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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

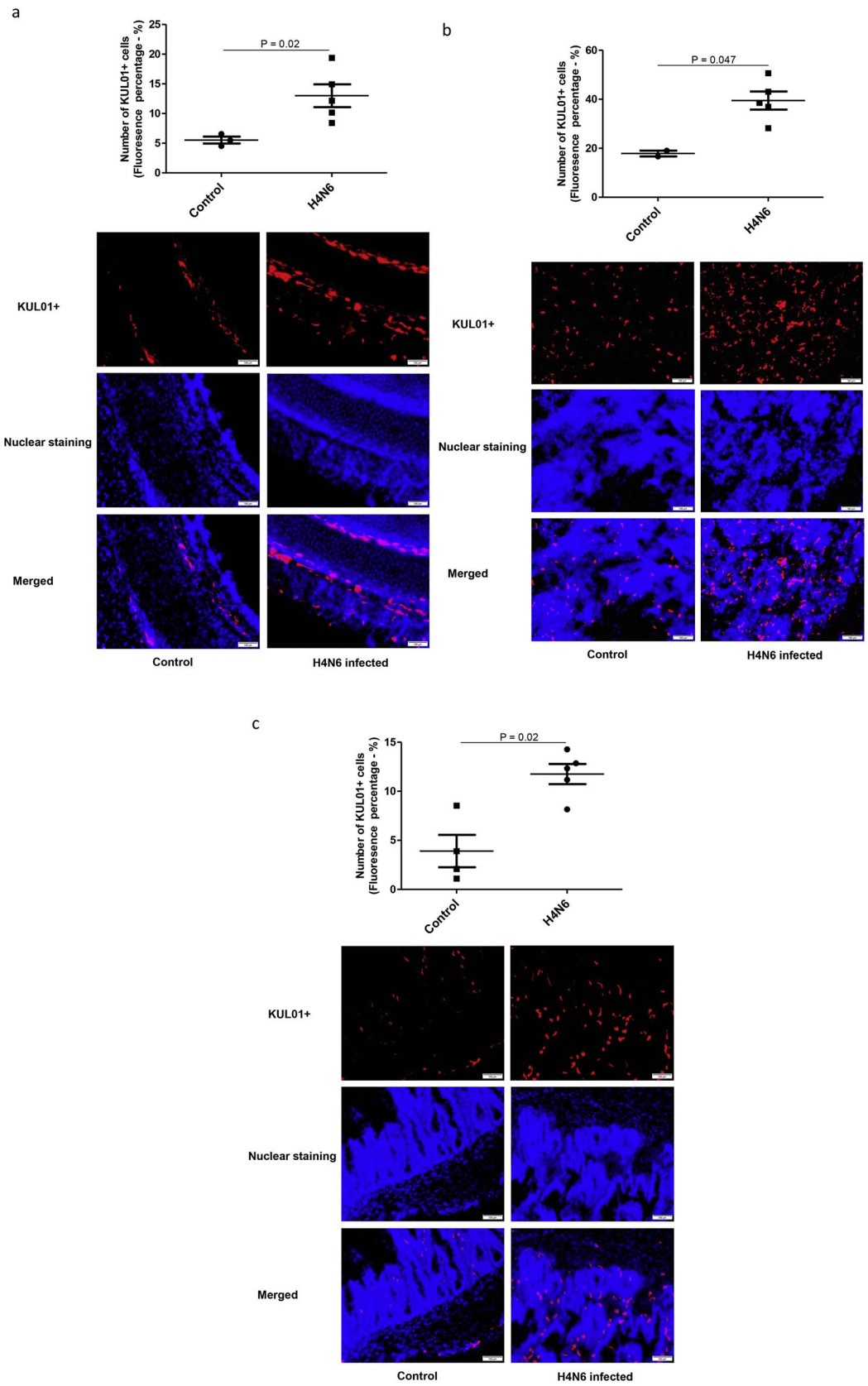

Fig. 1. H4N6 LPAIV infection increases the staining intensity of KUL01 + cells in trachea, lungs and duodenum of chicken. At 6 days of age, a group of chickens (n = 5) were infected with H4N6 LPAIV intra-nasally while another group was left as uninfected controls (n = 4). At 3 days post-infection, trachea, lung and duodenum were collected for immunofluorescent assay to quantify the staining intensity of KUL01 + cells. Representative images from trachea (a), lung (b) and duodenum (b) are shown. The Mann-Whitney *U* test was performed to identify group differences and the differences were considered significant at $P < 0.05$.

