Original Article

Enhancement of cell-mediated immune response in chickens by combination of TIR-TLR7 with inactivated Newcastle disease vaccine

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ABSTRACT

Introduction: Live and inactivated vaccines are wildly used against Newcastle disease (ND) which is a highly contagious and acute viral infection of domestic and wild birds. A higher and prolonged immune response is required to improve the control of the disease. The aim of this study was to evaluate the potential of the conserved TIR domain of an immune regulatory protein TLR7 (*i.e.* TIR-TLR7) as a biological adjuvant in enhancing cell-mediated immunity in vaccinated chickens against the inactivated ND virus (NDV) V4 strain antigen. **Methods:** NDV V4 strain was propagated in chicken embryonated SPF eggs, tittered and then inactivated by formalin. The amount of 10 µg of TIR-TLR7 was mixed with the NDV antigen before intramuscular administration. Fifty SPF chickens were divided in A-E groups (n=10), consisted of negative control, TIR-TLR7, inactivated NDV antigen, TIR-TLR7/inactivated NDV antigen in prime, and the same regimen in boost platform. The blood samples were collected at week intervals up to 6 weeks post-vaccination. Humoral response was measured by detection of specific NDV antibody titer using the HI test. The cell-mediated immunity was evaluated by measuring lymphocyte proliferation in splenocytes cell culture using MTT. **Results:** All immunized chickens with TIR-TLR7/inactivated NDV antigen had significant ($P < 0.05$) cell-mediated and HI responses to NDV compared to the control groups. No statistically-significant difference was observed between the prime and boost trials. **Conclusion:** The results indicated that the combination of TIR-TLR7 and inactivated NDV antigen gave a strong immune response at both the humoral and the cellular levels.

KEYWORDS: Newcastle disease, inactivated vaccine, TIR-TLR7, immune response.

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral infection of many avian species caused by a member of *Paramyxoviridae* family [1]. An extensive vaccination program including live and oil-emulsion inactivated vaccines with good management and strict biosecurity are implemented for commercial broiler and layer poultry farms, especially in ND endemic areas [2, 3]. Despite the advantages of inactivated whole virus vaccines such as safety, provoking strong humoral antibody levels, high coverage within the vaccinated populations and maintaining the immunity against ND virus (NDV), they require an adjuvant to enhance and modulate the immunogenicity of the antigen [4]. In order to enhance the potential efficacy of existing inactivated vaccines, to provide long-term protection, and to avoid the deleterious side-effects of traditional adjuvants, the use of alternative adjuvants should be emphasized. Different cytokines and immune regulatory proteins like chicken IL-18 and chicken IL-12 have been examined for their adjuvant effect on inactivated vaccines in chickens [5-7]. Toll-like receptors (TLRs) as immune regulatory proteins play a critical role in linking innate and adaptive immune responses and also participate in the functional polarization of naive lymphocytes during antigen presentation [8, 9]. Upon stimulation, TLRs support antigen presentation and promote the secretion of proinflammatory cytokines and interferons (IFNs) [8]. Among the TLRs, TLR7 and TLR9 are selectively expressed on plasmacytoid DCs (pDC) surface, known as IFN-producing cells and regulate the functions of adaptive immune responses [10]. The TLR signaling cascade is activated through an interaction of the cytoplasmic region of the interleukin-1 (IL-1) receptor (TIR) domain of TLR with TIR domain-containing adaptor proteins. The TIR domain has an important role in the initiation of TLR signaling, leading to activation of NF-κB and MAPK, and stimulation of type 1 IFN-inducible genes in DCs [8, 11].

In a more recent study, an addition of the conserved TIR domain of TLR7 (TIR-TLR7) has been applied to recombinant VP2 infectious bursal disease virus vaccine [12]. The results have indicated that TIR-TLR7 can significantly increase the

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humoral immune responses to the peptide vaccine, compared to chickens vaccinated with recombinant VP2 (11). This study was carried out to investigate the effects of TIR-TLR7 on the humoral and cell-mediated immunity (CMI) responses at the time of vaccination with inactivated NDV vaccine.

MATERIALS and METHODS

Virus antigen preparation

NDV V4 strain (GenBank accession No. MH603396) was propagated in allantoic cavities of 9- to 11-day-old embryonated specific pathogen free (SPF) chicken eggs (Razi Institute, Karaj, Iran), according to the OIE standard protocol [13]. The infected allantoic fluid was harvested and clarified by centrifugation at 8,000 × g for 20 min at 4°C. The prepared antigen was titrated by hemagglutination assay (HA) using 1% (v/v) chicken red blood cells [13]. At the titer of $10^{10.3}$ mean embryo infectious doses (EID50/ml), the harvested antigen was inactivated with a 0.1% final concentration of formalin (Merck) in PBS, following incubation for 16 h at 37°C. Complete inactivation of the virus was confirmed through 3 passages in 10-day-old embryonated SPF chicken eggs by observing the survival rate and assaying hemagglutination inhibition (HI) titer.

