCCCTTCGATGCCT
	Reverse	TGTTGCCAAGTAACAGGATGCC
chTLR16	Forward	TTGCTTGCACGTCTTCGACAT
	Reverse	TTAGGAAGACCGTGTCCAGGTG
chTLR21	Forward	GCAGCTAGCCGCTCCTTTT
	Reverse	CCTTCTTCTTCCTCCTCCTCTCC
chGAPDH	Forward	TGCTGTGCTGGCCATCTTT
	Reverse	GCTTTGGACGTGCTCCAAA

are representative of one of two replicates and with cDNA isolated from the indicated tissues of five embryos of 19-day old chickens.

2.4. Quantitative real-time PCR analysis

Cytokine mRNA levels in the isolated tissue samples were measured by RT-qPCR as described previously [38]. Briefly, RT-qPCR was performed using the Reverse Transcriptase qRT-PCR Master Mix-Kit (Erurogentec, Seraing, Belgium). For each condition, 50 ng of DNaseI (Fermentas) treated RNA was used as template. The sets of primers and probes that were used are listed in Table 2 [38]. The amplification reactions were performed in a Roche Light-Cycler® 480 using the following conditions: Reverse Transcription step at 48 °C (30 min), followed by 10 min at 95 °C, 40 sequential cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was run in duplicate. Transcript levels were normalized to those for the chicken housekeeping gene *chGAPDH*. For each gene, results were expressed as fold change in mRNA level of the immunized embryos compared to the non-injected controls according to Schmittgen & Livak [39] using the formula: (1) ΔC_t target gene – ΔC_t GAPDH for each sample, (2) ΔC_t target gene treated – ΔC_t target gene control. The fold change in mRNA for each gene transcript was determined using the formula: Fold change = $2^{-\Delta(\Delta Ct \text{ gene treated} - \Delta Ct \text{ gene control})}$. To calculate the relative expression levels for FliC-injected and mock-treated, the transcript levels in samples of individual embryos were compared with the mean value of the group of non-injected embryonated eggs, yielding a mean ± SEM value for the mock-treated and FliC-injected groups. The SEM values thus represent the variation in fold difference between individual chickens.

2.5. Cell culture, transfection, and gene reporter assay

The HeLa-57A cell line, stably transfected with a NF-κB luciferase reporter construct [40], was generously provided by Dr. R. T. Hay (Institute of Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, U.K.). Cells were routinely propagated in 25-cm^2 tissue culture flasks (Corning) in DMEM with 10% FCS at 37 °C in a 5% CO_2 atmosphere.

For transfection, cells were propagated in 24-well tissue culture plates in DMEM with 10% FCS until 70% confluence was reached (~24 h). Then, cells were transiently transfected with plasmid pTracer 3xFLAG-chtlr5 or (as control) empty vector, in DMEM without FCS using FuGENE HD (Roche Diagnostics) at a lipid to DNA ratio of 3:1, as previously described [29]. After 4 h of incubation (37 °C), the medium was replaced with fresh medium containing DMEM with 10% FCS. Functional assays were performed at 48 h post-transfection.

ChTLR5 signaling was essentially assessed as described [41]. In brief, transfected cells were stimulated with 10 ng ml^{-1} of *Salmonella* flagellin (or solvent) for 5 h, rinsed with PBS, and immediately lysed in reporter lysis buffer (Promega). Firefly luciferase activity was measured with a luciferase assay system (Promega) using a luminometer (TD-20/20, Turner Designs). Purified flagellin showed no contamination with DNA or TLR2 ligands and low amounts of LPS activity as previously indicated by chTLR21, chTLR2/16 and chTLR4 reporter assays [42].

Table 2
Quantitative RT-PCR primers and probes used in this study [38].

RNA target	Primer/probe	Sequence (5′-3′)
chGAPDH	Forward	GCCGTCCTCTCTGGCAAAG
	Reverse	TGTAAACCATGTAGTTCAGATCGATGA
	Probe	(FAM)-AGTGGTGGCCATCAATGATCC-(TAMRA)
chIL-8	Forward	GCCCTCCTCCGGTTTCAG
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	(FAM)-TGCTCTGTCGCAAGGTAGGACGCTG-(TAMRA)
chIL1-β	Forward	GCTCTACATGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)
chINF-β	Forward	ACAACTTCCTACAGCACAACAACTA
	Reverse	GCCTGGAGGCGGGACATG
	Probe	(FAM)-TCCCAGGTACAAGCACTG-(TAMRA)
chIL-6	Forward	GCTCGCCGGCTTCGA
	Reverse	GGTAGGTCTGAAAGGCGAACAG
	Probe	(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)

