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# Upregulation of chicken TLR4, TLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella* in vitro

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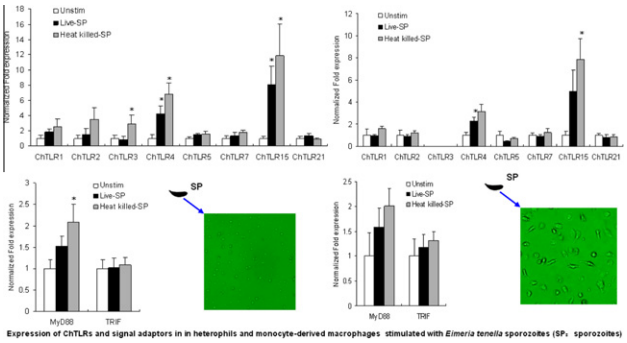
## HIGHLIGHTS

- The expression of ChTLR4, ChTLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella* for 2h were upregulated significantly.
- The heat-killed *E. tenella* sporozoites had ability of stimulating higher expression of ChTLR4, ChTLR15 and MyD88 than live sporozoites.
- ChTLR4 and ChTLR15 maybe involved in recognizing of *E. tenella*, by triggered MyD88-dependent signal pathway, and played a role in chickens infected with *E. tenella*.

## GRAPHICAL ABSTRACT




Expression of ChTLRs and signal adaptors in in heterophils and monocyte-derived macrophages stimulated with *Eimeria tenella* sporozoites (SP: sporozoites)

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## ABSTRACT

Coccidiosis, caused by *Eimeria* parasites, is a major parasitic disease responsible for great economic losses in the poultry industry. Toll-like receptor (TLR) family is one of the most important innate immune receptors, which involved in pathogen detection by initiating host responses, and it plays important roles in the reduction and clearance of pathogens. Very little information is available about the roles of chicken TLRs (ChTLRs) during *Eimeria tenella* infection. In the current study, mRNA expression of ChTLRs and associated signal adaptors in heterophils and monocyte-derived macrophages stimulated with *E. tenella* in vitro were measured by real-time quantitative polymerase chain reaction. The results showed that ChTLR4 and ChTLR15 expression were increased significantly in heterophils and monocyte-derived macrophages following live *E. tenella* sporozoites stimulation. The heat-killed *E. tenella* sporozoites stimulated higher expression of ChTLRs and signal adaptors than live sporozoites, the expression of ChTLR4, ChTLR15 and MyD88 in heterophils and monocyte-derived macrophages stimulated with heat-killed *E. tenella* sporozoites were up-regulated significantly than unstimulated cells. The results suggest that ChTLR4 and ChTLR15 are involved in response to *E. tenella* infection, and may operate in a MyD88-dependent manner for host defense.



## 1. Introduction

Avian coccidiosis, caused by multiple species of genus *Eimeria*, is one of the most common and expensive poultry diseases (Shirley et al., 2007). The economic losses due to coccidiosis worldwide were estimated to be more than three billion US dollars per annum (Williams, 1999). While conventional disease control methods have relied on prophylactic chemotherapy, alternative methods are needed due to increasing governmental restrictions on the use of commercial coccidiostats, and the continual emergence of drug resistant parasites in the field. Inoculation with anticoccidial vaccines is a practical alternative to drugs for coccidiosis control (Del et al., 2011). Since the first live, nonattenuated vaccine

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(Coccivac) was introduced in the USA, vaccines against avian coccidiosis had been available for more than 50 years. Nevertheless, the large-scale manufacture of live vaccines, especially attenuated vaccines, is expensive because oocysts usually have to be obtained from specific pathogen-free (SPF) chicken, purified from faeces and formulated to high quality. The long-term goal is to develop a new generation of vaccine based upon recombinant antigens. However, despite the years of investigation in identifying and characterizing parasite antigens, no successful approach in developing recombinant vaccines have been reported (McDonald and Shirley, 2009). In order to develop novel vaccines, we should pay more attention to the host-parasite interactions that lead to host resistance and make a better understanding on the mechanisms of immunity to *Eimeria* spp. infection.

There are two types of immune mechanisms, innate immunity and adaptive immunity, involved in the responses against *Eimeria tenella* infection (Dalloul and Lillehoj, 2006). Innate immunity is the first line of defense against invading pathogens (Medzhitov, 2001). During the early stage of infection, innate immune system of the host can rapidly detect and respond to protozoan parasite infection via innate immune receptors (Gazzinelli and Denkers, 2006). A recent paper has demonstrated that broiler breeders with an efficient innate immune response are more resistant to *E. tenella* (Swaggerty et al., 2011). The toll-like receptor (TLR) family is one of the most important innate immune receptors in vertebrates, which is responsible for recognizing conserved components of pathogens, typically called pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs, will trigger TLR signaling, including MyD88-dependent and TRIF-dependent pathways, and induce expression of cytokines such as interferons (IFNs), tumor necrosis factor (TNF) and IL-6 (Kawai and Akira, 2007; Hong et al., 2006). Ten chicken toll like receptors (ChTLRs) have been identified, and five of them (ChTLR2a, 2b, 4, 5 and 7) have clear orthologs to those found in mice and humans. ChTLR15 is unique in the avian species (Brownlie and Allan, 2011; Temperley et al., 2008). However, the previous studies have focused on the adaptive immune responses to *Eimeria*, only a few reports mentioned the relationship of innate immunity and *Eimeria* infection (Sumners et al., 2011; Zhang et al., 2012). It was reported that ChTLR3, ChTLR4 and ChTLR15 were highly expressed during *E. praecox* infection in mucosa of the duodenum and jejunum (Sumners et al., 2011), and more recently, ChTLR1La, ChTLR4, ChTLR5, ChTLR7 and ChTLR21 were found highly expressed in the cecum of three-week-old SPF chickens infected with *E. tenella* (Zhang et al., 2012). However, the studies above were performed in vivo, and the number of bacteria including *Salmonella typhimuriu* (Baba et al., 1993; Baba et al., 1992), *Salmonella enteritidis* (Qin et al., 1995) and *Clostridium perfringens* (Baba et al., 1992; Dykstra and Reid, 1978), which adhesion to the cecal mucosal surface, may affect the innate immune responses to *E. tenella*.

