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***In situ* hybridization to detect and localize signature cytokines of T-helper (Th) 1 and Th2 immune responses in chicken tissues**

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Abstract

The avian immune system has been shown to possess a repertoire of cytokines directing T-helper (Th) 1 and Th2 types of immune responses similar to that in mammals. The objective of this study was to establish *in situ* hybridization (ISH) for the localization of mRNA of selected signal cytokines, chicken interferon- γ (ChIFN- γ), chicken interleukin (ChIL)-4 and ChIL-13 in fixed tissues. RNA probes were generated to hybridize to 488, 318, and 417 bp of the respective target mRNA. Probe concentrations ranging from 100 ng/ml to 400 ng/ml were shown to be suitable to label cells that expressed these cytokines. The specificity of every probe was verified using the respective sense probe. ChIFN- γ , ChIL-4 and ChIL-13 positive cells were observed in the lymphocytic infiltrations of liver and in the periarteriolar lymphatic sheaths of spleen collected from specific-pathogen-free chickens. ISH of these cytokines in a severely inflamed liver due to infiltration with the parasite *Histomonas meleagridis* revealed the expression of both ChIFN- γ and ChIL-13 mRNA in the mononuclear infiltrates. In conclusion, ChIFN- γ , ChIL-4 and ChIL-13 mRNA were efficiently localized by ISH, which supplies a valid technique to characterize immune responses in fixed tissues.

Keywords

In situ hybridization; RNA probes; ChIFN- γ ; ChIL-4; ChIL-13

1 Introduction

The T-helper (Th) 1/Th2 paradigm, first demonstrated in murine models (Mosmann et al., 1986; Mosmann and Coffman, 1989), has been central to our understanding of adaptive immune responses as well as protective and deleterious types of immune reactions in mammals. This concept has been shown to extend to the avian immune system. Functional

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Conflict of interest

None

genes of signature cytokines hall-marking Th1- and Th2-type responses in mammals, particularly interleukin (IL)-4, IL-12, IL-13, IL-18 and interferon- γ (IFN- γ), have been identified in chicken (Avery et al., 2004; Balu and Kaiser, 2003; Degen et al., 2004; Digby and Löwenthal, 1995; Schneider et al., 2000). In addition, the existence of a Th1 like system was shown *in vitro* through IL-18-induced proliferation and IFN- γ secretion of chicken splenic CD4⁺ T cells (Göbel et al., 2003). Another study also found Th1 and Th2 like responses *in vivo*, characterized by elevated expression of IFN- γ , IL-4 and IL-13 following infection of birds with a virus (Newcastle disease virus) and a helminth (*Ascaridia galli*), representing intracellular and extracellular pathogens, respectively (Degen et al., 2005). However, the avian Th1/Th2 system has some peculiarities, for instance the absence of immunoglobulin E and functional eosinophils, which are involved in the mammalian Th2 response (Kaiser and Stäheli, 2014).

Studies that reported changes in chicken Th1/Th2 cytokine expressions following infection have mainly used quantitative reverse transcription PCR (qRT-PCR) (Kaiser and Stäheli, 2014) whereas histological assays received little attention. However, studies in mammals have benefited for long from utilizing *in situ* hybridization (ISH) to decipher associations of cytokine expression with inflammation (McNicol and Farquharson, 1997; Rutar et al., 2015). A recent study (Kuribayashi et al., 2013) showed the potentials of ISH in localizing and displaying the involvement of chicken cytokines (IL-6) in inflammatory responses using tissue sections.

Non-radioactive ISH using *in vitro* transcribed digoxigenin (DIG) labeled RNA probes is a sensitive tool to localize cells that express the mRNA of cytokines (Meltzer et al., 1998). The availability of genomic information for chicken interferon- γ (ChIFN- γ), chicken interleukin (ChIL)-4 and ChIL-13 has made establishing ISH with such probes possible. The technique, however, depends heavily on the careful selection of probes and optimization of ISH protocols, pertinent to the target tissue and molecule. The objective of this study was therefore to test and validate DIG-labeled RNA probes and produce an optimized protocol for ISH of ChIFN- γ , ChIL-4, and ChIL-13 in formalin-fixed paraffin-embedded tissue sections in which the respective cytokine mRNA positive cells can be localized.