2.6. Measurement of FliC-specific IgY antibodies

Flagellin-specific IgY antibodies were detected using Maxisorb 96-wells ELISA plates coated (16 h, 4 °C) with 2.5 μg ml^{-1} of FliC protein in carbonate-bicarbonate buffer. Plates were washed 5 times with PBS-T (10 mM phosphate buffer, 150 mM NaCl, 0.05% Tween 20, pH 7.4), blocked (2 h) with 5% FCS diluted in PBS, and incubated (1 h, 20 °C, constant shaking) with serial dilutions (1:20–1:2560) of chicken sera in assay buffer (5% FCS, 0.5%Tween 20 in Tris-buffered saline). After rinsing of the wells with PBS-T, goat-anti-chicken IgY-Fc antibody conjugated to horse reddish peroxidase (HRP) (AAI29P, AbD Serotec, dilution: 1:2500 in assay buffer) was added. After 1 h of incubation, the wells were rinsed with PBS-T and HRP substrate (3,3′,5,5′-Tetramethylbenzidine, TMB) was added. After 10 min of incubation (in the dark), the enzyme reaction was stopped by adding H_2SO_4. Absorption (450 nm) was measured using a Fluostar Omega spectrophotometer. Antibody titers for the sera of each of the hatched chickens were defined as the highest sera dilution giving statistically significant differences between the immunized and control group.

2.7. Statistical analysis

GraphPad Prism 6.05 software was used for all statistical analyses. Data were analyzed by multiple *t*-test using the Holm-Šidák method for comparison of cytokine transcript levels of FliC-injected, mock-injected and non-injected groups and for comparison of antibody levels of FliC-injected and mock-injected groups. Differences were considered to be statistically significant at $P < 0.05$.

2.8. Ethics statement

All procedures involving animals were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

3. Results

3.1. In ovo delivery of molecules

To investigate the fate of recombinant protein delivered into the amniotic fluid, we first monitored the tissue distribution of Patent Blue V (2.5%) after injection into 18-day old embryonated eggs. Macroscopic examination of the tissues of the embryos at day 1 post-injection revealed strong staining of the distal part of the intestine but not of other organs of the embryo. In these experiments, the length of the needle (22G, one-inch) that was used for *in ovo* delivery into the amniotic sac was critical as injection with needles of different length failed to reach into the amniotic sac or resulted in local staining of the embryo at the injection site without accumulation of the stain in the chicken gut.

3.2. Expression of Toll-like receptors in the embryonic gut

To enable a rational choice of a TLR agonist as *in ovo* vaccine adjuvant, we next investigated the expression of TLR genes in gut and spleen tissue isolated from five non-injected 19-day-old embryonated eggs from SPF chickens. RT-PCR transcript analysis on extracted tissue RNA demonstrated that both organ tissues contained transcripts encoding chTLR1b, chTLR2t1, chTLR2t2, chTLR3, chTLR4, chTLR5, chTLR7, and chTLR21 (Fig. 1). There were no consistent differences in the detection of transcript between proximal, distal, or caecal gut tissue (not shown). ChTLR15 and chTLR16 (TLR1a) appeared predominantly present in spleen tissue but weakly expressed in gut tissue (Fig. 1). All RNA samples used for cDNA synthesis were demonstrated to be free from genomic DNA contamination by the absence of transcripts in the non-reverse transcribed samples (data not shown). Together, the TLR transcript results suggest that most TLR receptors are present at the main intestinal antigen delivery site in 19-day old embryos.

3.3. Expression and purification of recombinant Salmonella FliC

As chTLR5 was found to be expressed at the late embryonic stage, we choose the FliC flagellin protein of *Salmonella* Enteritidis to test the immune response after *in ovo* immunization with purified protein to embryonated eggs. from SPF chickens. The FliC protein carries intrinsic chTLR5 stimulating (adjuvant) activity [29]. The recombinant protein, which was cloned with *N*-terminal His-tag to enable purification, migrated on SDS-PAGE as a single protein band with the expected apparent molecular mass of 64 kDa. The protein band reacted with anti-FliC antibodies in western blots (Fig. 2A). As the protein tended to aggregate in saline buffer, stock solutions were dissolved in 4 M urea. TLR activation assays using chTLR5 transfected HeLa 57A cells that carry an NF-κB luciferase reporter gene demonstrated that the purified FliC protein stimulated NF-κB activity in a chTLR5 dependent fashion (Fig. 2B), confirming its intrinsic TLR5 stimulating activity [29].

Next, we injected 20 μg of the purified FliC protein into 18-day old embryonated eggs. RT-PCR analysis on RNA isolated from the gut and spleen tissue of the embryos isolated at one day post-injection showed grossly similar TLR expression profiles as observed for the non-injected embryonated eggs of this age with exception of chTLR15 (Fig. 1B). The transcript of the TLR15 gene appeared with a much stronger intensity in the gut tissue after the injection of FliC. ChTLR15 is activated by microbial proteases [43] but its contribution to the immune response is unknown. ChTLR16 (TLR1a) remained weakly expressed in the gut tissue after flagellin exposure. Injection of FliC into the eggs did not influence the subsequent hatching and survival of the chickens.

