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Short communication

Class B CpG ODN stimulation upregulates expression of TLR21 and IFN-γ in chicken Harderian gland cells

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ABSTRACT

This study aimed to evaluate the response of Harderian gland (HG) cells after in vitro stimulation with class B synthetic oligodeoxyribonucleotides (ODN) containing CpG motifs. This knowledge is of importance for the development of mucosal vaccines for poultry, such as eye-drop or spray vaccines, to determine if class B CpG ODN can act as an vaccine adjuvant or as a prophylactic treatment mainly against respiratory disease viruses. The relative expression of Toll-like receptor 21 (TLR21), interferon (IFN)-γ, interleukin (IL)-1β and IL-10 genes were quantified at 1, 3, 6 and 18 h post-stimulation of HG cells from 5-week-old birds. In addition, it was also investigated if expression of these genes was affected by the age of the birds (differences between 5- and 12-week-old birds), concentrations of ODN or cell preparation method used. Class B CpG ODN induced upregulation of TLR21 and IFN-γ mRNA expression levels at 1 h post-stimulation depending on concentration of ODN used but only in HG cells isolated from young birds.

1. Introduction

Bacterial DNA containing unmethylated CpG motifs is one of the key immunostimulatory pathogen-associated molecular patterns (PAMPs) (Wagner, 2002). Synthetic oligodeoxynucleotides (ODN) containing CpG motifs can activate host defense responses, with class B CpG ODN being potent B cells activators (Krieg, 2002). In mammals, Toll-like receptor (TLR) 9 recognizes CpG motifs and thereafter mediates the induction of cell signalling pathways (Akira and Takeda, 2004). The functional counterpart of TLR9 in the chicken is TLR21 (Brownlie et al., 2009; Keestra et al., 2010). In mammalian B cells, TLR-mediated responses facilitate antibody isotype-switching, induce the production of immunoglobulins, cytokines and promote proliferation (Barr et al., 2007; Jegerlehner et al., 2007; Giordani et al., 2009). Chicken B cells express TLRs 2, 3, 4, 5, 7 and 21 at the transcript levels (Iqbal et al., 2005; Han et al., 2010), proliferate in response to CpG ODN, especially Bu-1 enriched cells, and produce cytokines as well as molecules associated with antigen presentation (CD80, MHCII) (Wattrang, 2009).

CpG ODN act as immunostimulants in the chicken, leading to increased protection against *Escherichia coli*, *Salmonella* Typhimurium or *Salmonella* Enteritidis (Taghavi et al., 2008, 2009; Mackinnon et al., 2009). CpG ODN can act as a vaccine adjuvant when administered parenterally (Wang et al., 2009; Mallick et al., 2011, 2012). CpG ODN can also adjuvant the chicken's immune response when given

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orally (Ameiss et al., 2006). Birds receiving a single dose of CpG ODN and BSA had consistently higher IgY, IgM, and IgA titres in the serum and in specific areas of the intestine, when compared to the non-immunized and BSA only groups. Levels of BSA-specific antibody produced depended on the concentration of CpG ODN used, ranging from 5 to 100 μg, and differed in time. Injection of CpG ODN with FIA and BSA increased IgY levels both pre- and post-boost, led to an early increase in IgM after stimulation with at least 20 μg of CpG ODN, but resulted in no difference in IgA levels in the serum (Vleugels et al., 2002). Thus, the route of CpG ODN administration influences B cell responses.

The chicken Harderian gland (HG) is part of the eye-associated lymphoid tissues, located in the orbit behind the eye. The HG shows the structure of a typical secondary lymphoid organ, with many B and plasma cells, germinal centres, and T cell-dependent interfollicular regions with scattered T cells and macrophages. It plays an important role in adaptive mucosal immune response upon ocular exposure to avian pathogens. With respect to Ig-producing B cells in the HG, the literature is unclear. Jeurissen et al. (1989) described IgY plasma cells only in birds older than 6 weeks. Jalkanen et al. (1984) showed the majority of B cells in the HG of 10-week-old chickens were IgY^+, followed by IgM^+ cells, with only a small number of IgA^+ cells, whereas Oláh et al. (1996) reported numerous IgM- and IgA-producing plasma cells but rarely IgY^+ plasma cells. Thus different Ig isotypes seem to predominate in the HG, depending on the bird's age.