2 Materials and methods

2.1 Generating RNA probes for the detection of ChIFN- γ , ChIL-4 and ChIL-13

For the purpose of probe synthesis, spleen tissue was collected aseptically from a non-infected specific-pathogen-free (SPF) chicken immediately after euthanization. The tissue was stabilized with RNAlater® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was then extracted using RNeasy® Mini kit (Qiagen). The cDNA of the respective cytokines was synthesized by conventional reverse transcription (RT)-PCR using QIAGEN OneStep RT-PCR kit and gene specific primers (Table 1). Reverse transcription PCR was performed using DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription was carried out at 50°C for 30 minutes. After denaturation of the reverse transcriptase at 95°C for 15 minutes, thermal cycling conditions were performed for 40 cycles of 95°C for 30 seconds, gradient of 55.1°C to 58.6°C for 30 seconds and 72°C for 1 minute. Final extension was allowed at

72°C for 10 minutes. The PCR products were then separated in 1.5% Agarose gel and purified using QIAquick® Gel Extraction Kit (Qiagen). The respective cDNAs were ligated into pCR® 4-TOPO® vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The sequence and orientation of the cloned products were then verified by sequencing (LGC Genomics, Augsburg, Germany).

Plasmids validated to contain the cDNA of cytokines were linearized with *SpeI* or *NotI* restriction enzymes (Thermo Scientific, Dreieich, Germany). Depending on the orientation of the inserts, T7 or T3 RNA polymerases were used to generate both sense and antisense DIG-labeled RNA probes. The *in vitro* synthesis of the probes was performed using DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

The probe yield was quantified using NanoDrop spectrophotometer (Thermo Scientific) and the size of the transcribed probes was verified by gel electrophoresis. Furthermore, the efficiency of the DIG-labeling was assessed by immunoblotting dilution series of DIG-labeled RNA probes according to the manufacturer's recommended protocol given in the DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics), with some modifications. Briefly, 2 µl of serially diluted RNA solution was spotted to a positively charged nylon membrane strip and cross linked using UV. Then, the strip was incubated in a blocking solution which consisted of 50% of Buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl), 0.3% TRITON®-X (Merck, Darmstadt, Germany) and 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in DEPC treated water, for 30 minutes. The blocking solution was then replaced by anti-DIG-AP-antibody (Roche Diagnostics) diluted 1:100 in blocking solution; the strip was allowed to incubate for 30 more minutes. Afterwards, it was rinsed two times in Buffer I diluted in equal volumes of DEPC-treated water for 15 minutes each, followed by equilibration in Buffer II (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM MgCl₂, final pH 9.5) for 10 minutes. For visualizing the spots, a color reaction was carried out by incubating the strip in a colorimetric substrate solution comprising of Buffer II, 0.45 mg/ml of 4-nitro blue tetrazolium chloride (NBT) and 0.175 mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics) in the dark until dark spots were observed.

2.2 Control samples for *in situ* hybridization

Fresh spleen and liver samples collected from five non-infected SPF chickens as well as an archived liver sample from *Histomonas meleagridis* infected chicken with liver lesions obtained from a previous infection experiment (Zahoor et al., 2011) were employed for establishing the method. Samples from non-infected SPF chicken were chosen on the basis of the respective mRNA expression confirmed by qRT-PCR in one of the non-infected birds. The tissues were fixed in 4% neutral buffered formaldehyde (SAV liquid production, Flintsbach, Germany) for 72 h at 4°C and preceded to routine histological tissue processing and paraffin embedding under RNase-free condition. The sample from the infected bird originated from 10 days post infection, a time point when expression of ChIFN-γ, ChIL-4 and ChIL-13 mRNA by qRT-PCR in liver was observed in a previous study (Powell et al., 2009).

