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Research Article

Flow cytometric characterization of Marek's disease virus infected macrophages and dendritic cells at later time points

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ABSTRACT

Marek's disease virus (MDV) infects APCs (antigen-presenting cells: macrophages and/or dendritic cells [DCs]), B cells and activated T cells *in vivo*. MDV is a highly cell-associated virus which makes *in vitro* studies difficult. A *de novo in vitro* MDV-APC infection model has therefore been developed to carry out extensive studies regarding the MDV-APC interactions in the context of resistance or susceptibility to Marek's disease (MD). Using this model, MDV-macrophage interactions were previously studied at 1 dpi (day post infection). This study was aimed to characterize MDV-APC interactions by flow cytometry at later time points (3 and 5 dpi) following infection with MDV. Chicken bone marrow-derived macrophages and DCs from MD resistant (6_1) and susceptible (7_2)line were infected with MDV *in vitro* for 3 and 5 days. Flow cytometric study revealed that macrophages from line 7_2 had a higher infection rate than those of line 6_1 on both the days. On the other hand, no difference was observed regarding infection rate between DCs of the two chicken lines. The findings of this study boost up the previous perception of macrophages playing an important role in exerting resistance to MD.

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1. INTRODUCTION

Marek's disease virus (MDV) was primarily described as a lymphotrophic alphaherpes virus for a long time. Though, apart from immune cells, MDV replicates well *in vitro* in non-lymphoid cells such as fibroblasts, kidney cells and embryonic skin cells (Churchill and Biggs, 1967; Cook and Sears, 1970; Nazerian and Purchase, 1970; Dorange *et al.*, 2000). As described in the well-established 'Cornell model' of MDV infection, the *in vivo* infection takes place by the cell-free virus wrapped in dander (Calnek, 2001) and phagocytic cells from the lungs carry the virus to the spleen and other lymphoid tissues following infection through respiratory tract. Virus is then presumed to pass to the lymphocytes where it causes lytic infection of B lymphocytes and lytic or latent infection in T cells. It was thought that T cells play a crucial role to spread the virus in various visceral organs and also in nerve tissues and that is how pathological lesions emerge after infection. However, the role of innate immune cells, especially APCs (antigen-presenting cells: macrophages and/or dendritic cells [DCs]), cannot be ignored. APCs are important

cells of the innate immune system and play a crucial role at early stages of microbial infection. The early differential viraemia (Burgess and Davison, 2002) and gene expression profiles (Smith *et al.*, 2011) of MDV-infected MD-resistant and susceptible lines support the hypothesis that innate immunity plays a pivotal role in determining resistance to MD. However, very little is known about the early stages of MDV infection.

Genetic resistance to MD is a complex trait, as genes from the MHC and also from outside of the MHC are involved. However, two chicken inbred lines, 6_1 and 7_2 which are highly resistant and susceptible to MD, respectively, are MHC-congenic (Cole, 1968), which shows that the resistance to MD is largely determined by genes outside of the MHC. Researches have been carried out to determine the basis of resistance or susceptibility between the two lines, such as differences in the virus titre (10-fold higher in susceptible compared to resistant birds) in splenocytes (Lee *et al.*, 1981) and differential cytokine expression in splenocytes (IL-6 and IL-18 were expressed in susceptible but not in resistant birds) (Kaiser *et al.*, 2003) during the cytolytic phase of MDV infection. Differential gene expression was observed in splenocytes of these two lines at 3 dpi (Smith *et al.*, 2011) and by this time MDV infection occurs in APCs, B and activated T cells. MDV infection of macrophages *in vitro* in lines 6_1 and 7_2 were studied previously as early as 1 dpi (Chakraborty *et al.*, 2019). However, MDV-APC interaction has not yet been studied at later time points. This study was therefore aimed to infect APCs of lines 6_1 and 7_2 by MDV *in vitro* and then to characterize the infected cells by flow cytometry on 3 and 5 dpi in order to reveal the pattern of infection of APCs by MDV at these time points.