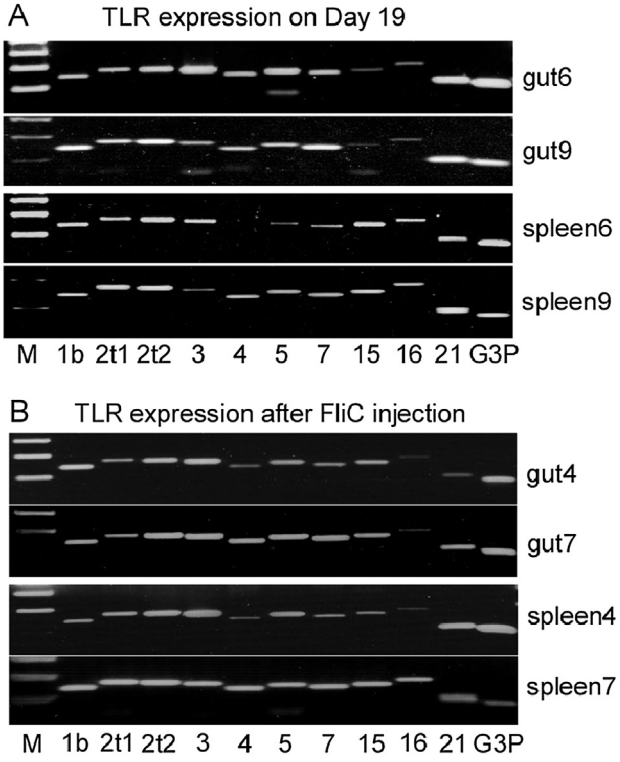

Fig. 1. Expression profile of the chicken TLR repertoire. Reverse transcription polymerase chain reaction (RT-PCR) was performed on the indicated tissues of (A) non-injected and (B) FliC-injected embryonated eggs at embryonic day 19 to determine TLR gene expression. PCR products were separated on 2% agarose gels and visualized with ethidium bromide. For each tissue, two representative results from five embryos from SPF chickens are shown. Numbers on the horizontal axis represent the TLR class. M indicates 100, 200 and 300 base pair DNA markers. G3P: GAPDH.

3.4. Effect of FliC injection on cytokine gene expression

We also tested for changes in the expression of inflammatory genes in the gut and spleen tissue after the *in ovo* delivery of FliC protein to embryonated eggs from SPF chickens. Quantitative RT-PCR transcript analysis on RNA isolated at day 1 post-injection revealed that FliC induced a strong upregulation (30–75-fold) of chIL-6 and chIL-8 (CXCL8-CXCLi2) mRNA levels in both the small intestine and caecal tissue when compared to the levels present in the mock-injected embryos (Fig. 3). The upregulation was not observed when the same volume of buffer was injected into the eggs. The injection of the flagellin did not induce significant changes in transcript levels of the genes in spleen tissue (Fig. 3). Additional comparative transcript analysis demonstrated minimal differences in chIL-1β and chINF-β transcripts between the FliC-injected and mock-injected embryonated eggs for all tested tissues (Fig. 3). Overall, the results indicate that *in ovo* delivery of FliC protein to 18-day chicken embryos induces a potent and specific intestinal innate immune response at one day post-injection.

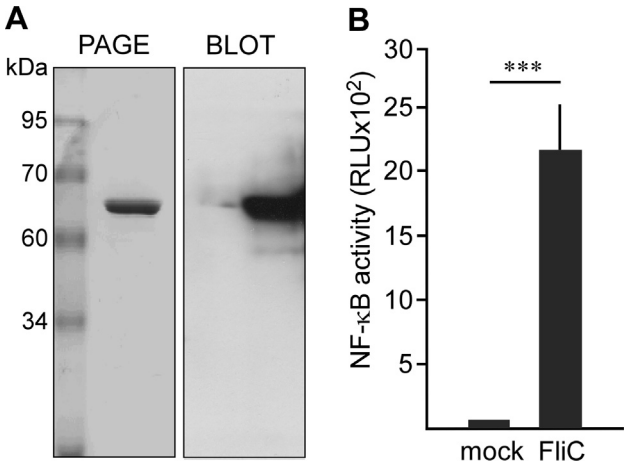

Fig. 2. Purity and function of recombinant *Salmonella* FliC. (A) SDS-PAGE and Western blot of His-tagged *Salmonella* FliC antigen used for immunization. Protein was visualized with Coomassie Brillant Blue (gel) and FliC-specific antisera (blot). (B) NF-κB activation of HeLa-57A cells transfected with chTLR5 after exposure (5h) to purified FliC or solvent. Stimulation of NF-κB is expressed as relative light units (RLU). Data are the mean ± SEM of three experiments. Molecular mass is indicated in kilodaltons (kDa). The asterisk indicates statistical significance (**P < 0.01).