2.3 *In situ* hybridization

Paraffin sections of 4 μm thickness were prepared using Microme HM360 rotary microtome (Microme, Walldorf, Germany), mounted on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and allowed to adhere overnight at 37°C. For ISH, the sections were first deparaffinized in Neo-Clear® (Merck), and rehydrated in a series of graded alcohols (100%, 96% and 70%), for 5 minutes in each solvent. They were then pre-treated with 2.8 $\mu\text{g}/\text{ml}$ of proteinase K (Invitrogen, Carlsbad, CA, USA) in 0.05 M Tris-HCl (pH 7.5), at 40°C for 30 minutes. Following this, rinsing and dehydration was performed in DEPC-treated water followed by 95% and 100% ethanol. After a brief period of air drying, the sections were covered with a hybridization mix composed of 50% formamid, 4x standard saline citrate buffer (SSC), Herring's Sperm DNA (500 $\mu\text{g}/\text{ml}$), 1x Denhardt's solution, 10% dextran sulfate, and the digoxigenin (DIG)-labeled RNA probe diluted in DEPC treated water. For obtaining an optimal staining of cytokine mRNA expressing cells, a varying concentration of RNA probes ranging from 5–1000 ng per milliliter of the hybridization mix were used. Finally, the slides were heated at 95°C, for six minutes, followed by a quick cooling on ice. Hybridization was allowed to continue overnight at 40°C, in a humidified chamber.

On the next day, post-hybridization stringency washes were performed in four steps: first by incubating the slides in 2x SSC at room temperature for 30 minutes, followed by RNase A (20 $\mu\text{g}/\text{ml}$) (Roche Diagnostics) treatment in a solution mix of 0.5 M NaCl, 5 mM Tris-HCl (pH 7.5) and 1 mM EDTA, at 40°C for 30 minutes. Further washing steps for two times in 1x and 0.1x SSC at room temperature for 10 minutes in each.

For immunological detection of the hybridized probes, the sections were first incubated in a blocking solution of 50% of Buffer I, 0.3% TRITON®-X and 5% normal goat serum in distilled water, for 30 minutes in a humidified chamber. The blocking solution was then replaced by anti-DIG-AP-antibody diluted 1:100 in the blocking solution mix, incubation was allowed for 1 hour at room temperature. Afterwards, post-immunological-hybridization wash and equilibration was performed in three steps. First unbound antibody was removed by washing the slides in 1 to 1 dilution of Buffer I in distilled water, two times, 15 minutes each and lastly equilibration in Buffer II for 10 minutes.

Chromogenic detection of the hybrid-immune-complexes was conducted by incubating the sections with colorimetric substrate solution composed of Buffer II, 0.45 mg/ml NBT, 0.175 mg/ml BCIP and Levamisol (240 $\mu\text{g}/\text{ml}$) (Sigma, Deisenhofen, Germany). Reaction was allowed in a humidified chamber under dark conditions. Coloring was monitored every 30 minutes until 2 hrs and 4 hrs, 6 hrs, 8 hrs and 16 hrs afterwards. After overnight incubation, the reaction was stopped in Tris-EDTA (pH 8.0) for at least 10 minutes. The slides were then rinsed in distilled water and briefly counterstained with Haematoxyline (Merck). They were mounted under cover slips (Menzel-Gläser) with Aquatex® aqueous mounting medium (Merck). The sections were inspected under Olympus BX53 microscope and documented with an Olympus DP72 camera (Olympus Corporation, Tokyo, Japan).

2.4 Quantitative reverse transcription polymerase chain reaction

To examine the expression of ChIFN- γ , ChIL-4, and ChIL-13 in non-infected tissue samples, a pilot qRT-PCR analysis of the cytokines was performed. Spleen and liver samples were stabilized, and total RNA was extracted as described in the probe generation work flow. Amplification and detection of specific products was performed using one step qRT-PCR with TaqMan chemistry. The reaction was performed on Agilent Mx3000 P qPCR system (Agilent Technologies, Santa Clara, CA, USA) using Brilliant III Ultra-Fast QRT-PCR Master Mix kit (Agilent Technologies). Previously published primers and probes were employed for amplification and quantification of ChIFN- γ (Rothwell et al., 2004), ChIL-4, ChIL-13 and 28S rRNA (Avery et al., 2004). The thermal profile was one cycle at 50°C for 10 min and 95°C for 3 min followed by 37 cycles at 95°C for 5 sec and 60°C for 20 sec. Fluorescence was detected and reported at each cycle during the 60°C step. 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 (R_n). The Ct values of the cytokines were normalized for every sample with the Ct value of the reference gene 28S rRNA by using the following formula: $Ct + ((N't - C't) \times S/S')$ where $N't$ is the mean Ct for 28S rRNA among all samples, $C't$ is the mean Ct for 28S rRNA in the sample, S and S' are the slopes of the regressions of the standard plots for the cytokine mRNA and the 28S RNA, respectively (Chrastek et al., 2014; Powell et al., 2009).

