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Low pathogenic avian influenza virus infection increases the staining intensity of KUL01 + cells including macrophages yet decrease of the staining intensity of KUL01 + cells using clodronate liposomes did not affect the viral genome loads in chickens

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ABSTRACT

The effect of depletion of macrophages using clodronate liposomes as well as macrophage response following viral infections have been studied in various mouse-virus infection models, but they have not been extensively studied in chickens relevant to virus infections. When we infected day 6 chickens with H4N6 low pathogenic avian influenza virus (LPAIV), we observed that H4N6 LPAIV infection increased the staining intensity of KUL01 + cells in trachea, lungs and duodenum of chickens at 3 days post-infection. Then, we used clodronate liposomes intra-abdominally in 5 day-old chickens and found significant reduction of staining intensity of KUL01 + cells in trachea and duodenum but not in lungs at 4 days post-treatment. When we infected the clodronate liposome and PBS liposome treated chickens with H4N6 LPAIV intra-nasally at day 6, we found no effect on H4N6 LPAIV genome loads in trachea, lungs and duodenum of chickens. This study indicates that although KUL01 + cell intensity are increased in respiratory and gastrointestinal tissues in chickens following H4N6 LPAIV infection, the decrease of KUL01 + cell intensity using clodronate liposomes did not change the H4N6 LPAIV genome loads in any of the examined tissues suggesting that KUL01 + cells may not be critical during H4N6 LPAIV infection in chicken.

1. Introduction

The innate immune system, which mounts potent, nonspecific and broadly effective host responses, is equipped with an array of immune cells. One of the key immune cells indispensable in this regard is the macrophages. In addition to phagocytic activities of macrophages against various microbes and harmful substances, they also act as antigen presenting cells and a source of cytokines and chemokines facilitating the induction of antigen specific adaptive immune responses (Arango Duque and Descoteaux, 2014). In avian species, mobilization of macrophages into the site of infection contribute more than resident macrophages in phagocytic activity (Maina, 2002).

Liposome encapsulated clodronate (dichloromethylene diphosphonate or CL2-MDP) has been widely used to deplete macrophages (Benoit et al., 2006; Kameka et al., 2014a; Leemans et al., 2001). Once phagocytosed by macrophages, clodronate liposomes get accumulated in the cytosol, resulting in apoptosis and depletion of macrophages (van Rooijen et al., 1996). It has been shown that the use of clodronate liposomes significantly depletes macrophages in chickens (Jeurissen et al., 1998). The effect of depletion of macrophages using clodronate liposomes has been studied against various virus infections in mouse models, such as measles (Roscic-Mrkic et al., 2001) and influenza (Tate et al., 2010). In chickens, increased Marek's disease virus genome load in the blood and spleen following treatment with clodronate liposomes has been shown (Rivas et al., 2003). However, macrophage depletion using clodronate liposomes has not been extensively studied during other viral infections in chickens.

Macrophage recruitments following viral infections in chickens such as Marek's disease virus, infectious bursal disease virus, adenovirus and infectious bronchitis virus infections have been shown previously (Abdul-Careem et al., 2009; Abdul-Careem et al., 2008; Fulton et al., 1993; Kameka et al., 2014b; Nakamura et al., 2001). However, studies

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that investigated macrophage response in chickens following avian influenza virus infection are scarce (Cornelissen et al., 2013; Rebel et al., 2011). Avian macrophages have been shown to be targeted for viral replication during infectious bronchitis corona virus (Amarasinghe et al., 2017), Marek's disease virus (Barrow et al., 2003; Chakraborty et al., 2017), avian influenza virus (Barjesteh et al., 2014), infectious laryngotrcaheitis virus (Calnek et al., 1986) and reovirus (Swanson et al., 2001) infections. Avian macrophages are also have been shown to elicit antiviral response depending on nitric oxide (NO) production against avian influenza virus (Abdul-Cader et al., 2017), infectious laryngotracheitis virus (Haddadi et al., 2013) and Marek's disease virus (Xing and Schat, 2000). Following avian influenza virus infection, macrophages secrete pro-inflammatory cytokines and chemokines, facilitating the development of immune response, which further reduce the replication and spread of avian influenza virus in the host (Herold et al., 2006; Peschke et al., 1993; Seo et al., 2004).

The effect of macrophage depletion on avian influenza virus replication in chickens has not been studied. Kim et al. reported that the depletion of macrophages using clodronate liposomes in pigs, results 40% mortality after infection with H1N1 influenza virus while zero mortality in infected control pigs (Kim et al., 2008). Similarly, Tate et al. have shown that the depletion of macrophages in a mouse model of influenza virus infection leads to severe viral pneumonia in H3N2 influenza virus infected mice (Tate et al., 2010). We hypothesized that LPAIV infection will increase the staining intensity of KUL01+ cells, which include macrophages, monocytes and interdigitating cells (Mast et al., 1998) in respiratory and gastrointestinal tracts. We also hypothesized that the decrease of the staining intensity of KUL01 marker + cells following intra-abdominal administration of clodronate liposomes will augment replication of low pathogenic avian influenza virus (LPAIV) in respiratory and intestinal tracts of chickens.