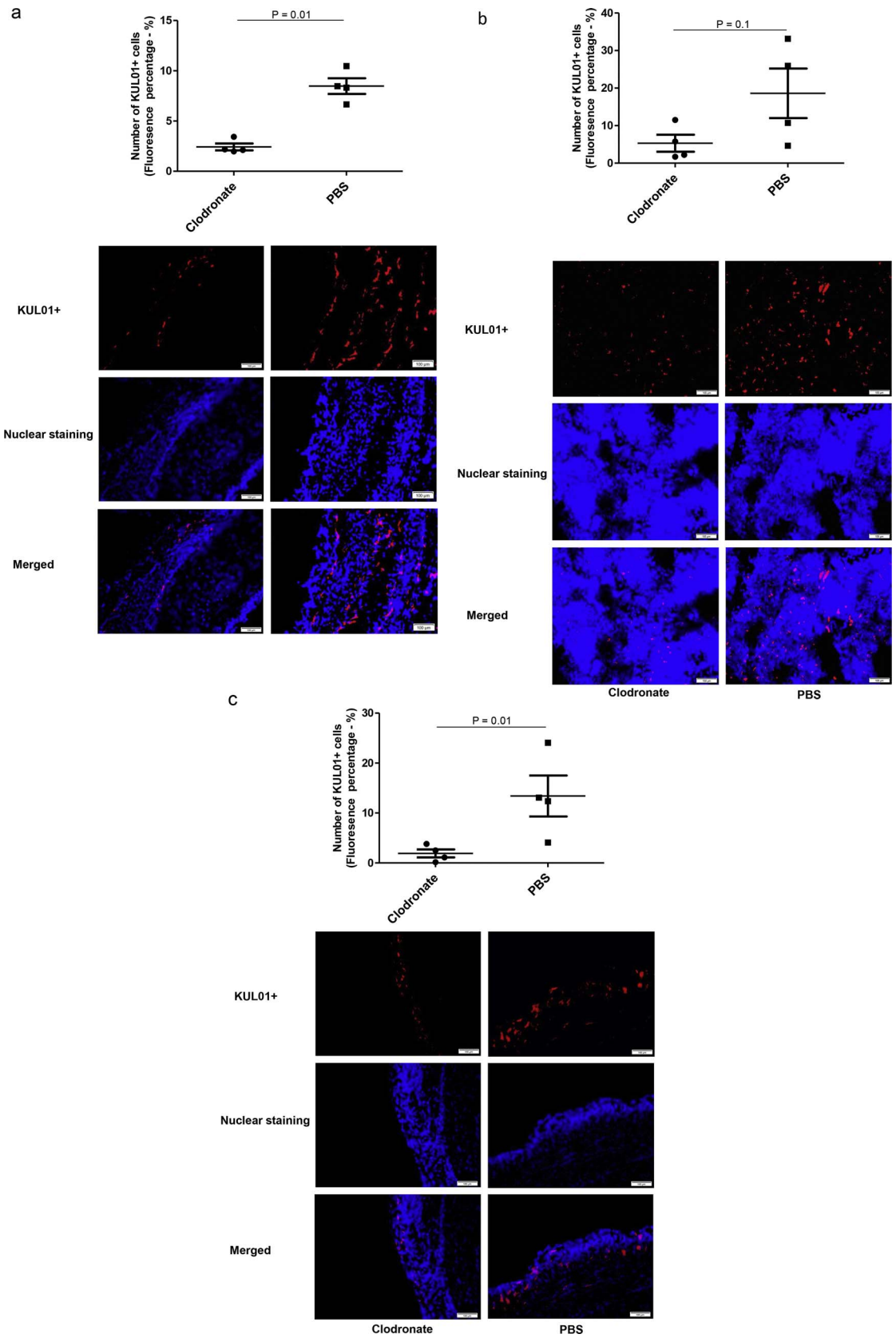

Fig. 2. Clodronate liposomes decrease the staining intensity of KUL01 + cells in trachea and duodenum of chicken. At 5 days of age, the chickens were treated with clodronate liposomes (n = 4) or PBS liposomes (n = 4) intra-abdominally. At 4 days post-treatment, trachea, lung and duodenum were collected. The immunofluorescent assay was performed for the quantification of the staining intensity of KUL01 + cells. Representative images obtained following the immunofluorescent assay for the quantification of the staining intensity of KUL01 + cells in trachea (a), lung (b) and duodenum (c) are shown along with quantitative data. The Mann-Whitney *U* test was performed to identify group differences and the differences were considered significant at $P < 0.05$.

