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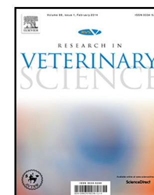

Administration of TLR7 agonist, resiquimod, in different types of chicken induces a mixed Th1 and Th2 response in the peripheral blood mononuclear cells

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ABSTRACT

This study evaluated the variation in immune response in peripheral blood mononuclear cells (PBMCs) of broiler, White Leghorn (WL) and Kadaknath breeds of chicken following administration of TLR7 agonist, resiquimod (R-848). Expression of different immune related genes viz., interferon-β (IFN-β), IFN-γ, IL-1β, IL-4, TLR7 and iNOS was assessed by quantitative real time PCR over a period of 24 h. The results indicated that there was a significant up-regulation in the relative expression of immune response genes post R-848 administration ($P < 0.01$). In conclusion, the transcriptional expression of IFN-β, IFN-γ, IL-1β, IL-4, iNOS and TLR7 genes in the PBMCs was significantly up-regulated over 24 h in broiler, WL and Kadaknath breeds of birds after the administration of R-848. Overall, R-848 induced a mixed Th1 and Th2 response in PBMCs of chicken origin *ex vivo*.

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Toll-like receptors (TLRs), a type of pattern recognition receptors (PRRs), are evolutionarily conserved membrane-bound and germ-line encoded receptors present in a wide range of species from insects to vertebrates. TLRs recognize pathogen-associated molecular patterns (PAMPs) on micoorganisms (Akira, 2001) and induce innate immunity in host cells through activation of transcription factors for production of pro-inflammatory cytokines, chemokines, type I interferons (IFNs), reactive oxygen and nitrogen intermediates. In addition, TLRs interlink innate and adaptive immune responses through activation of co-stimulatory molecules (Janeway and Medzhitov, 2002). Agonists of TLRs have been investigated against different diseases and cancers as adjuvants or stand-alone agents, and, several agonists are currently under clinical trials (Chaung et al., 2012; Chen et al., 2007; Yu et al., 2012). Presently, 10 members of TLR family have been identified in chicken (*Gallus gallus*) viz., TLR1La, TLR1Lb, TLR2a, TLR2b, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 (Chen et al., 2013). The nucleic acid recognizing TLRs is known to induce antiviral activity and cognate agonists are chiefly employed against viral diseases in animal models (Diebold et al., 2004; Lee et al., 2003; Lund et al., 2004). TLR7 plays a crucial role in antiviral immunity by recognizing viral-derived ssRNA especially enriched with GU (Guanosine-Uridine) or poly-U rich sequences, up-regulating type I IFNs and pro-inflammatory cytokines, and inducing co-stimulatory molecule expression (Heil et al., 2004; Hemmi et al., 2002; Kawai and Akira, 2010; Yamamoto et al., 2002).

Imidazoquinolines such as imiquimod (R-837), resiquimod (R-848) and loxoribine are known synthetic TLR7 agonists. Several studies *in vitro* have confirmed the actions of these compounds in inducing immune response genes like TNF-α, Type I IFNs, IFN-γ, IL-6, IL-12 etc. in a wide range of cells and activation of cells such as B cells, dendritic cells (DCs) and PBMCs (Bekeredjian-Ding et al., 2005; Hackstein et al., 2011; Hayashi et al., 2003; Paul et al., 2012; Sabbatucci et al., 2011). In addition, TLR7 agonists are currently used as prophylactic agents in different diseases and as anticancer agents due to simultaneous activation of several immune pathways (Hemmi et al., 2002; Smits et al., 2010). Resiquimod, a more potent agonist than imiquimod, has been reported to have antiviral and antitumor properties in animal models and shown to induce endogenous cytokine production via TLR7 in mouse and TLR7 and 8 in humans (Dockrell and Kinghorn, 2001; Harrison et al., 1988; Hemmi et al., 2002; Jurk et al., 2002; Sidky et al., 1992). Despite the fact that the effects of R-848 have been widely studied in mammals, its effect

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Table 1
Details of quantitative real time PCR primers.