2. MATERIALSAND METHODS

Chickens and the virus

The chickens used in this study were layer chicken line J, an intercross-bred from 9 lines, originally inbred from Brown Leghorn chickens at the Poultry Research Centre, Edinburgh. They were bred and conventionally raised at The Roslin Institute (http://www.narf.ac.uk/chickens/lines). The virus, CVI988 UL41 eGFP (enhanced green fluorescent protein), was generated from a BAC (Bacterial artificial chromosome) construct of vaccine strain CVI988 (Rispens) of MDV serotype 1, in which the UL41 gene was replaced with eGFP under control of the murine phosphoglycerol kinase promoter (Wasson, 2011). UL41 is a non-essential gene for MDV replication and a UL41-deletant mutant replicates as well as the parental strain *in vitro* (Gimeno and Silva, 2008). The presence of eGFP will therefore indicate MDV replication (Figure 1).

Cell cultures

Chicken embryos of 9 to 11 days old were used to collect chicken embryo fibroblasts (CEF) which were then cultured in T_{175} flasks at 38.5°C with 5% CO_2 in CEF medium consisting of M-199 medium (Gibco) containing 10% (v/v) tryptose phosphate broth (Invitrogen), 2.7% (v/v) $NaHCO_3$ (Sigma-Aldrich), 1% (v/v) pen-strep (Sigma-Aldrich), 0.5% (v/v) gentamycin (Sigma-Aldrich), 0.001% (v/v) fungizone (amphotericin B, 250 µg/ml) (Thermo Scientific), and 0.5-10% (v/v) foetal bovine serum (FBS) (Gibco) depending on CEF confluency in culture flasks. The MDV-BAC virus was initially grown and propagated in CEF cultures as previously described (Petherbridge *et al.*, 2003). MDV-infected CEF were then grown in large numbers and pooled together to obtain a high virus titer. Pooled infected CEF were resuspended in freezing media (FBS, RPMI-1640 and DMSO), aliquoted (250-500 µ l/cryovial) and stored at -80°C until further use.

Chicken bone marrow-derived macrophages (BMDM) and dendritic cells (BMDC) were cultured from chicken bone marrow cells isolated from 3 to 6-week-old birds as described previously (Garceau *et al.*, 2010; Wu *et al.*, 2010). Cells were cultured for 4 days in T_{75} flasks at 41°C with 5% CO_2 using RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (PAA) (for BMDM), 10% heat-inactivated chicken serum (CS) (for BMDC), 1% L-glutamine and 0.1% pen-strep. Recombinant chicken interleukin-4 (chIL-4) and granulocyte-macrophage colony stimulating factor (chCSF-2 or GM-CSF) were added to the BMDC cultures at optimal dilution of each cytokine, whereas recombinant chCSF-1 was added to the BMDM cultures. In order to obtain approximately 1×10^7 BMDM or BMDC at harvest, bone marrow (BM) cells were seeded at a concentration of approximately 1×10^6 cells/ml.

Co-culture infection experiments, FACS (fluorescent activated cell sorting) and flow cytometry

Due to the cell-associated nature of MDV, infected CEF were used to infect phagocytes. Prior to the infection of phagocytes, previously frozen virus was propagated in large numbers in CEF cultures. On the day of phagocyte infection, infected CEF were harvested by 2.5% trypsin (diluted in PBS), pelleted by centrifugation (500×g for 5 min) and resuspended in FACS buffer (PBS and 1% BSA).

Immunofluorescent staining of infected CEF was carried out as described previously (Balic *et al.*, 2014) using anti-CD45 (clone AV53, isotype IgG1, The Pirbright Institute) and a goat anti-mouse IgG1 conjugated with Alexa Fluor (AF) 647 as secondary antibody. Gr 13.1 (ovine NKp46; kindly provided by Dr. Timothy Connelley, The Roslin Institute) was used as isotype control. The $eGFP^{+}CD45^{-}$CEF were sorted using FACSAriaTM III cell sorter (BD Biosciences). Data analyses were carried out using FACSDiva v 6.1.3 software.