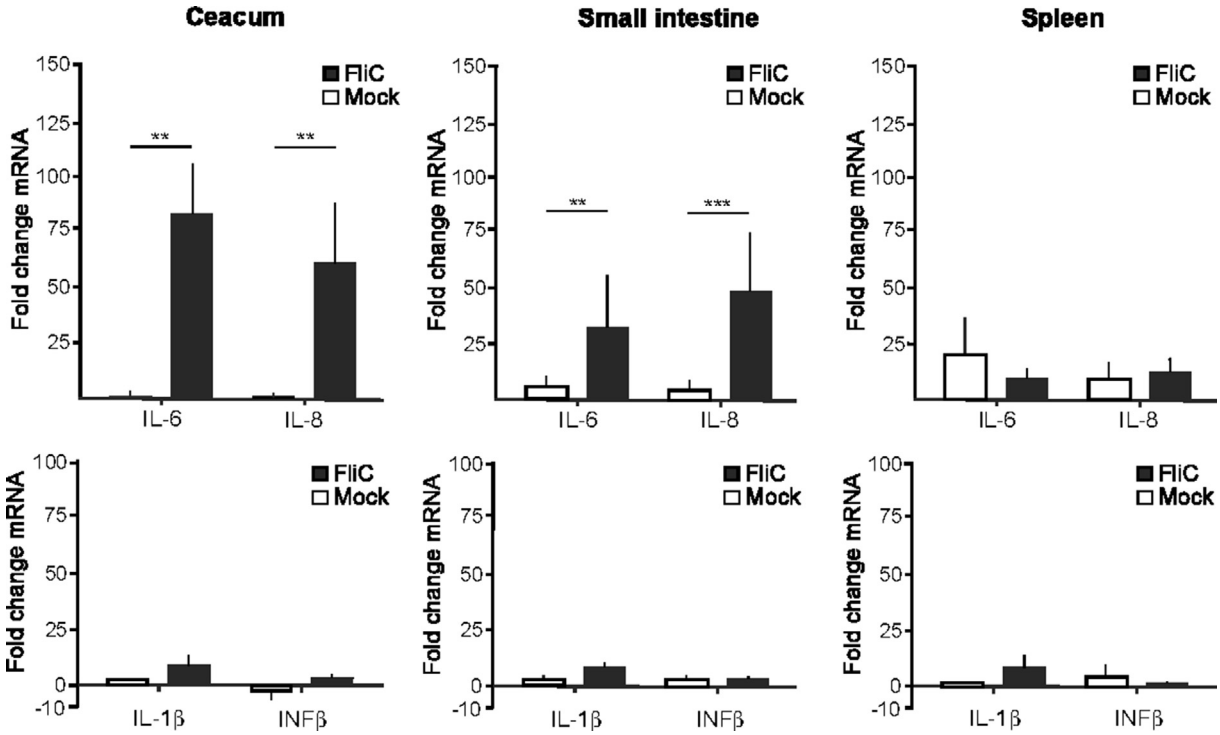

Fig. 3. Expression of chIL-8 (CXCLi2), chIL-6, ch-IL1β, and chINFβ transcripts. Expression of the inflammatory genes in the indicated tissues at one day after *in ovo* delivery of purified FliC to 18-day old embryonated eggs from SPF chickens as determined by RT-qPCR. Results for each of the indicated genes are expressed as the mean ± SEM fold difference between the average mRNA level in the indicated tissues of four FliC- and mock-injected embryonated eggs compared to non-injected controls. Significant differences in ΔmRNA values between the FliC- and mock-injected groups were analyzed using log transformed data as described in Section 2 and are indicated by asterisks. ***P < 0.005; **P < 0.01.

3.5. Antibody response after in ovo protein delivery

To investigate the effect of *in ovo* immunization with FliC protein on the generation of an antibody response, the presence of FliC-directed IgY antibodies was followed in time. Sera were collected at day 11 and day 21 post-hatch from the FliC-injected, mock-injected, and non-treated experimental groups of embryonated eggs. In the first experiment, embryonated eggs from SPF chickens were used to exclude an effect of possible maternal FliC-specific antibodies. The presence of anti-FliC antibodies was detected by enzyme-linked immunosorbent assay (ELISA) using purified FliC as antigen. Mean antibody titers were defined as the highest sera dilution giving statistically significant differences between the FliC-injected and mock-injected groups. Results showed a mean antibody titer of 1:1280 at day 11 post-hatch for the FliC-injected group (Fig. 4A). At day-21 post-hatch, FliC antibody levels had dropped to a mean titer of 1:160 (Fig. 4B). Repetition of the experiment with embryonated eggs from a commercial chicken flock yielded similar results including the decline in antibody titer in the period between day-11 and day 21 (Fig. 4C and D). In this experiment, the mean titer for the FliC-injected group declined from 1:640 on day 11 to 1:80 on day 21. Overall, the results indicate that i*n ovo* delivery of purified flagellin with TLR5 stimulating activity induces a potent immune response that can easily be detected at day-11 post-hatch but that has declined at day 21 of age.

4. Discussion

The present study was designed to evaluate the ability of chicken embryos to mount an immune response after *in ovo* delivery of *Salmonella* flagellin. Evidence is provided that antigen injected into the amniotic fluid of 18-day old fertilized eggs reaches the intestine of the embryo and evokes a local cytokine response within 24 h, and gives rise to the development of flagellin-specific antibodies that can be detected until at least day 21 post-hatch. The results indicate that at the late embryonic stage the chicken immune system is sufficiently mature to respond to *in ovo* injected protein antigens. This implies that *in ovo* immunization with protein-based subunit vaccines may be a feasible approach to limit bacterial infections early after hatching.