3 Results and Discussion

Digoxigenin labeled RNA probes designed to hybridize to ChIFN- γ , ChIL-4 and ChIL-13 mRNA were efficient to localize cells expressing the cytokines in spleen and liver. Cells expressing ChIFN- γ mRNA were stained at a probe concentration of 400 ng/ml whereas ChIL-4 and ChIL-13 mRNA expressing cells could be labeled at a probe concentration of 100 ng/ml. As illustrated in Figs. 1 and 2, all the probes showed distinctive and specific staining results, however, an overnight incubation of the color substrate was necessary to find clearly identifiable positively labeled cells. DIG-labeled sense RNA probes showed no signal. In addition, same tissue sections incubated only with DEPC-treated water in place of the probes were used as an additional negative control and showed no signal (data not shown).

In spleen sections (Fig. 1A), ChIFN- γ , ChIL-4 and ChIL-13 mRNA positive cells were mainly distributed in the periarteriolar lymphoid sheaths (PALS). Chicken spleen in general harbors various T-cell subtypes and the PALS in particular is enriched with CD3⁺ lymphocytes that mostly express CD4 (Oláh et al., 2014), one of the cellular sources of Th1/Th2 cytokines in naive organisms (Mosmann and Coffman, 1989). In liver sections (Fig. 1B), ChIFN- γ , ChIL-4 and ChIL-13 mRNA positive cells were present exclusively among the mononuclear cells surrounding the portal areas. These leukocytes in the liver which have the ability to express IFN- γ , IL-4 and IL-13 mRNA presumably represent similar lymphocyte populations as found in the spleen. Nonetheless, various cell types have been shown to be the physiological sources of these cytokines in the mammalian immune system. Double staining approaches using cell surface markers will be a prospective

approach to identify the cell subsets which express the mRNA of these cytokines in the avian tissue.

The expression of these cytokines in leukocytes of non-infected birds is somewhat similar to previous observations. Spontaneous secretion of ChIFN- γ protein was observed in unstimulated splenocyte cultures from some SPF birds (Prowse and Pallister, 1989; Weiler and von Bülow, 1987). Moreover, another study showed comparable levels and simultaneous expressions of ChIFN- γ , ChIL-4 and ChIL-13 mRNA in spleen tissues collected from non-infected birds by semi-quantitative RT-PCR (Degen et al., 2005). Karr et al. (1995) also observed *in vitro* murine Th0 cells that co-express Th1 (IFN- γ) and Th2 (IL-4) mRNA simultaneously, by ISH in cellular preparations.

In this study, the qRT-PCR assay substantiated the ISH staining by confirming the detection of all the target cytokines' mRNA in the non-infected spleen and liver tissues (Fig. 3). Nevertheless, the results obtained by qRT-PCR and ISH should not be quantitatively compared in a direct way due to the intrinsic difference in the detection system of both techniques. There is a likelihood of variation in the expression assays which can be either due to fewer presence of the target cell population in a structurally heterogeneous tissue sample (De et al., 2010; Rosa et al., 2013) or low expression of the mRNA in individual cells.