A total of 2×10^{6} sorted infected CEF were used to infect BMDM and BMDC on day 4 of culture in T_{75} flasks at an infection ratio of 1:5 (CEF:BMDM or BMDC) in RPMI-1640 medium containing 2-10% FBS (Gibco) (serum percentage was determined according to the confluency of CEF in culture flask), 1% pen-strep and 1% L-glutamine. In addition, medium for BMDC was supplemented with 5% CS. Co-cultured cells were incubated at 41ºC with 5% CO_2 for 3 days and harvested for downstream experiments, such as flow cytometry or cell-sorting. For flow cytometry, cells were harvested with 100 mM EDTA in PBS, pelleted by centrifugation and resuspended in PBS containing 1% BSA and 0.1% sodium azide. Immunofluorescent staining was carried out using a macrophage marker (clone KUL01, isotype IgG1, SouthernBiotech) and anti-CD45. KUL01 was recently identified as a mannose receptor (Staines *et al.*, 2014). Cells were stained for flow cytometric analysis as described above and analyzed using a FACS Calibur (BD Biosciences). Viable cells were gated based on 7-AAD (7-aminoactinomycin D, Life Technologies) staining and the resulting data were analyzed with FlowJo software.

3. RESULTS

Infection and subsequent flow cytometric characterization of BMDM from the inbred lines

Chicken BMDM from lines 6_1 and 7_2 were grown separately in three T_{75} flasks to carry out flow cytometric experiments on 3 and 5 dpi. Meanwhile, MDV-infected CEF were grown in two T_{175} flasks for each experiment. On day 4 of culture, macrophages were infected with pre-sorted GFP^{+} CEF at an infection ratio of 1:5 (CEF:BMDM).

Following infection, cells were observed daily under fluorescence microscope and it was noted that the number of GFP^{+} cells was gradually reduced in both the lines as the time progressed (Figure 2). Cells were harvested on 3 and 5 dpi from both lines for flow cytometric analyses after staining with KUL01, CD45 and Gr 13.1 (isotype control). At least 10^{6} viable cells were counted from each sample.

The number of infected macrophages on 3 dpi was found in 4% in line 6_1 and 10% in line 7_2, indicating a large difference (2.5 times) regarding the rate of MDV infection between resistant and susceptible lines

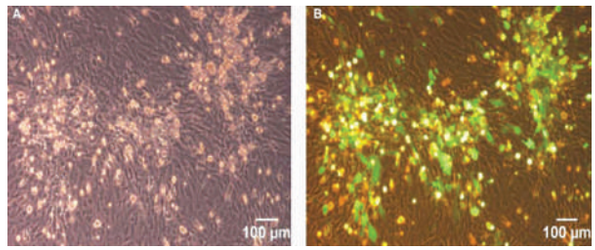

Figure 1. MDV plaques formed in CEF culture visualized under **(A)** bright field and **(B)** fluorescence microscope

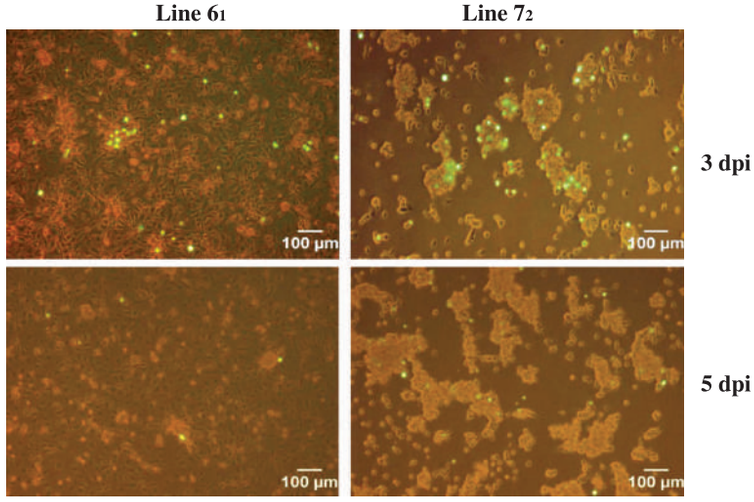

Figure 2. Infection of macrophages with pre-sorted GFP+ CEF in inbred lines. The ratio of infection was 1:5 (CEF:BMDM). Left panel: Line 6_1 macrophages on 3 and 5 dpi. Right panel: Line 7_2 macrophages on 3 and 5 dpi.

