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The *in vitro* and *in ovo* responses of chickens to TLR9 subfamily ligands

Kristie A. Jenkins [a], John W. Lowenthal [a,*], Wayne Kimpton [b], Andrew G.D. Bean [a]

[a] CSIRO Livestock Industries, Australian Animal Health Laboratories, Private Bag 24, Geelong, Victoria 3220, Australia
[b] Department of Veterinary Science, The University of Melbourne, Melbourne, Victoria, Australia

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ABSTRACT

Although Toll-like receptors (TLRs) have been well characterised in mammals, less work has been carried out in non-mammalian species, such as chickens. In this study the response of chicken cells to the TLR9 subfamily of ligands was characterised *in vitro* and *in ovo*. It was found that even though chickens appear to have only one functional receptor to represent the TLR9 subfamily, stimulation of chicken splenocytes with TLR7 and TLR9 ligands induced proinflammatory cytokine production and cell proliferation, similar to that observed when the homologous mammalian receptors are stimulated. Furthermore, we demonstrated that the *in ovo* administration of these TLR ligands elicits a response, such as cytokine production, that can be detected post-hatch. The current knowledge of the action of TLR ligands in mammals, in conjunction with their immunomodulating ability shown in this study, draws attention to their potential use as therapeutic agents for the poultry industry.

1. Introduction

Toll-like receptors (TLRs) were first identified in mammals in 1997 [1] and to date, several TLRs have been identified, 12 in mice (TLRs 1–9 and 11–13) and 10 in humans (1–10) [2]. In mammals, TLRs can be divided into 5 subfamilies: subfamily-2, -3, -4, -5 and -9 based on phylogenetic analysis of amino acid (aa) sequences [3]. The TLR9 subfamily, which consists of TLRs 7–9, is believed to form an evolutionary cluster based on sequence homology and their requirement for endosomal maturation to detect their ligands [4–6]. Furthermore, this TLR subfamily also appears to play an important role in viral recognition. Moreover, the expression of the TLR9 subfamily in intracellular compartments, such as endosomes, is essential for their ability to recognise nucleic acid derivatives, which is important in viral recognition [6–8]. For instance, a number of viruses invade cells by receptor-mediated endocytosis resulting in viral contents being exposed to the cytoplasm by fusion of viral and endosomal membranes. Virus degradation in the endosomal compartment results in the release of particles including the TLR9 subfamily ligands, nucleosides such as single stranded ribonucleic acid (ssRNA) and CpG-DNA [8]. Therefore, the localisation of this TLR subfamily within intracellular components is vital to the appropriate recognition of the ligands. Furthermore, unlike the majority of TLRs which recognise distinct pathogen associated molecular patterns (PAMPs) which are clearly "non-self" molecules [9], TLRs 3, 7–9 recognise nucleosides, which are also present in the host [6,7]. Therefore, it is believed that it is abnormal localisation of these nucleosides within the endosome, rather than structure or unique motifs that triggers the response through the TLR9 subfamily members [10,11]. This notion is supported by the finding that TLR7 mediates a response to polyuridylic acid (polyU), a simple motif likely to be present in non-viral RNAs found in the endosome [10]. Additionally, the presence of extracellular ribonucleases in interstitial fluids ensures that little if any self ssRNA reaches the endosomal compartments of antigen presenting cells where the TLRs detect their ligands [10]. Therefore, TLR9 subfamily monitoring of endosomal nucleosides represents a vital step in the anti-viral immune response.

The natural ligand for TLR9 is unmethylated CpG-dinucleotide containing sequences, which are more common in pathogen genomes than in vertebrate genomes [12,13]. Originally TLR9 was thought to recognise unmethylated CpG-DNA from bacteria alone [12]; however, it is now known to recognise genomic viral DNA also and is therefore an important recognition receptor for both viral and bacterial infections [14]. In mammals the activation of the TLR9 subfamily by its ligands is known to result in the production of various cytokines such as IL1, IL6, IL12, IL18 and IFNα [10,15]. The production of these cytokines can result in the maturation of immature DCs, B cell proliferation, the induction of nitric oxide (NO) secretion and the activation of NK cells, monocytes and macrophages [16,17]. If the same holds true in chickens, then stimulation of the immune system with TLR9 ligands may lead to a new therapeutic strategy for disease control in poultry.