Target gene	Primer sequence (5′-3′)	Product size (bp)	Reference
IFN-γ	F: TGAGCCAGATTGTTTCGATG R: CTTGGCCAGGTCCATGATA	152	Nang et al., 2011
IFN-β	F: GCTCACCTCAGCATCAACAA R:-GGGTGTTGAGACGTTTGGAT	187	Nang et al., 2011
IL-1β	F: GGATTCTGAGCACACCACAGT R: TCTGGTTGATGTCGAAGATGTC	272	Nang et al., 2011
iNOS	F: AGGCCAAACATCCTGGAGGTC R: TCATAGAGACGCTGCTGCCAG	371	Sundaresan et al., 2005
β-actin	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	110	Fan et al., 2013
IL-4	F: GGAGAGCATCCGGATAGTGA R: TGACGCATGTTGAGGAAGAG	186	Nang et al., 2011
TLR7	F: AGAGACTGGCTTCCAGGACA R: CAGCTGAACATACCGGGACT	219	Nang et al., 2011

in chicken has not been explored. Only a few studies have been done in chicken model *in vitro*. Recently, we demonstrated the R-848 induced expression of IFN-β, IFN-γ, IL-4, IL-1β and iNOS transcripts *in vitro* in chicken PBMCs (Ramakrishnan et al., 2014). Hence, the present study was undertaken to investigate the induced immune response in broiler, White Leghorn (WL), indigenous Kadaknath breeds. following administration of R-848 in the PBMCs.

We used three week old birds (n = 20, per breed) of broiler, White Leghorn (WL) and Kadaknath breeds after getting approval from the institute animal ethics committee of Indian Veterinary Research Institute, Izatnagar. Commercially available TLR7 agonist, R-848 (InvivoGen, USA), was injected (50 μg/bird) intramuscular and blood sampling (1 ml/bird) was done randomly at 0, 4, 12 and 24 h post-treatment (6 birds at each interval) with heparin @ 20 IU ml^{-1}. Isolation of PBMCs was done as described previously (He et al., 2003). Cell viability was checked by trypan blue dye exclusion method and around 1×10^6 viable cells from each sample was preserved in 1 ml of Ribozol (AMRESCO®, USA) at −80 °C. Total RNA was extracted from PBMCs as per manufacturer's guidelines and eluted in 20 μL nuclease free water (NFW). The purity and quantity of RNA were determined by Nano-drop® Spectrophotometer Analyzer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per manufacturer's protocol and stored at −20 °C until use.

The effect of R-848 on different immune response genes viz., IFN-β, IFN-γ, IL-1β, IL-4, iNOS and TLR7 in PBMCs (*ex vivo*) was analyzed by quantitative real-time PCR in CFX96 Touch™ (Bio-Rad, USA) system following SYBR green chemistry (Qiagen, The Netherlands). The earlier reported oligonucleotide primers (Table 1) were used in this study. Each sample was run in duplicate and the total reaction volume was 20 μL containing QuantiTect SYBR qPCR master mix (2X) 10 μL, template cDNA 2 μL (1:10 diluted with NFW), gene specific forward and reverse primer (0.5 μL each, 20 picomole) and nuclease free water 7 μL. Real-time PCR conditions were one cycle 95 °C for 5 min, 40 cycles of 94 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s, and 94 °C for 30 s one cycle, then 55–94 °C ramp for melt curve analysis to check the amplicon specificity. Two independent experiments were conducted to check the repeatability and for each gene, the cycle threshold (Ct) value at each time point was normalized to the respective endogenous control, β-*actin*, and the results were expressed as fold change in mRNA expression relative to the control (Pfaffl, 2001). Time effect within a breed was analyzed by orthogonal contrast model (0 h versus other time points). Fisher's LSD was used as post-hoc test. Significance was set at 5%. Data are presented as mean ± SE (n = 6). SPSS version 20 was used for data analysis.