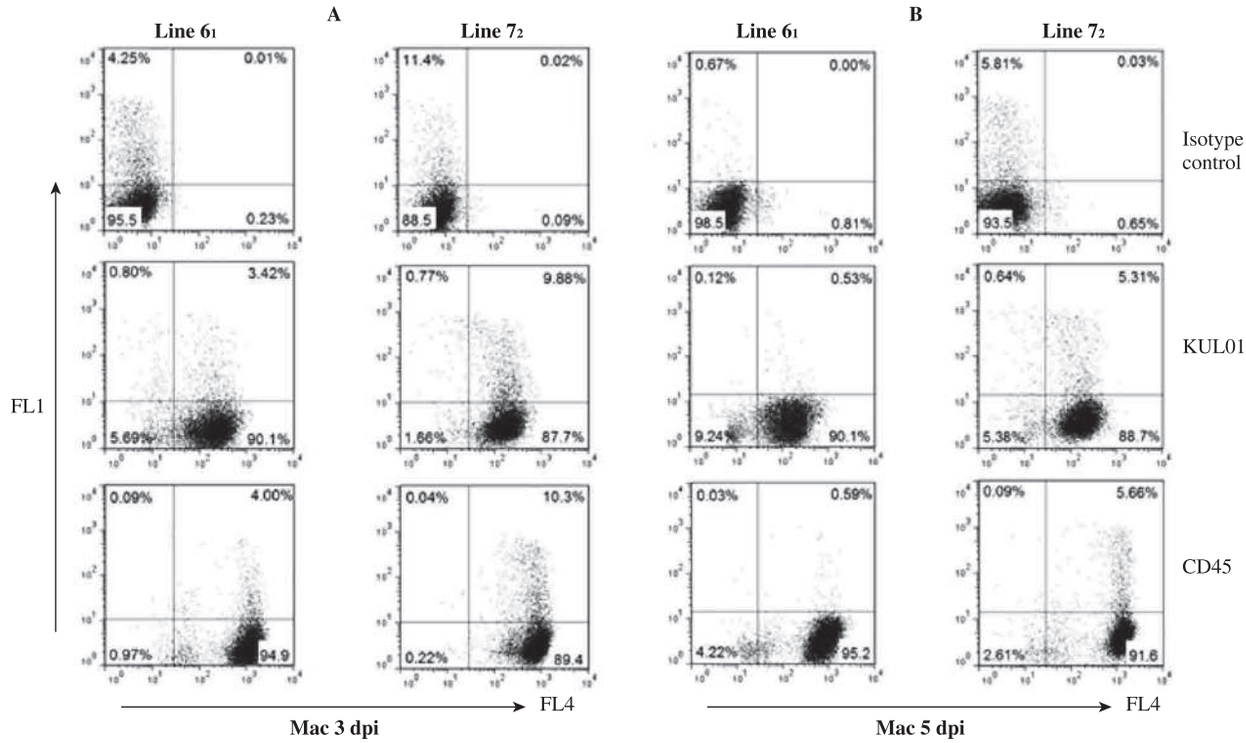

Figure 3. Flow cytometric characterization of macrophages (Mac) following co-culture with MDV-infected CEF in two inbred lines on **(A)** 3 dpi and **(B)** 5 dpi. Macrophages were infected with pre-sorted MDV-infected GFP$^+$ CEF at 1:5 ratio (CEF:BMDM). Live cells were analyzed in FL1/FL4 dot plots to detect KUL01$^+$ and CD45$^+$ macrophages compared to isotype control (Gr 13.1). FL1 shows the fluorescence of GFP (green)-encoded MDV and FL4 shows the fluorescence of Alexa Fluor 647 (red) at the surface of the cells. Distribution of cells in antibody stained plots, FL1$^-$FL4$^-$: uninfected CEF; FL1$^+$FL4$^-$: infected CEF; FL1$^-$FL4$^+$: uninfected macrophages and FL1$^+$FL4$^+$: infected macrophages.