TIR-TLR7 oligonucleotide adjuvant synthesis

The cDNA encoding TIR-TLR7 containing the IL-1 receptor was ordered to be synthesized (Metabion international AG, Germany) according to the Ebrahimi et al study [12]. The amount of 10 µg of the oligonucleotide was used as an adjuvant in the immunization trial.

Chicken immunization trial

Three-week-old SPF chickens (Razi Institute, Karaj, Iran) were divided into 5 groups (10 birds/each). The chickens were cared according to the Council for International Organization of Medical Sciences for Biomedical Research involving animals guideline [14]. Three groups were served as controls and received only TIR-TLR7 or inactivated NDV antigen or were left without antigen/adjuvant. Two groups received TIR-TLR7/inactivated NDV antigen in a prime and booster platform as mentioned in Table 1. Each group of birds was housed separately in isolation units and maintained under management conditions with feed and water *ad libitum*. The amount of 0.1 ml of samples, regarding the groups, was administrated intramuscularly.

Table 1. The grouping of SPF chickens used in this study and their administration trials

Groups	Administration
A	Negative control
B	TIR-TLR7 adjuvant
C	Inactivated NDV antigen
D	Inactivated NDV antigen and TIR-TLR7 adjuvant prime
E	Inactivated NDV antigen and TIR-TLR7 adjuvant boost, 14 days after the prime

Humoral response monitoring by HI assay

Blood samples were collected from each group at two-week intervals until the end of the trial (6 weeks). The levels of ND antibodies were measured using the HI test in collected sera. Briefly, 50 µl two-fold dilutions of each serum in PBS were prepared in round-bottomed 96-well plates and the same amount of 4 HAU of NDV was added to each well and incubated at room temperature. Then cRBCs were added and incubated at 4°C. The reciprocal of the last serum dilution showing complete inhibition of hemagglutination activity was considered as the HI titer expressed as the $\log_2$.

Lymphocyte proliferation assay

Chickens were euthanized, 6 weeks after the first administration. Spleen from each group was aseptically removed and minced. Splenocytes were prepared at a concentration of 1×10^6 cells/ml in RPMI-1640 (Sigma, Aldrich) and 100 µl cells/well were transferred into flat-bottomed 96-well plates. Spleen cells were stimulated with equal volumes of medium supplemented with fetal calf serum containing 10^6 EID_{50}/mL of whole inactivated V4 NDV strain and 20 µg/mL of phytohemagglutinin (PHA; Sigma, Aldrich). Negative controls received 100 µl RPMI 1640 medium only. After 72 h of incubation, 20 µl fresh MTT (3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide, thiazolyl-blue) were added for a final incubation of 4 h. The supernatant from each well was carefully removed and 100 µl of dimethyl sulfoxide (DMSO) was added to solubilize MTT formazan crystals. The absorbance was measured at a wavelength of 570 nm after incubating the plates for 30 min at 37°C. Proliferation responses were calculated by mean OD of stimulated splenocytes-mean OD of blank/mean OD of unstimulated splenocytes, expressed as stimulation index (SI).

Statistical analysis

The data were statistically analyzed using one-way ANOVA test (SPSS ver. 11). The difference was considered significant at a value of $P < 0.05$.

RESULTS

The embryonated SPF chicken eggs injected with the formalin-treated NDV were survived after 120 h. No HA titer was detected, indicating that the formalin-treated virus was completely inactivated after 3 passages in chicken eggs. The level of antibody production increased rapidly in groups that were administered by inactivated NDV antigen and TIR-TLR7 adjuvant at their both prime and boost. The treatment groups D and E showed a significant increase in HI antibody titer from the second week post-vaccination which lasted until the end of the trial. The titers were still detectable at a high level, ranging from 5.14 to 6.51 log2. The NDV antibody titer was negative in the control groups A and B ($P < 0.05$; Fig. 1).

CMI derived from the inactivated NDV antigen with and without TIR-TLR7 was evaluated by their ability to activate chicken splenocytes. No stimulating effect was observed with the inactivated viral antigen; however, all vaccinated chickens had specific CMI responses to NDV. The results demonstrated that chickens receiving TIR-TLR7 had significantly-increased CMI against NDV, compared to inactivated NDV antigen group (Fig. 2).

