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# *In ovo* administration of Toll-like receptor ligands encapsulated in PLGA nanoparticles impede tumor development in chickens infected with Marek's disease virus

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ABSTRACT

One of the economically important diseases in the poultry industry is Marek's disease (MD) which is caused by Marek's disease virus (MDV). The use of current vaccines provides protection against clinical signs of MD in chickens. However, these vaccines do not prevent the transmission of MDV to susceptible hosts, hence they may promote the development of new virulent strains of MDV. This issue persuaded us to explore alternative approaches to control MD in chickens. Induction of innate responses at the early stage of life in the chicken may help to prevent or reduce MDV infection. Further, prophylactic use of Toll-like receptor ligands (TLR-Ls) has been shown to generate host immunity against infectious diseases. In this regard, encapsulation of TLR-Ls in Poly(D, L-lactic-co-glycolic acid) (PLGA) may further enhance host responses by controlled release of TLR-Ls for an extended period. Hence, in the current study, protective effects of encapsulated TLR4 and TLR21 ligands, LPS and CpG, respectively, were investigated against MD. Results indicated that administration of encapsulated CpG and LPS first at embryonic day (ED) 18, followed by post-hatch at 14 days-post infection (dpi) intramuscularly, diminished tumor incidence by 60% and 42.8%, respectively at 21dpi compared to the MDV only group. In addition, analysis of cytokine gene profiles of interferon (IFN)-α, IFN-β, IFN-γ, inducible nitric oxide synthase (iNOS), interleukin (IL)-1β, IL-18 and IL-10 in spleen and bursa of Fabricius at different time points suggests that TLR-Ls possibly triggered host responses through the expression of IL-1β and IL-18 to reduce tumor formation. However, further studies are needed to explore the role of these pro-inflammatory cytokines and other influencing elements like lymphocytes in the hindrance of tumor development by TLR-Ls treatment in chickens.



## 1. Introduction

Marek's disease virus (MDV) infects chickens via the respiratory route and causes Marek's disease (MD) which is characterized by T cell lymphoma and immunosuppression. Following infection, MDV spreads to lymphoid organs and causes lysis of B and T cells. This initial cytolytic phase, which takes place around 2-7 days post-infection (dpi), is followed by the latent phase which occurs around 7-10dpi [1]. In the next phase, which is the late cytolytic and immunosuppressive phase, MDV is reactivated from latency and continues to the transformation and proliferation phase which leads to tumor formation in internal organs and skin. Further, the infectious form of MDV produced in the feather follicular epithelium is shed into the environment with feather dander and transmitted by inhalation of dust particles.

Marek's disease is currently controlled by vaccination. However, vaccines are not able to control MDV shedding. Both innate and adaptive immune mechanisms are involved in control of MDV infection. The innate components of the immune system promptly act against MDV infection and activate adaptive immune responses. Toll-like receptors (TLRs) are an indispensable part of the innate mechanisms. Similar to other herpesviruses [2], MDV may also be detected by TLR2, TLR3, TLR7 and TLR21 (the avian

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counterpart of TLR9). An increased expression of TLR3 and TLR7 in the lungs of MDV-infected chickens supports this notion [3]. Similar to mammalian models, TLR-Ls such as CpG and lipopolysaccharide (LPS), which is detected by TLR4, can trigger innate defense mechanisms *in vitro*, *in ovo* and *in vivo* in chickens [4–6] and elicit host responses against MDV in chickens [7,8].

Enhancement of innate responses during the neonatal period may provide improved immune protection in chickens. Young chicks are exposed to MDV in the first few days of life, therefore, it is important to confer early protection prior to maturation of adaptive immunity. In this regard, previous studies have revealed that *in ovo* administration of TLR-Ls provide protective immunity and control replication of microbial pathogens such as infectious laryngotracheitis virus [9], infectious bronchitis virus [10] and *Escherichia coli* [11]. However, no study has investigated the *in ovo* administration of TLR-Ls conferring protective immunity against MDV in young chicks.

There are limitations to the use of TLR-Ls as antimicrobial agents or adjuvants, such as their short half-life and rapid elimination from the body which can reduce their efficacy [12]. To overcome these limitations, TLR-Ls can be encapsulated into poly(D, L, lactic-co-glycolic acid) (PLGA) as nanoparticles [13]. This facilitates slow release of encapsulated TLR-L from PLGA nanoparticles upon hydrolysis in tissues or within cells following uptake. Therefore, in this study, we evaluated the efficacy of TLR-Ls encapsulated PLGA nanoparticles against MD when these ligands were administered *in ovo* and post-hatch.