The strategy of *in ovo* vaccination as a method to protect young birds against infections is well established. The procedure is successfully used worldwide to deliver live attenuated vaccines that limit the spread of viral diseases such as Marek's disease and Newcastle disease [4,44,6,26]. To our knowledge, *in ovo* immunization with protein subunit vaccines is much less developed and not commercially applied, possibly for vaccine efficacy, the need of adjuvant, or economic reasons. We investigated the feasibility of this approach by analysis of the evoked local intestinal innate response and the antigen-specific antibody kinetics after *in ovo* delivery of a recombinant protein with intrinsic adjuvant activity. We used *Salmonella* flagellin as an antigen as this protein is immunogenic in adult chickens [45]. This immunogenicity may be partly attributed to the intrinsic activity of *Salmonella* flagellin to activate chTLR-5 [29,46,28,47]. Our results indicate that *in ovo* immunization with recombinant flagellin results in a potent adaptive immune response. This finding seems at variance with the reported incomplete development of the secondary immune organs in late embryonic and neonatal chickens [48]. However, our results are in line with the response observed after *in ovo* delivery of non-replicating adenovirus-vector based vaccines [7,8], heat-killed *Campylobacter* organisms [49], and recombinant *Eimeria* proteins [9,11]. These results strongly suggest that the chicken immune system at the late embryonic stage is sufficiently mature to respond to subunit vaccine antigens.

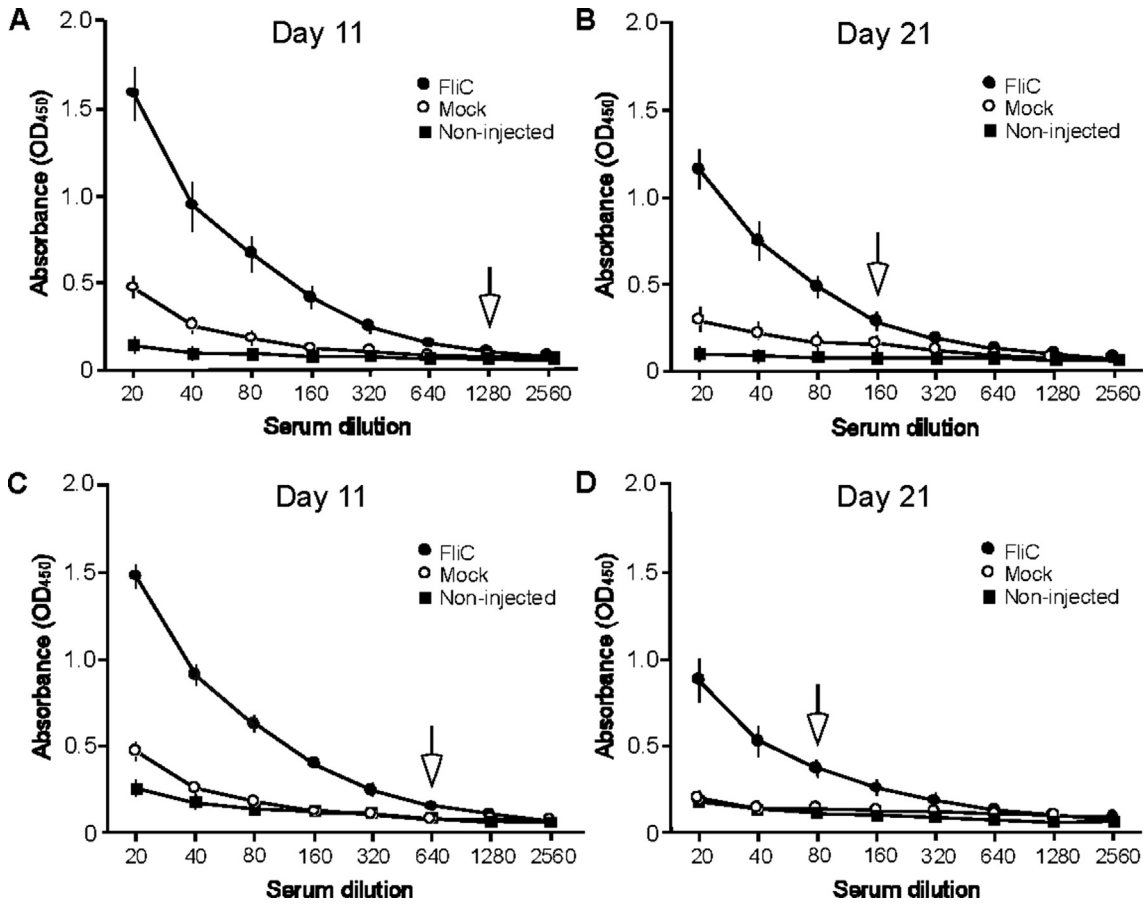

Fig. 4. FliC-specific IgY reactivity in serially-diluted sera of FliC immunized embryos at day 11 and day 21 post-hatch as measured by ELISA. Both sera from hatched chicken from eggs of (A, B) SPF and (C, D) commercial flocks were tested. Sera from chicken hatched from mock-injected or non-injected embryonated eggs served as controls. The numbers on the X-axis represent the reciprocal of the serum dilution. Data represent mean ± SEM values of the reactivity of individual chicken sera. The highest dilutions which yielded a statistically significant difference in antibody reactivity between the FliC- and mock-injected groups are indicated with arrows.