The SI equal to or greater than 2 was considered as significant proliferation evidence. The mean of SI levels in the mitogen-stimulated cell culture obtained from group E was significantly higher, compared to the same culture that was obtained from the inactivated NDV antigen group ($P < 0.05$). There was no significant difference in the levels measured between stimulation with either prime or boost vaccinations.

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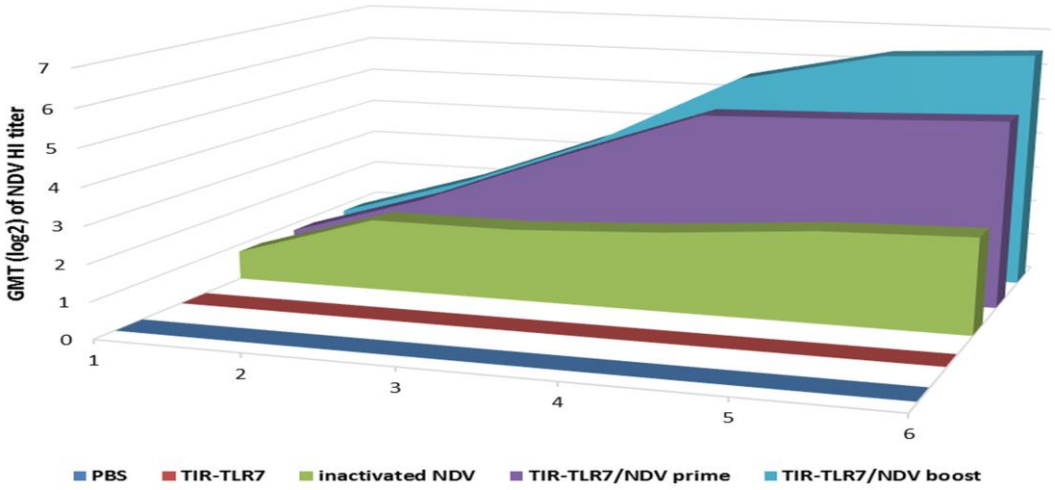

Fig. 1. Post-vaccination geometric mean antibody titer (log_2) of chickens vaccinated by inactivated NDV V4 antigen with and without TIR-TLR7 at different weeks post-vaccination. A single dose of TIR-TLR7/inactivated NDV antigen induced the HI titers significantly higher than titers from the inactivated NDV antigen control group ($P < 0.05$). HI titers increased to an average 6.51 log2 after the boost, indicating TIR-TLR7 improved the NDV antibody response. The PBS and TIR-TLR7 control groups did not show HA activity and had similar results.

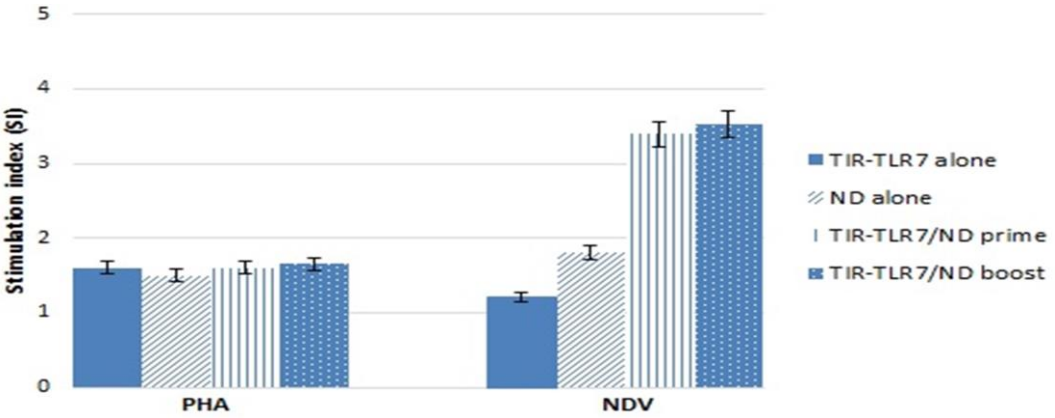

Fig. 2. The mitogenic response of splenocytes of SPF chickens, stimulated with inactivated NDV V4 antigen with and without TIR-TLR7. Spleens from the treated and the control chickens were stimulated by the antigen and phytohemagglutinin (PHA). The cell proliferation levels were then detected by MTT. The monitoring of lymphocyte proliferation showed significant differences at *P* value less than 0.05.