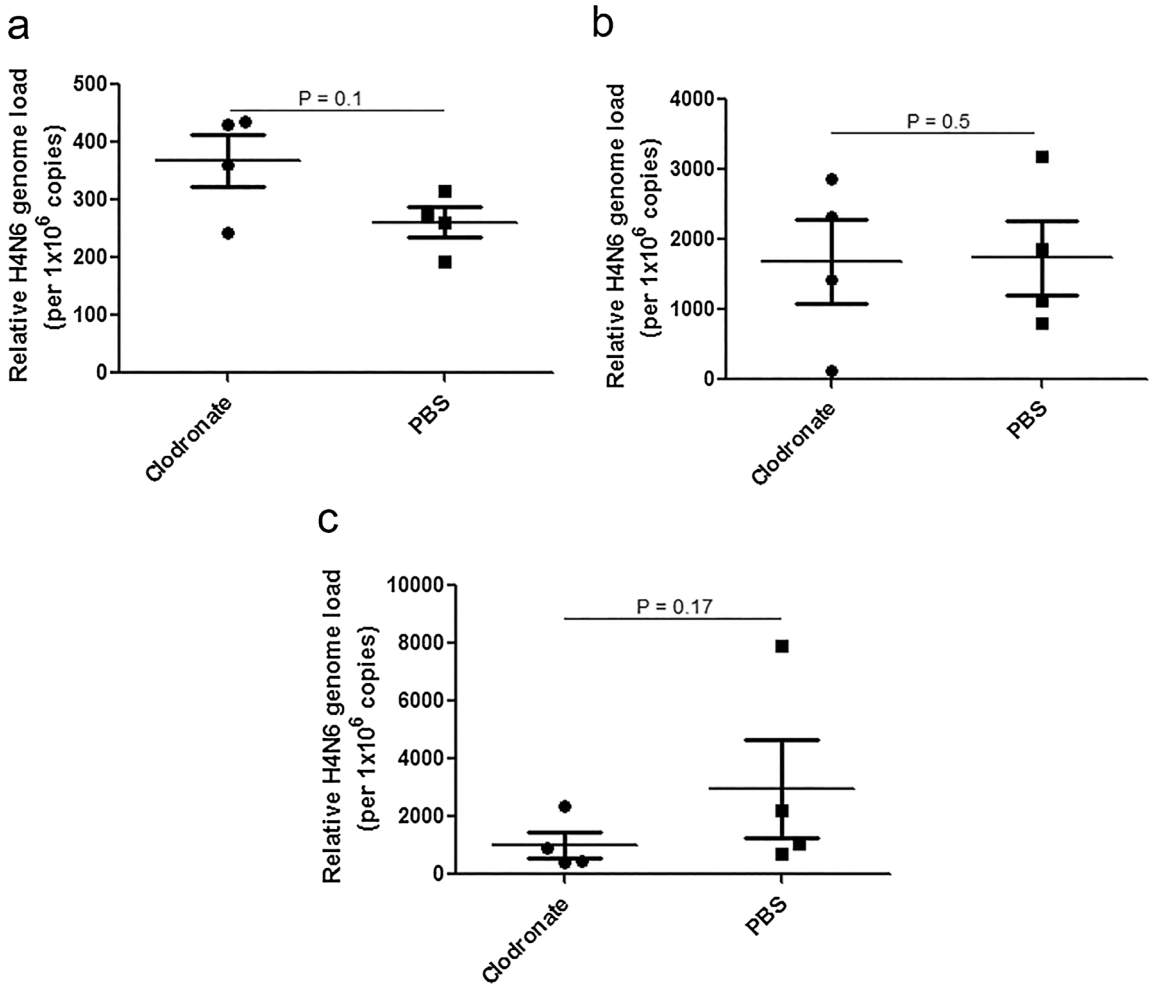

Fig. 3. Reduction of the staining intensity of KUL01+ cells with clodronate liposomes did not change H4N6 infection in trachea, lungs and duodenum. At 5 days of age, the chickens were treated with clodronate liposomes (n = 4) or PBS liposomes (n = 4) intra-abdominally. After 24 h, all chickens were infected with H4N6 LPAIV intra-nasally. At 3 days post-infection, trachea (a), lung (b) and duodenum (c) were collected. Real-time PCR assay was performed for H4N6 LPAIV genome quantification. The Mann-Whitney *U* test was performed to identify group differences and the differences were considered significant at $P < 0.05$.

activate mouse mononuclear cells (Lund et al., 2004). Furthermore, Lee et al. have shown that activation of TLR7 induces differentiation of myeloid-derived suppressor cells into one of the KUL01+ cells, macrophages (Lee et al., 2014).

3.2. Clodronate liposomes decrease the staining intensity of KUL01+ cells in trachea and duodenum of chicken

We observed that intra-abdominally delivered clodronate liposomes in 5 day-old chickens significantly reduced the staining intensity of KUL01+ cell populations in trachea and duodenum but not in lung at 4 days post-treatment when compared to the controls that received PBS liposomes (Fig. 2a and c, $P < 0.05$; Fig. 2b, $P > 0.05$).