* Corresponding author. Tel.: +61 3 5227 5000; fax: +61 3 5227 5555.
E-mail address: John.Lowenthal@csiro.au (J.W. Lowenthal).

Abbreviations: CEF, chicken embryo fibroblasts; ch, Chicken; ChIL6, chicken interleukin 6; chloro, chloroquine; dph, days post-hatch; E18, embryonic day 18; EF-DNA, endotoxin free E. coli DNA; IFN, interferon; loxo, loxoribine; PAMP, pathogen associated molecular pattern; SE, standard error; SPF, specific pathogen free; TLR, Toll-like receptor.

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In contrast to mammals, in which the TLR9 subfamily has been well characterised, in non-mammalian species, such as chickens, these TLRs have not been as comprehensively researched. Prior to the identification of chicken TLRs (ChTLRs), chickens were reported to respond to what are now known as ligands for the TLR9 subfamily, such as CpG motifs [18,19]. However, the release of the chicken genome sequence allowed the identification of ChTLRs based on sequence similarity to mammalian TLRs. Intriguingly, examination of the chicken genome revealed that chickens seem to have only one functional representative of the TLR9 subfamily, making the ligand response unclear. To date, bioinformatics searches of the chicken genome have not identified an orthologue to TLR9 [20–22]. The genes that surround TLR9 in other genomes, such as those of humans and Xenopus can be identified in the chicken genome. However, the region between these genes does not contain an identifiable TLR9 orthologue [21,22]. Therefore, although the presence of TLR9 in the chicken genome cannot be ruled out, if it is present it does not appear to be syntenic like mammalian TLRs [23]. Furthermore, in chickens TLR8 has been shown to be disrupted by multiple introns, rendering it non-functional [24]. Consequently, the only functional TLR9 subfamily receptor to be identified to date in chickens is TLR7 [20–22]. Interestingly, there appears to be two splice variants of ChTLR7 (ChTLR7^{S1} and ChTLR7^{S2}) [24]. This has lead to the speculation that either ChTLR7, or another TLR, such as the novel chicken TLR15 or TLR21, may elicit the response to the mammalian TLR9 ligands [22,25,26].

The observed differences between chicken and mammals with regards to TLRs, allows us to increase our understanding of the function of the TLR9 subfamily. In the present study, the effect of synthetic TLR9 subfamily ligands on cytokine production, cell proliferation and the requirement for endosomal maturation to elicit these responses was studied *in vitro* and *in ovo*. This type of analysis may allow us to uncover novel forms of therapeutics and immune-modulators.

2. Methods

2.1. Experimental chickens

Specific pathogen-free (SPF) hybrid white leghorn chickens were purchased from SPAFAS (Woodend, Victoria, Australia). These chickens were raised in flexible plastic isolators in the Australian Animal Health Laboratories Small Animal Facility in Geelong, and were fed fumigated feed and acidified water. All collection of tissues and animal experimentation was performed with CSIRO Animal Ethics Committee approval.

2.2. Preparation of splenocytes

Spleens were removed from 3- to 5-week-old SPF chickens and individual single cell suspensions prepared, strained through a 70 μm nylon cell strainer and were transferred onto pre-warmed Lymphoprep solution (Axix-Shield, Norway). The tubes containing the Lymphoprep and single cell suspensions were centrifuged at $1100 \times g_{max}$ for 20 min at 21 °C with no brake. The splenic mononuclear cell layer was collected and washed twice in PBS. The cells were resuspended in 10 mL of media (DMEM) containing 5% (v/v) Foetal Calf Serum (FCS) and cell counts were performed. Subsequently, cells from individual spleens were diluted to give a final concentration of 1×10^6 cells/mL in the assays.