## 2. Materials and methods

### 2.1. Chicken eggs

Specific pathogen free eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Canada) and incubated at recommended temperature and relative humidity at Arkell Poultry Research Station, University of Guelph. All experiments were conducted in accordance with the guidelines of the University of Guelph's Animal Care Committee. At embryonic day (ED) 18, TLR-Ls were administered into the eggs and they were set for hatching. Day old chicks were transported to the animal isolation facility of Ontario Veterinary College, University of Guelph.

### 2.2. TLR-Ls and virus

LPS from *Escherichia coli* O111:B4 and synthetic Class B CpG ODN 2007 with phosphorothioate backbone were purchased from Sigma-Aldrich (Oakville, ON, Canada). *In vivo* propagated, very virulent strain of MDV (RB1B), which was provided by Dr. K.A. Schat [14], was used to infect chicks.

### 2.3. Encapsulation of TLR-Ls

LPS and CpG were encapsulated in PLGA, (Resomer® RG 503H, Sigma-Aldrich) using the modified double emulsion solvent evaporation method to generate PLGA nanoparticles as described in our previous studies [5,6]. The encapsulation efficiency of the PLGA nanoparticles for both TLR-Ls were determined as in our previous studies [5,6]

### 2.4. Experimental design

Three independent experiments were performed to examine the protective effect of encapsulated TLR-Ls (LPS and CpG) against MDV infection. The purpose of the first experiment was to identify the suitable form of TLR-Ls (free or encapsulated) and the day of *in ovo* administration (ED18 or ED19) of TLR-Ls that can provide protection against MDV infection. In the first experiment, four groups of ED18 embryos were injected via the amniotic route with 20 μg soluble or encapsulated LPS (ELPS), or 25 μg soluble or encapsulated CpG (ECpG) and were designated as 18LPS, 18ELPS, 18CpG, 18ECpG groups. ED19 embryos were similarly inoculated with the above formulations and were designated as 19LPS, 19ELPS, 19CpG, 19ECpG groups. Mock PLGA nanoparticles were administered to ED18 embryos (18PLGA). Each group had 11-12 embryonated eggs. All day-old chicks, except the PBS group were infected with 250 plaque-forming units of very virulent RB1B MDV/chick via intra-abdominal route. TLR-Ls sham treated, but MDV-infected group (MDV only) was used as a positive control group. The experiment was terminated at 21dpi. Based on the results of the tumor incidence, encapsulated TLR-Ls were selected for subsequent experiments.

The second experiment was designed to determine whether a single or a double dose of encapsulated TLR-Ls could provide protective immunity against MDV. In this experiment, the first dose of ELPS or ECpG or both (ELPS + ECpG) TLR-Ls or PLGA were administered to ED18 embryos. All day-old chicks were infected with MDV as indicated above. The groups that received the second dose of ELPS or ECpG or combination of above, or PLGA were injected at 14dpi through the intramuscular route. The experimental groups were designated as ECpG, ELPS, ECpG + ELPS, 2ECpG, 2ELPS, 2(ECpG + ELPS), PLGA, 2PLGA, MDV only and PBS groups and each group had 15 ED18 embryos. The dose and volume of TLR-Ls remained identical to the first experiment. The experiment was terminated at 21dpi and tumor incidence was recorded. Based on the results, double doses of encapsulated TLR-Ls were selected to use in the subsequent experiment.

The third experiment was designed to address the potential immunological mechanisms involved in the protective effect of encapsulated TLR-L treatments against MDV infection. ELPS or ECpG or PLGA was initially administered to ED18 embryos. Day-old chicks were infected with MDV as mentioned above. The second dose of ELPS or ECpG or PLGA was administered at 14dpi. The groups were designated as 2ECpG, 2ELPS, 2(ECpG + ELPS), 2PLGA, MDV only and PBS groups and each group had 32-33 ED18 embryos. At 4, 10 and 21 dpi, spleen and bursa of Fabricius (BF) and body weights (BW) were recorded and samples from spleen, BF and feathers were collected. Tumor incidence in chickens was recorded at 21dpi.

### 2.5. DNA and RNA extraction

Genomic DNA was extracted from feather tips as described previously [15]. One hundred nanograms of DNA was used in real-time PCR. RNA was extracted as described previously [6]. The quantity and quality of DNA and RNA were determined using the NanoDrop® ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE).

### 2.6. Real-time PCR

Real-time PCR was performed using SYBR green dye in a LightCycler 480 II to quantify MDV genome copy numbers and cytokine gene expression (Roche Diagnostics, Laval, Quebec) as described previously [4,15]. Primer sequences of target and reference genes are listed in Table 1. The primers were synthesized by Sigma–Aldrich Canada (Oakville, ON).

### 2.7. Statistical analysis

Relative expression was calculated using LightCycler 480 II advanced relative quantification software in relation to chicken

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