To our knowledge, the role of innate immunity induced by innate immune cells with *E. tenella* stimulation in vitro has not yet been investigated. Chicken heterophils and macrophages are innate immune cells, and are of great importance for the clearance and destruction of both intracellular and extracellular pathogens (Dalloul and Lillehoj, 2006; Gordon and Taylor, 2005; He et al., 2003; Kogut et al., 2001; Kogut et al., 2005; Swaggerty et al., 2003, 2011). The determination of the initial events associated with innate immune response induced by heterophils and macrophages to *E. tenella* stimulation is of great importance for the understanding of the innate immune mechanism against coccidiosis. The objectives of this study were to determine the ChTLRs and signal adaptors associated with *E. tenella* stimulation in heterophils and monocyte-derived macrophages isolated from chickens.

## 2. Materials and methods

### 2.1. Experimental animals

Fourteen indigenous 2 months old village chickens were obtained from local farmers in Rongchang county, Chongqing, and rear under clean condition. The chickens were given cold boiled water and fed without any anti-coccidial drugs. *Eimeria* oocysts in feces were detected by flotation method with saturated salt to make sure that the chickens were free from any *Eimeria* spp. infection before blood collection.

### 2.2. Purification of E. tenella sporozoites

Sporozoites were isolated and purified from sporulated oocysts of *E. tenella* (Rongchang strain) as previously described (Dulski and Turner, 1988). Briefly, the sporulated oocysts were washed with sterilized distilled water (1200 × *g*, 8 min), and sterilized in 8% sodium hypochlorite for 10 min. The sporulated oocysts were washed in sterilized distilled water (1200 × *g*, 8 min) till the sodium hypochlorite were removed, and were resuspended in ice-cold PBS. The oocysts were broken by tissue homogenizer. Breakage was observed by microscope at 5 min intervals till roughly 70% of the oocysts were broken. The suspension was pelleted at 2000 × *g* for 10 min. The pellet was resuspended in 50% Percoll (Sigma Chemical Co. St Louis, MO), and the sporocysts were pelleted at 10000 × *g* for 10 min. The pelleted sporocysts were resuspended in the excysting solution containing 0.25% trypsin, 4% taurocholate and 10 mM $MgCl_2$, and incubated for 45 min at 41 °C. The sporozoites were pelleted, washed in PBS, then resuspended in 55% Percoll and pelleted at 10000 × *g* for 10 min. Resuspended the sporozoites in PBS and divided into two 1.5 ml microfuge tubes. Sporozoites in the first microfuge tube were killed in boiling water for 5 min, and sporozoites in the other tube were without any treatment. The sporozoites were pelleted at 2000 × *g* for 10 min, and resuspended in RPMI1640 (Gibco Life Technologies, Paisley, Scotland, UK) (with 100 U/ml ampicillin and 100 μg/ml streptomycin). The heat-killed and live sporozoites were pelleted at 10000 × *g* for 10 min, then resuspended in RPMI1640 again and adjusted to a concentration of $8 \times 10^6$ sporozoites/ml. The sporozoites were stored at 4 °C until use.

### 2.3. Isolation and stimulation of heterophils

Avian heterophils were isolated and purified from the peripheral blood following the procedure as previously described (Kogut et al., 2005), and with a few modifications. Briefly, disodium ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood was mixed with 1% methylcellulose (25 centiposes; Sigma Chemical Co., St Louis, MO) at a 1.5:1 ratio and centrifuged at 25 × *g* for 10 min. The serum and buffy coat layers were retained and suspended in $Ca^{2+}$, $Mg^{2+}$-free Hanks' balanced salt solution (HBSS, 1:1; Sigma Chemical Co. St Louis, MO). The suspension was layered over a discontinuous Ficoll- Histopaque (Sigma Chemical Co. St Louis, MO) gradient (specific gravity 1.077 g/mL over specific gravity 1.119 g/mL), and centrifuged at 250 × *g* for 30 min. After centrifugation, the heterophils in the 1.077/1.119 interfaces and 1.119 band were collected and washed twice with RPMI 1640 medium and resuspended in fresh RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Gibco Life Technologies, Australia), 100 U/ml ampicillin and 100 μg/ml streptomycin. Cell viability was determined by trypan blue exclusion (Fig. 1). The purity of the heterophil suspensions was assessed by microscopic examination of heterophil smears by Wright staining. Heterophil preparations