Akaki et al. (1997) previously demonstrated that transfer of IgA^+ HG cells resulted in preferential migration of HG-derived IgA^+ B cells into caecal tonsils, suggesting that the HG not only plays a role in local immunity in the eye but may also have a systemic mucosal role. The role of the HG in adaptive immune responses following ocular exposure to avian pathogens has been previously studied (Jalkanen et al., 1984; Jeurissen et al., 1989; Baba et al., 1988; Van Ginkel et al., 2009). In the latter study, birds immunized with adenovirus expressing the AIV haemagglutinin H5 gene generated influenza-specific antibody not only in tears but also in the serum.

Although the humoral responses in the chicken HG have been described, the TLR-mediated responses to class B CpG ODN have yet to be elucidated. To this end, we hypothesized that chicken HG cells express TLR21 and respond to its ligand by up-regulating cytokine transcripts. As such, the objective of the present study was to examine the response of HG cells after in vitro stimulation with class B CpG ODN.

2. Materials and methods

2.1. Animals

Layer type J-line chickens were bred and hatched at the Roslin Institute, reared in floor pens and water and feed were provided ad libitum. The 5-week-old ($n = 16$) and 12-week-old birds ($n = 6$) came from four and two different hatches, respectively. HGs were collected and single-cell suspensions prepared by gently squeezing the HG through a 40 μm nylon cell strainer. Leukocytes were isolated either with or without density gradient centrifugation for 20 min at $300 \times g$ using Histopaque 1.077 (Sigma–Aldrich, Poole, UK), and the cells washed and re-suspended in RPMI medium containing 10% FBS in a total volume of 400 μL. The HG cells were seeded into 96-well plates (50 μL/well) for in vitro stimulation with TLR ligands.

2.2. TLR ligands

The synthetic class B CpG ODN (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) and non-CpG ODN (5′-TGCTGCTTGTGCTTTTGTGCTT-3′) were purchased from TIB Molbiol, Syntheselabor GmbH (Berlin, Germany), both with a phosphorothioate backbone. Both ODN were resuspended in sterile phosphate-buffered saline (PBS, pH 7.4) and diluted to their working concentrations in RPMI medium.

2.3. Induction of gene expression

The synthetic class B CpG ODN was used to stimulate HG cells. Control groups received either non-CpG ODN or RPMI medium. After purification, cells were stimulated by class B CpG ODN at two different concentrations (5 and 50 μg/mL) whereas those isolated without density gradient centrifugation were stimulated with 50 and 500 μg/mL, in all cases in a total volume of 50 μL/well. At 1, 3, 6 and 18 h post-stimulation cells were harvested for RNA extraction.

2.4. RNA extraction and quantitative real-time RT-PCR

Total RNA from the HG cells was isolated using an RNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. IL-1β, IL-10, IFN-γ and TLR21 mRNA levels were quantified using TaqMan qRT-PCR as described previously (Hughes et al., 2007; Harrison et al., 2007a,b).

Primers and probes are described in Table 1. The qRT-PCR was performed using the TaqMan FAST Universal PCR master mix and one-step qRT-PCR master mix reagents (Applied Biosystems, Cheshire, UK). Amplification and detection of specific products were performed using the Applied Biosystems 7500 Fast Real Time PCR System with the following cycle profile: one cycle of 48 °C for 30 min, one cycle of 95 °C for 20 s, 40 cycles of 95 °C for 3 s, and 40 cycles of 60 °C for 30 s. Quantification was based on the increased fluorescence detected by the 7500 Fast Sequence Detection System as described previously (Wu et al., 2010).

2.5. Data analysis

Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold (ΔRn). The Ct values for IL-1β, IL-10, IFN-γ and TLR21 for each sample were normalized using the Ct value of the 28S rRNA product for the same sample. Normalized Ct values were calculated using the formula $Ct + ((N_t' - C_t') \times S/S'$ where N_t' is the mean Ct for 28S RNA among all samples, C_t' is the mean Ct for 28S RNA in the sample and S and S' are the slopes of the regressions of the standard plots for the cytokine mRNA and the 28S RNA, respectively. The data are presented as fold difference from levels in the medium-only control group and