Both the intestinal accumulation of injected materials [50,51] and the increased cytokine transcript levels in the intestinal tissue at 24 h after *in ovo* antigen delivery indicate that the delivered materials enter the intestine of the embryo via the oral or cloacal route. The induction of mucosal chIL-6 and chIL-8 (CXCL8-CXCLi2) transcripts indicates that naive embryonic intestinal tissue (i.e., not previously exposed to microbiota) is capable to respond to the delivered antigen. This effect is likely mediated through TLR5 as after flagellin purification other chTLR stimulating activity is either non-detectable (chTLR2 and chTLR21) or very low (chTLR4) [42] while chickens lack the TLR4-MyD88-independent activation pathway [17]. Furthermore, to our knowledge intracellular receptors for flagellin appear lacking in chickens. Activation of recombinant chTLR5 by *Salmonella* FliC promotes chIL-6 and chIL-8 gene transcription [29]. Additionally, our RT-PCR results clearly demonstrate intestinal chTLR5 expression at day 19 of embryonic development. Previous analysis of TLR expression during chicken embryogenesis indicates the presence of transcripts as early as day 3 of embryonic development [34] although expression patterns vary during subsequent embryonic development [33,34]. Notably, *in ovo* delivery of flagellin protein at day 18 did not increase chIL-6 or chIL-8 transcript levels in spleen tissue, despite the presence of chTLR5. This suggests that the injected flagellin may not have been transported beyond the intestinal delivery site. Alternatively, it can be argued that the reported immature status of spleen immune tissue of the embryo [47] prevented an appropriate response.

The strong humoral immune response towards bacterial flagellin measured at day-11 post-hatch clearly demonstrates that *in ovo* immunization of chicken embryos with a purified highly immunogenic protein with intrinsic adjuvant activity also evokes a potent adaptive immune response. This result implies that not only the innate immune system but also the adaptive immune system is sufficiently mature at the late embryonic stage to respond to a single injection with purified antigen that carries intrinsic adjuvant activity. This is important as the strategy of protein subunit immunization deviates from the successful *in ovo* immunization with live attenuated vaccines. After delivery of these vaccines, the virus may still replicate *in vivo* resulting in enhanced and prolonged exposure to the antigen. The recent use of bacterial flagellin as a constituent in viral vaccine [23] underlines the potency of the adjuvant activity of flagellin in the generation of a protective immune response.

A potential disadvantage of *in ovo* immunization with isolated protein antigens without additional boosting early post-hatch may be the rapid waning of antibody titers. Indeed, analysis of the sera of chickens collected at days 11 and 21 post-hatch revealed persistent but gradually declining flagellin antibody titers. Yet, flagellin-specific antibodies could be detected for up to at least three weeks post-hatch, indicating that *in ovo* injection results a relatively long lasting humoral response. The observed gradual decline in anti-flagellin titer may reflect a reduced antibody production but may also be attributed to a dilution of the antibodies due to the rapid growth of the broiler chicken. If needed, boosting of the chicken with isolated flagellin (e.g. via drinking water) or by natural antigen exposure during the first weeks of life may reverse the waning of antibody levels [49]. Of note, successful

and commercially applied live attenuated vaccines against viral diseases are considered to protect broilers for the duration of 4–6 weeks, when desired, longer protection can be achieved with re-vaccination.

Overall, our results indicate that *in ovo* immunization with recombinant bacterial flagellin protein evokes a potent humoral immune response that can be detected for up to at least three weeks post-hatch. This finding combined with those obtained for non-replicating virus vaccines and *Eimeria* proteins indicate that *in ovo* application of bacterial subunit vaccines is a feasible approach. Major target organisms of bacterial *in ovo* vaccines may be the pathogenic *E. coli* species and the dominant zoonotic pathogens *Salmonella* Enteritidis and *Campylobacter jejuni*. *In ovo* vaccination is attractive because of the low vaccine dose and the proven history of being safe and suitable for mass vaccination [52]. A major challenge remains to identify conserved antigens that together with flagellin provide sufficient cross-protection between strains.

Acknowledgments

We thank Dr. R.T. Hay (Institute of Biomolecular Sciences, School of Biomedical Sciences, University of St. Andrews, Scotland, UK) for providing the HeLa-57A cell line. The authors are grateful to T.G. den Ouden, F. Weites and M. Kranenburg for assistance with the animal experiments. The work was funded by The Danish Council for Strategic Research (Grant Number: CamVac 09-067131) and the European Community's Seventh Framework Programme FP7/2007–2013. (Grant Number: CamCon 244547).