The results of time dependent gene expression within a breed are depicted in Fig. 1. Overall, R-848 injection significantly ($P < 0.01$) up-regulated the expression of IFN-β in all three breeds of birds than that of control (0 h). The expression of IFN-γ was also significantly ($P < 0.01$) up-regulated in all the three breeds at 24 h post-stimulation than that of control. In addition, IFN-γ was significantly elevated both at 4 and 12 h in WL birds. IL-1β expression was significantly ($P < 0.01$) up-regulated at all the intervals in broiler birds; however, it was up-regulated at 4 and 24 h in WL and at 12 and 24 h in Kadaknath birds after administration of R-848 in comparison to control. Expression of iNOS was significantly ($P < 0.01$) up-regulated at 4, 12 and 24 h in both WL and Kadaknath than that of control (0 h); while it was significant ($P < 0.01$) only at 24 h interval in broiler birds. Similarly, IL-4 expression was significantly up-regulated at 4, 12 and 24 h in WL ($P < 0.01$) and Kadaknath ($P < 0.05$) while the same was significant at 12 ($P < 0.05$) and 24 h ($P < 0.01$) in broiler birds. The expression of TLR7 was significantly ($P < 0.01$) up-regulated in all the three breeds at 4 h post-stimulation and at 12 h post-stimulation only broiler breed showed significant ($P < 0.05$) up-regulation. Kadaknath birds had shown maximum TLR7 expression at 24 h post-stimulation, while at the same period, broiler ($P < 0.01$) and WL ($P < 0.05$) birds also showed significant up-regulation.

The present study was aimed to ascertain the variation in immune related genes expression post R-848 administration in the PBMCs of three breeds of chicken *ex vivo*. Apart from typical broiler and layer breeds, Kadaknath was chosen as it is one of the popular and well recognized native chicken breeds of India known for its black color meat (Arora et al., 2011). Several reports have shown the

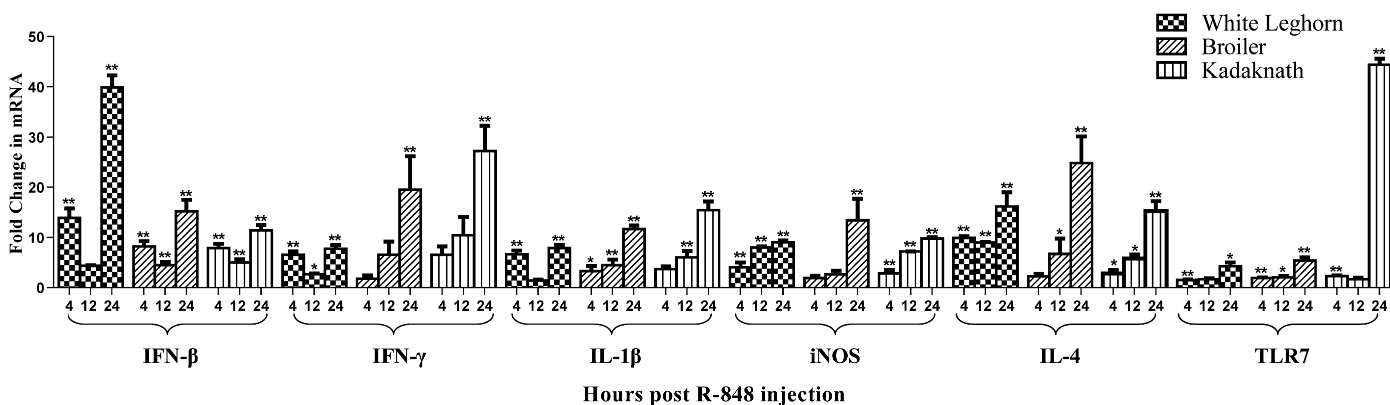

Fig. 1. Relative mRNA expression of immune related genes in the PBMCs of different types of chicken following administration of R-848. Time dependent variation was analyzed within a breed by one-way ANOVA. Each bar represents fold change mean ± SE. ** indicates significance at $P < 0.01$ and * indicates significance at $P < 0.05$ as compared to control (0 h).

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