2. Materials and methods

2.1. Animals, virus and reagents

The Veterinary Science Animal Care Committee (VSACC) and Health Science Animal Care Committee (HSACC) have approved the use of specific pathogen free (SPF) eggs, embryos, and chickens used in all our experimental procedures. The eggs were purchased from the Canadian Food Inspection Agency (CFIA), Ottawa, Canada and incubated (60–70% relative humidity and 37.2–37.6 °C temperature depending on the stage of the incubation) at Health Research Innovation Center (HRIC), University of Calgary. A LPAIV, A/Duck/Czech/56 (H4N6), propagated in the embryonated chicken eggs, was used in the studies. The titer of H4N6 was determined by plaque assay using Madin-Darby Canine Kidney (MDCK) cells. Clodronate liposomes (Foundation Clodronate Liposomes, Amsterdam, Netherland) were used for decreasing the staining intensity of KUL01 + cells in chickens as described earlier (Kameka et al., 2014a) and phosphate buffered saline (PBS) liposomes were used as a control.

2.2. Experimental design

2.2.1. Evaluating the effect of H4N6 LPAIV infection on the staining intensity of KUL01+ cell populations in chickens

Six day-old chickens (n = 5) were infected with 2.7×10^5 PFU of H4N6 LPAIV per chicken intra-nasally with uninfected controls (n = 4). At 3 days post-infection, the chickens were euthanized and the trachea, lungs and duodenum were collected. The collected samples were preserved in optimum cutting temperature (OCT, VWR International, Mississauga ON, Canada) and immunofluorescent assay was performed to quantify the staining intensity of KUL01+ cells as described earlier (Abdul-Cader et al., 2017).

2.2.2. Evaluating the effect of reduction of the staining intensity of KUL01+ cells against H4N6 LPAIV infection in chickens

We delivered 0.5 mL (5 mg/ml) of clodronate liposomes (n = 8) or PBS liposomes (n = 8) intra-abdominally to each 5 day-old chicken. At day 6, subsets of chickens (clodronate liposome, n = 4 and PBS liposome, n = 4 treated groups) of both groups were infected with 2.7×10^5 PFU of H4N6 LPAIV per chicken intra-nasally with uninfected controls (n = 4 per group). At 3 days post-infection, the chickens were euthanized and trachea, lung and duodenum were collected. Previously, it has been shown that lower gastrointestinal tract is also a site of LPAIV replication (Slemons and Swayne, 1990) as has been the duodenum (Wang et al., 2016). From the tissue samples collected, RNA extraction was done to quantify the genome loads of H4N6 LPAIV. A portion of samples were preserved in OCT and immunofluorescent assay was performed to quantify the staining intensity of KUL01+ cells as described earlier (Abdul-Cader et al., 2017).

2.3. Data analyses

2.3.1. Image J analysis of fluorescent signals

For the quantification of staining intensity of KUL01+ cells in the tissues, five areas with highest DyLight® 550 fluorescent signals and corresponding nuclear stained 4,6-diamidino-2-phenylindole (DAPI) areas were captured under 20X magnification from each tissue section. Then, these images were subjected to fluorescent intensity quantification using Image-J software (National Institute of Health, Bethesda, Maryland, USA). The resultant fluorescent intensities for DyLight® 550 positive signals were expressed relative to the total nuclear stained areas as a percentage.

2.3.2. Statistical analysis

To identify group differences, we analyzed all the data (GraphPad Prism Software 5, La Jolla, CA, USA) using non-parametric test, Mann-Whitney *U* test due to low number of animals per group. Before being analyzed each set of data, the outlier test was conducted using the Grubbs' test (GraphPad software Inc., La Jolla, CA, USA). The differences between groups were considered significant at $P \leq 0.05$.

3. Results and discussion

3.1. H4N6 LPAIV infection increases the staining intensity of KUL01 + cells in trachea, lungs and duodenum of chicken

First, we questioned whether H4N6 LPAIV infection contributes to the increase of staining intensity of KUL01+ cells including macrophage populations in trachea, lungs and duodenum. When we infected 6 day-old chickens with H4N6 LPAIV intra-nasally, we found that, at 3 days post-infection, H4N6 LPAIV infection significantly increased the staining intensity of KUL01+ cells in trachea (Fig. 1a, $P < 0.05$), lungs (Fig. 1b, $P < 0.05$) and duodenum (Fig. 1c, $P < 0.05$) compared to the uninfected control chickens. In trachea, the staining intensity of KUL01+ cells distributed mainly mucosal and submucosal areas with some distribution between cartilages and serosal surface. In the lungs and duodenum, the KUL01+ cells mainly distributed throughout the lung parenchyma and mucosa respectively (Fig. 1a–c).

Increased recruitment of KUL01+ cells following viral infections other than avian influenza virus in chickens are shown (Abdul-Careem et al., 2009; Abdul-Careem et al., 2008; Fulton et al., 1993; Kameka et al., 2014b; Nakamura et al., 2001). Similarly, it has been shown that avian influenza virus infection also recruit KUL01+ cells in chickens (Cornelissen et al., 2013; Rebel et al., 2011). The recruitment of KUL01 + cells following viral infections may potentially be due to the availability of pathogen associated molecular patterns (PAMPs) of H4N6 LPAIV during the infection, increasing the recruitment of these cells. It has been previously shown that toll-like receptor (TLR)7 of the innate immune system recognizes PAMPs of influenza virus and