References

[1] Sharma JM. Embryo vaccination with infectious bursal disease virus alone or in combination with Marek's disease vaccine. Avian Dis 1985;29:1155–69.
[2] Wakenell PS, Sharma JM. Chicken embryonal vaccination with avian infectious bronchitis virus. Am J Vet Res 1986;47:933–8.
[3] Reddy SK, Sharma JM, Ahmad J, Reddy DN, McMillen JK, Cook SM, et al. Protective efficacy of a recombinant herpesvirus of turkeys as an in ovo vaccine against Newcastle and Marek's diseases in specific-pathogen-free chickens. Vaccine 1996;14:469–77.
[4] Avakian AP, Wakenell PS, Grosse D, Whitfill CE, Link D. Protective immunity to infectious bronchitis in broilers vaccinated against Marek's disease either *in ovo* or at hatch and against infectious bronchitis at hatch. Avian Dis 2000;44:536–44.
[5] Ricks CA, Avakian AP, Bryan T, Gildersleeve R, Haddad E, Ilich R, et al. *In ovo* vaccination technology. Adv Vet Med 1999;41:495–515.
[6] Negash T, Al-Garib SO, Gruys E. Comparison of in ovo and post-hatch vaccination with particular reference to infectious bursal disease. Vet Q 2004;26:76–87.
[7] Toro H, Tang DC, Suarez DL, Zhang J, Shi Z. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. Vaccine 2008;26:2640–6.
[8] Avakian AP, Poston RM, Kong FK, Van Kampen KR, Tang DC. Automated mass immunization of poultry: the prospect for nonreplicating human adenovirus-vectored *in ovo* vaccines. Expert Rev Vaccine 2007;6:457–65.
[9] Ding X, Lillehoj HS, Quiroz MA, Bevensee E, Lillehoj EP. Protective immunity against *Eimeria acervulina* following in ovo immunization with a recombinant subunit vaccine and cytokine genes. Infect Immun 2004;72:6939–44.
[10] Dalloul RA, Lillehoj HS, Klinman DM, Ding X, Min W, Heckert RA, et al. *In ovo* administration of CpG oligodeoxynucleotides and the recombinant microneme protein MIC2 protects against *Eimeria* infections. Vaccine 2005;23:3108–13.
[11] Lillehoj HS, Ding X, Dalloul RA, Sato T, Yasuda A, Lillehoj EP. Embryo vaccination against *Eimeria tenella* and *E. acervulina* infections using recombinant proteins and cytokine adjuvants. J Parasitol 2005;91:666–73.
[12] Kobierecka PA, Wyszyńska AK, Gubernato J, Kuczkowski M, Wiśniewski O, Maruszewska M, et al. Chicken anti-*Campylobacter* vaccine – comparison of various carriers and routes of immunization. Front Microbiol 2016;7:740.
[13] Hilton LS, Bean AG, Lowenthal JW. The emerging role of avian cytokines as immunotherapeutics and vaccine adjuvants. Vet Immunol Immunopathol 2002;85:119–28.
[14] Asif M, Jenkins KA, Hilton LS, Kimpton WG, Bean AG, Lowenthal JW. Cytokines as adjuvants for avian vaccines. Immunol Cell Biol 2004;82:638–43.
[15] Savelkoul HF, Ferro VA, Strioga MM, Schijns VE. Choice and design of adjuvants for parenteral and mucosal vaccines. Vaccine (Basel) 2015;3:148–71.
[16] Boyd A, Philbin VJ, Smith AL. Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne zoonoses. Biochem Soc Trans 2007;35:1504–7.
[17] Keestra AM, de Zoete MR, Bouwman LI, Vaezirad MM, van Putten JPM. Unique features of chicken Toll-like receptors. Dev Comp Immunol 2013;41:316–23.
[18] Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol 2001;2:947–50.
[19] West AP, Koblansky AA, Ghosh S. Recognition and signaling by Toll-like receptors. Annu Rev Cell Dev Biol 2006;22:409–37.
[20] Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol 2015;109:14.12.1-10.
[21] Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity 2010;33:492–503.
[22] Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human. Immunol Rev 2011;239:178–96.
[23] Gupta SK, Deb R, Dey S, Chellappa MM. Toll-like receptor-based adjuvants: enhancing the immune response to vaccines against infectious diseases of chicken. Expert Rev Vaccines 2014;13:909–25.
[24] Genovese KJ, He H, Lowry VK, Nisbet DJ, Kogut MH. Dynamics of the avian inflammatory response to *Salmonella* following administration of the Toll-like receptor 5 agonist flagellin. FEMS Immunol Med Microbiol 2007;51:112–7.
[25] Chaung HC, Cheng LT, Hung LH, Tsai PC, Skountzou I, Wang B, et al. *Salmonella* flagellin enhances mucosal immunity of avian influenza vaccine in chickens. Vet Microbiol 2012;157:69–77.
[26] Haygreen L, Davison F, Kaiser P. DNA vaccines for poultry: the jump from theory to practice. Expert Rev Vaccines 2005;4:51–62.
[27] Fu J, Liang J, Kang H, Lin J, Yu Q, Yang Q. Effects of different CpG oligodeoxynucleotides with inactivated avian H5N1 influenza virus on mucosal immunity of chickens. Poult Sci 2013;92:2866–75.
[28] Iqbal M, Philbin VJ, Withanage GS, Wigley P, Beal RK, Goodchild MJ, et al. Identification and functional characterization of chicken Toll-like receptor 5 reveals a fundamental role in the biology of infection with *Salmonella enterica* serovar Typhimurium. Infect Immun 2005;73:2344–50.
[29] Keestra AM, de Zoete MR, Van Aubel RA, van Putten JPM. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol 2008;45:1298–307.
[30] Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A, et al. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 2009;46:3163–70.
[31] Keestra AM, de Zoete MR, Bouwman LI, Van Putten JPM. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. J Immunol 2010;185:460–7.
[32] Michailidis G, Theodoridis A, Avdi M. Transcriptional profiling of Toll-like receptors in chicken embryos and in the ovary during sexual maturation and in response to *Salmonella* Enteritidis infection. Anim Reprod Sci 2010;122:294–302.
[33] Meade KG, Higgs R, Lloyd AT, Giles S, O'Farrelly C. Differential antimicrobial peptide gene expression patterns during early chicken embryological development. Dev Comp Immunol 2009;33:516–24.
[34] Kannaki TR, Reddy MR, Verma PC, Shanmugam M. Differential Toll-like receptor (TLR) mRNA expression patterns during chicken embryological development. Anim Biotechnol 2015;26:130–5.
[35] van Aubel RA, Keestra AM, Krooshoop DJ, van Eden W, van Putten JPM. Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells. Mol Immunol 2007;44:3702–14.
[36] Tabor S, Richardson CC. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci U S A 1987;84:4767–71.
[37] Han Y, Niu M, An L, Li W. Involvement of TLR21 in baculovirus-induced interleukin-12 gene expression in avian macrophage-like cell line HD11. Vet Microbiol 2010;144:75–81.
[38] Vaezirad MM, Keestra AM, de Zoete MR, Koene MG, Wagenaar JA, van Putten JPM. Invasive behavior of *Campylobacter jejuni* in immunosuppressed chicken. Virulence 2017;8:248–60.
[39] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101–8.
[40] Rodriguez MS, Thompson J, Hay RT, Dargemont C. Nuclear retention of IκBα protects it from signal-induced degradation and inhibits nuclear factor κB transcriptional activation. J Biol Chem 1999;274:9108–15.
[41] Voogdt CG, Bouwman LI, Kik MJ, Wagenaar JA, van Putten JPM. Reptile Toll-like receptor 5 unveils adaptive evolution of bacterial flagellin recognition. Sci Rep 2016;6:19046.
[42] Radomska KA, Vaezirad MM, Verstappen KM, Wösten MM, Wagenaar JA, van Putten JPM. Chicken immune response after *in ovo* immunization with chimeric TLR5 activating flagellin of *Campylobacter jejuni*. PLoS One 2016;11:e0164837.
[43] de Zoete MR, Bouwman LI, Keestra AM, van Putten JPM. Cleavage and activation of a Toll-like receptor by microbial proteases. Proc Natl Acad Sci U S A 2011;108:4968–73.
[44] Gagic M, St Hill CA, Sharma JM. *In ovo* vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis 1999;43:293–301.
[45] Ochoa-Repáraz J, Sesma B, Alvarez M, Jesús Renedo M, Irache JM, Gamazo C. Humoral immune response in hens naturally infected with *Salmonella* Enteritidis against outer membrane proteins and other surface structural antigens. Vet Res. 2004;35:291–8.