Furthermore, ISH was performed in *H. meleagridis* infected liver tissue sections. As shown in Fig. 2, ChIFN- γ and ChIL-13 mRNA positive cells were observed in inflammatory infiltrates comprising areas in the liver sections with lesions. Cells expressing ChIL-4 mRNA could not be detected (data not shown). In chickens, *H. meleagridis* can cause severe inflammation of the liver and the caecum following extracellular infiltration of the tissue. In this study, a marked cellular infiltration of both ChIFN- γ and ChIL-13 mRNA expressing cells were noticed. In an affected liver, increased levels of cytokine mRNA expression could reflect the elevated number of immune cells (Powell et al., 2009). In addition to T-lymphocytes, macrophages and heterophils are also known to infiltrate into the liver after infection with the protozoa (McDougald, 2008). In a relevant but not directly related study, investigating delayed type hypersensitivity, eosinophils have also been shown to modulate inflammatory responses in chicken (Abdul-Careem et al., 2008). As mentioned earlier, immunohistochemical and special stain approaches can be a potential area for further investigation to gain an insight on the cellular sources of the cytokines as well as cells infiltrating lesions.

The absence of ChIL-4 ISH signal in the infected liver sections could be due to the low expression level of the cytokine in this infection model. Using qRT-PCR, it was previously shown that IL-13 mRNA was the main Th2 marker cytokine expressed during *H. meleagridis* infection, not IL-4 which usually predominates a Th2 response in mammals (Powell et al., 2009). Infection experiments with *Ascaridia galli* or *Heterakis gallinarum* also revealed IL-13 as the dominantly expressed cytokine in chickens compared to IL-4 by semiquantitative RT-PCR and qRT-PCR (Degen et al., 2005; Schwarz et al., 2011a; Schwarz et al., 2011b).

Chromogenic ISH as a technique to study cytokine responses provides a unique approach by localizing cells expressing the cytokine genes. Through the application of counterstaining, lesions and cellular relationships associated with cytokine mRNA expressing cells and the general alteration of tissues in response to an infectious agent can be correlated. The technique also allows quantification of positively labeled cells and is generally applicable to computer-based imaging analyses such as TissueFAXS (TissueGnosticsGmbH, Vienna, Austria), by which leukocytes can be enumerated on the basis of labeling (Paudel et al., 2015). By using appropriate reagents and standardized protocols, multiple label co-analysis can be employed to identify the phenotype of the cytokine mRNA expressing cells. Apart from spleen and liver sections reported in this study, the technique may be adapted to other organs.

In conclusion, ISH for the detection of ChIFN- γ , ChIL-4 and ChIL-13 mRNA was employed and will be useful to show the role of these cytokines in the organ/tissue response to homeostatic or different infectious stimuli and their relation to physiological or pathological changes in tissue sections.

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Fig. 1.

ISH of ChIFN- γ (a), ChIL-4 (b) and ChIL-13 (c) mRNA in non-infected spleen (A) and liver (B) sections. Cytokine mRNA expressing cells were labeled in sections treated with antisense probes (panels a, b & c). Signals were observed in cells located mainly at the PALS of spleen and lymphoid infiltrations of liver (dark purple, shown in arrows). Sense probes used as negative control for ChIFN- γ (d), ChIL-4 (e) & ChIL-13 (f) showed no signal in same regions of the respective tissues (arrow heads). Bars = 100 μ m.

Fig. 2.
ChIFN- γ (a) and ChIL-13 (b) expressing cells in *H. meleagridis* infected liver tissue.
Positive cells were seen in the inflammatory infiltrates of sections treated with the antisense probe (arrows). Sense probes (c & d) showed no ISH signal in the inflammatory infiltrates (arrow heads). Bars = 100 μ m.

Fig. 3.

Relative gene expression of ChIFN- γ , ChIL-4 and ChIL-13 mRNA determined by qRT-PCR in non-infected liver and spleen tissues. The results are expressed in 37-Ct values after normalization with 28S rRNA.

Table 1

Primers used for RT-PCR to synthesize the cDNA for RNA probe generation.

RNA Target	Primer sequence (5'-3') ^a	Accession	Product size (bp)
ChIFN- γ	F: ACTTGCCAGACTTACAACCTTGR: CAATTGCATCTCCTCTGAGAC	NM_205149	488
ChIL-4	F:GTGCTTACAGCTCTCAGTG R:TGGAAGAAGGTACGTAGGTC	NM_001007079	318
ChIL-13	F: ATGCACCGCACACTGAAG R:TCAGTTTGCAGCTGTGGC	NM_001007085	417

^aF: forward primer; R: reverse primer.