2.3. In vitro effect of TLR ligands

Assays to examine cytokine production in response to TLR ligand stimulation were set up in Nunclon™ 6-well plates. The TLR ligands, Loxoribine (loxo), ODN 2006 and endotoxin free *Escherichia coli* DNA (EF-DNA) (Invivogen, USA) were used to stimulate splenocytes as prepared above. Unstimulated cells were also included as controls. The plates were incubated at 37 °C overnight in a humidified cell incubator with 5% CO_2. Supernatants were then harvested and stored for future analysis in cytokine bioassays and enzyme-linked immunosorbent assay (ELISA), while the cell pellets were stored in RNA later for subsequent real time PCR analysis of cytokine mRNA levels.

To determine the requirement for endosomal acidification for the induction of IL6 production, chloroquine (InvivoGen, USA) was used. Assays were carried out as described above except that cells were pre-treated with 100 μM of chloroquine for 30 min prior to ligand stimulation.

To examine the effect of the TLR ligands on cell proliferation, assays were set up in Nunclon™ 96-well plates. Serial twofold dilutions of ligand in 100 μL of DMEM containing 5% (v/v) FCS were carried out prior to the addition of 100 μL of cell suspension to each well. The plates were incubated at 37 °C for 24 h in a humidified cell incubator with 5% CO_2. The plates were then pulsed with 25 μL per well of ^{3}HT (1 uCi) (Amersham, UK), followed by an additional 6 h of incubation. Cells were harvested onto glass fibre filters (Wallac, Finland) using a 96 Mach III M (Tomtec, USA) manual harvester. Filters were then air-dried, prior to being saturated with 5 mL of Betaplate Scint scintillation fluid (Wallac, Finland) and sealed in sample bags (Wallac, Finland). Radioactivity was measured using a Luminescence Counter (Wallac, Finland).

2.4. ChIL6 bioassay

Levels of chicken IL6 (ChIL6) protein were determined using an IL6 dependent murine hybridoma cell line, 7TD1, the growth of which is strictly dependent on IL6 [27]. This assay has previously been shown to respond to ChIL6 [28] and was thus used to measure IL6 in the culture supernatants. 7TD1 cells were passaged in Nunclon™ 6-well plates containing 5 mL of 7TD1 media with 1 μg per well of recombinant ChIL6. Four days prior to IL6 bioassays, 7TD1 cells were expanded in a 75-cm^2 tissue culture flask containing 30 mL of 7TD1 media, 1 mL of log phase cells and 6 μL of recombinant ChIL6 [29]. Following 48 h of growth, the cells were starved by washing twice in PBS and once in 7TD1 media. The cells were then reseeded in 30 mL of fresh 7TD1 media without ChIL6. After 48 h of starvation the cells were diluted to 2×10^4 viable cells/mL. All samples were assayed in duplicate at a dilution of 1/9 in 96-well plates. The following controls were used, media alone control, ChIL6 (positive control) and ChIFNγ (negative control). The starved cells (100 μL) were added to each well and the plates were incubated at 37 °C for 48 h in a humidified cell incubator with 5% CO_2. ChIL6 production was then measured by cell proliferation as described above.

2.5. Measurement of IFN by a virus protection assay

Levels of IFN were measured using a virus protection assay as previously described [30]. In short, chicken embryo fibroblasts (CEF) cultures were prepared from 9-day-old embryos and diluted to 5×10^5 cells/mL in DME with 10% FCS and cultured for 72 h at 37 °C. The cells were then harvested and added 96-well plates at 5×10^4 cells per well in DMEM containing 10% (v/v) FCS and incubated for 24 h at 37 °C. All sample and control supernatants were assayed in duplicate at a dilution of 1/9 in 96-well plates and the cells were incubated at 37 °C overnight. The media was then removed and 100 μL of media containing Semliki Forest virus at 10^3 tissue culture infective dose 50% ($TCID_{50}$)/mL was added to