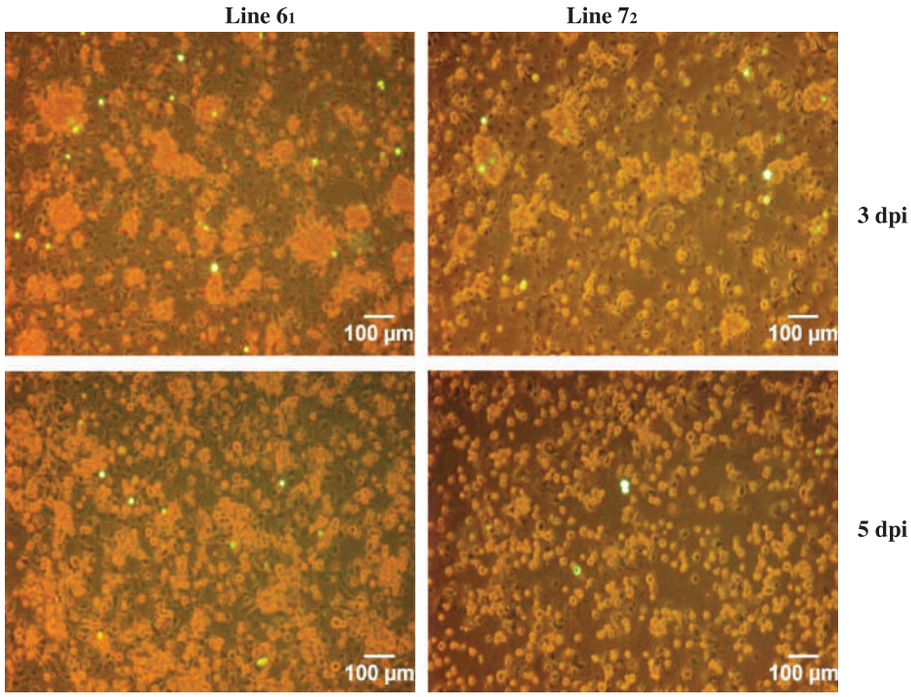

Figure 4. Infection of BMDC with pre-sorted GFP$^+$ CEF in inbred lines. The ratio of infection was 1:5 (CEF:BMDC). Left panel: Line 61 DCs on 3 and 5 dpi. Right panel: Line 72 DCs on 3 and 5 dpi.

(Figure 3A). On 5 dpi, flow cytometric analyses of live cells revealed that the number of infected macrophages was very few (around 0.6%) in the resistant line (6_1) compared to its susceptible counterpart (line 7_2, 5.5%) (Figure 3B).

Infection and subsequent flow cytometric characterization of BMDC from the inbred lines

Chicken BMDC were cultured in three separate T_{75} flasks from each of the two inbred lines to carry out flow cytometric experiments on 3 and 5 dpi. In parallel, MDV-infected CEF were also grown in two T_{175} flasks for each experiment and DCs were infected with pre-sorted GFP^+ CEF at a ratio of 1:5 (CEF:BMDC). It was observed under fluorescence microscope that the number of GFP^+ cells was sharply reduced at the later days of infection (Figure 4). Flow cytometric analyses were carried out on 3 and 5 dpi after staining with KUL01, CD45 and Gr 13.1 (isotype control) and at least 10^6 viable cells were counted from each sample.

No notable difference was observed on 3 dpi in terms of percentage of infected DCs between the two lines (Figure 5A). On 5 dpi, the number of infected DCs was very few (around 0.5%) and as observed previously on 3 dpi, there was no difference between resistant (6_1) and susceptible line (7_2) (Figure 5B).