DISCUSSION

It has been suggested that using the inactivated vaccines can give a broad protection against circulating NDVs in ND enzootic regions. Regardless of the necessity of the antigenic homology between the used vaccine and the field strains and adequate vaccination practices in the creation of appropriate immunity [2, 15, 16], the inactivated vaccines should be mixed with an adjuvant to confer strong protection against NDVs. Here, we examined the potential TIR-TLR7 adjuvant effects on the induction of specific immune responses against NDV in SPF chickens. Structurally, the TIR domain has 3 conserved sequence boxes. Box 3 located at the carboxyl terminus of MyD88 TIR domain is essential for IL-1β-induced signaling by formation of a signaling platform leading to the expression of IFN-stimulated genes, which have role in the initiation of innate and adaptive immune responses [11]. The preferentially trigger type I IFNs secretion by TLR7 depends on the TIR-TIR interaction mediated by NF-κB in pDCs [10, 17]. The signaling pathway directs the immune responses towards Th1 and Th2 immune responses through the activation of NF-κB, phosphorylation of IFN-inducible genes, as well as up-regulation of interleukins and type I IFNs [8, 9, 17]. The activated signal transduction pathway induces the transcription of NF-κB which involve in regulation of cytokine/chemokine genes expression by DCs. Activation of the transcription factor is critical for the subsequent development of adaptive immune responses [18]. Thus, any component that activates TLR signaling pathways can probably be considered as an adjuvant in combination with a vaccine antigen. Our results revealed that chickens vaccinated with NDV/TIR-TLR7 had higher CMI and HI antibody responses against NDV, compared to the group immunized with inactivated NDV alone. The induction of higher levels of humoral antibody responses after administration of IBDV/TIR-TLR7 may be in accordance with the ability of TLR7 in stimulation of B cells.

Consistent with this, numerous studies have shown that immune stimulator factors such as chIL-18, chIFN-α and chIL-12 modulate both CMI and humoral immunity in chickens when co-administrated with an avian vaccine [19-22]. Functionally, the co-stimulatory molecules for DCs could stimulate B cells differentiation, proliferation, maturation and maintenance and they could also stimulate T cell development, proliferation and

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homeostatic regulation. For example, it has been shown that ChIL-18 could increase CD4+ and CD8+ T cell populations, leading to production of IFN-γ, that is critical to CMI and IL-4 that enhances antibody production in chickens following co-immunization with NDV vaccine [19-22].The synergistic effects of IL-12 and IL-18 have been shown to result in effective differentiation of Th1 cells. It has been suggested that the deriving appropriate immune responses can be due to effective DCs stimulation to secrete IL-12 and IFN-γ [23].

In addition to TLR7-specific activating DCs that preferentially trigger IFNα secretion, TLR7 stimulates B cells to secrete immunoglobulin and produces IL-6 and TNFα. The activated TLR signaling which originates from the cytoplasmic TIR domain, regulates B-cell functions which results in isotype switching of immunoglobulin and promoting antibody response that influence humoral immune responses [13, 17]. The success of NDV vaccination is monitored by rising in antibody titer related to a strong humoral immunity. In our study, TIR-TLR7/inactivated NDV antigen could induce significant HI titers, compared to the inactivated NDV antigen control group which indicated that TIR-TLR7 has improved the NDV antibody responses.

Evidence shows that the activated TLR signaling also affects the upregulation of co-stimulatory molecules on DCs that are essential for T-cell activation and can enhance their ability to stimulate CMI responses [17, 24]. In immunization against NDV, the CMI contribute to decreased viral shedding and clearance due to the intracellular phase of NDV [9, 8]. This response is an important factor to show the development of protection in vaccinated chickens. The response detected after vaccination with an inactivated NDV vaccine, is not robust and sufficient to protect against virulent NDV challenge [8]. Thus, to derive the appropriate immunological memory responses, the vaccine requires companion immunomodulation agents to enhance the immunogenicity and protection against the virus infection.

The strength of TIR-TLR7 was its positive effect on inducing CMI responses in addition to the humoral antibody responses. Our results demonstrated that chickens receiving TIR-TLR7 mixed with inactivated NDV, had significantly increased CMI, compared to inactivated NDV vaccinated chickens. The raise was also detected in a group of chicken that had received TIR-TLR7 and inactivated NDV once. All chickens vaccinated with TIR-TLR7 and inactivated NDV antigen had specific CMI responses which developed HI antibodies. We observed a correlation between CMI value and HI antibody production after TIR-TLR7/inactivated NDV antigen vaccination. Our data suggest TIR-TLR7 can function as a potential vaccine adjuvant which is more effective at generating both humoral and CMI responses to inactivated NDV antigen. Further studies are needed to understand the detailed adjuvant activity of TIR-TLR7 mechanism.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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