In previous studies, it has been found that treatment of clodronate liposomes significantly decrease staining intensity of CV1-ChNL-74.2 antibody specific cells populations in chickens (Jeurissen et al., 1998). CV1-ChNL-74.2 antibody specific cells include macrophages, monocytes and interdigitating cells which are essentially similar to KUL01+ cells. In agreement with this finding, we observed decreased staining intensity of KUL01+ cells in this study in trachea and duodenum following intra-abdominal clodronate liposome treatment.

3.3. Decreasing the staining intensity of KUL01+ cells using clodronate liposomes had no effects on H4N6 LPAIV genome loads in trachea, lungs and duodenum

Since we observed that the clodronate liposomes significantly decreased the staining intensity of KUL01+ cells in trachea and duodenum, then we investigated to see whether this reduction in the staining intensity of KUL01+ cells influences the H4N6 LPAIV infection. We found that the clodronate liposome-mediated reduction of the staining intensity of KUL01+ cells was associated with no change of H4N6 LPAIV infection at 3 days post-infection (4 days post-treatment) in trachea (Fig. 3a, $P > 0.05$), lung (Fig. 3b, $P > 0.05$) and duodenum (Fig. 3c, $P > 0.05$).

KUL01+ cells including macrophages are important immune cells that can be beneficial in innate antiviral response against H4N6 LPAIV infection via several mechanisms. First, it is known that macrophages can lead to the production of a number of antiviral cytokines including IFNγ (Barjesteh et al., 2014; Dimier et al., 1998), IL-1β (Lavric et al., 2007) and release of reactive nitrogen species such as NO (Setta et al., 2012). Previously, it has been shown that NO originated from avian macrophages are inhibiting infectious laryngotracheitis virus (Haddadi et al., 2013), reo virus (Pertile et al., 1996) and Marek's disease virus (Xing and Schat, 2000) infections in vitro. We have previously observed that inhibition of H4N6 LPAIV replication is attributable to NO produced from KUL01+ cells (Abdul-Cader et al., 2017). Second, tracheal

macrophages can play a role in clearing H4N6 LPAIV infected cells as has been shown in mouse model of influenza infection (Hashimoto et al., 2007). However, the lack of significant differences in H4N6 LPAIV genome loads between clodronate liposome treated and controls could be two-fold. First, immune cell functions are redundant (Hamada et al., 2013) and it is possible that even the KUL01 + cell staining was decreased following clodronate liposome treatment, the functions of these cells may have been compensated by other intact immune cells in chicken. Second, it is possible that the number of animals used per group (n = 4) may not have provided adequate power in our experiments to show differences in H4N6 LPAIV genome loads in all the examined tissues in chickens although number of animals used are adequate for differentiating of KUL01 + cells in trachea and duodenum.

Although our investigations led to generation of significant new knowledge, there were some limitations. First, the monoclonal antibody used for the quantification of macrophages detects KUL01 mannose receptor and these receptors are also expressed by monocytes and dendritic cells of chicken (Mast et al., 1998) as such it is possible that the quantified macrophages may also include monocytes and dendritic cells. However, only macrophages and their precursor, blood monocyte will uptake the liposomes; since it has been shown that liposomes of the size > 200 nm will not be internalized by non-phagocytic cells and other immune cells such as granulocytes (Claassen et al., 1990). Dendritic cells are also unaffected upon administration of clodronate encapsulated-liposomes (van Rooijen and Hendrikx, 2010). However, we believe that KUL01 cell staining alone is not adequate for characterizing cells reduced by clodronate liposomes and further immune markers should have been included in the experiments. Second, we did not observe that intra-abdominal clodronate liposome treatment completely decrease the staining intensity of KUL01 + cells. Third, in our experiments we used a LPAIV strain H4N6 which does not lead to clinical signs due to the fact that it establishes low grade infection. Consequently, we resorted to real-time PCR technique to quantify this low grade H4N6 LPAIV infection in the observed tissues, which is more sensitive than the immunofluorescent assay (Landry et al., 2008; Perosa et al., 2013), although immunofluorescent assay would have given more meaningful data relevant to H4N6 LPAIV infection in the observed respiratory and gastrointestinal tissues.

In conclusion, we found that H4N6 LPAIV infection contributes to increase of the staining intensity of KUL01 + cell populations in trachea, lungs and duodenum of chickens. Although, we observed that intra-abdominal treatment of clodronate liposomes reduced the staining intensity of KUL01 + cells in trachea and duodenum of chickens, the decrease of the staining intensity of KUL01 + cells did not alter the H4N6 LPAIV genome load in examined tissues. Further investigations are warranted to investigate other roles of KUL01 + cells in the host responses elicited against H4N6 LPAIV infection.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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