[46] de Zoete MR, Keestra AM, Wagenaar JA, Van Putten JPM. Reconstitution of a functional Toll-like receptor 5 binding site in *Campylobacter jejuni* flagellin. J Biol Chem 2010;285:12149–58.
[47] Kogut MH, Iqbal M, He H, Philbin V, Kaiser P, Smith A. Expression and function of Toll-like receptors in chicken heterophils. Dev Comp Immunol 2005;29:791–807.
[48] Mast J, Goddeeris BM. Development of immunocompetence of broiler chickens. Vet Immunol Immunopathol 1999;70:245–56.
[49] Noor SM, Husband AJ, Widders PR. *In ovo* oral vaccination with *Campylobacter jejuni* establishes early development of intestinal immunity in chickens. Br Poult Sci 1995;36:563–73.
[50] Sorvari R, Naukkarinen A, Sorvari TE. Anal sucking-like movements in the chicken and chick embryo followed by the transportation of environmental material to the bursa of Fabricius, caeca and caecal tonsils. Poult Sci 1977;56:1426–9.
[51] Jochemsen P, Jeurissen SH. The localization and uptake of *in ovo* injected soluble and particulate substances in the chicken. Poult Sci 2002;81:1811–7.
[52] Tarpey I, Huggins MB. Onset of immunity following *in ovo* delivery of avian metapneumovirus vaccines. Vet Microbiol 2007;124:134–9.