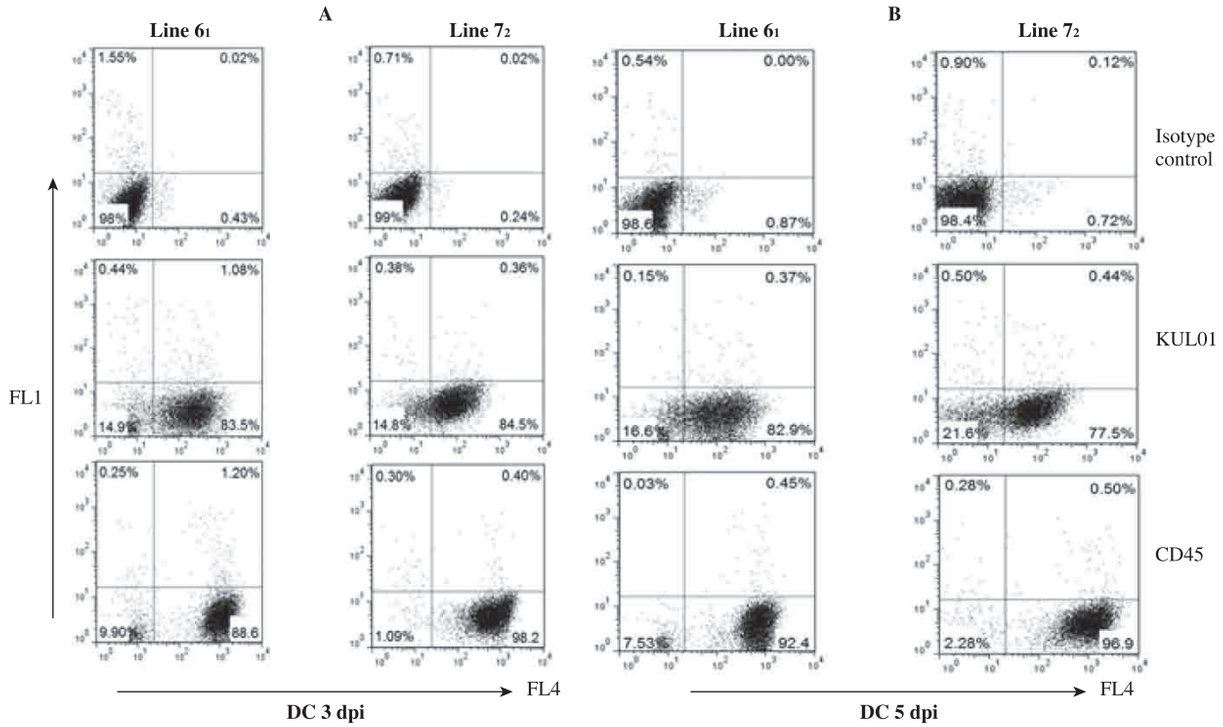

Figure 5. Flow cytometric characterization of DCs following co-culture with MDV-infected CEF in two inbred lines on **(A)** 3 dpi and **(B)** 5 dpi. DCs were infected with pre-sorted MDV-infected GFP^+ CEF at 1:5 ratio (CEF:BMDC). Live cells were analyzed in FL1/FL4 dot plots to detect $KUL01^+$ and $CD45^+$ DCs compared to isotype control (Gr 13.1). FL1 shows the fluorescence of GFP (green)-encoded MDV and FL4 shows the fluorescence of Alexa Fluor 647 (red) at the surface of the cells. Distribution of cells in antibody stained plots, $FL1^-FL4^-$: uninfected CEF; $FL1^+FL4^-$: infected CEF; $FL1^-FL4^+$: uninfected DCs and $FL1^+FL4^+$: infected DCs

4. DISCUSSION

To explore the pattern of infection of APCs by MDV at later time points, APCs from two inbred lines 6_1 and 7_2 were infected with MDV-infected CEF using a previously developed infection model (Chakraborty *et al.*, 2017) and subsequently characterized by flow cytometry. Despite sharing the same MHC genes, the chicken inbred line 6_1 is highly resistant and line 7_2 is highly susceptible to MD. The chicken inbred lines (6_1 and 7_2) show differences in viraemia level and gene expression profiles in splenocytes from the very early stages of MDV infection (Lee *et al.*, 1981; Smith *et al.*, 2011), suggesting that the inherent difference between two lines is due to differential responses of the innate immune system (Bumstead and Kaufman, 2004). The cells of the innate immune system, especially macrophages, play a crucial role during MDV infection. For example,

peritoneal macrophages isolated from MDV-infected chickens inhibited the formation of MDV plaques *in vitro* (Kodama *et al.*, 1979). Peritoneal macrophages also showed more phagocytic activity and plaque-inhibiting activity following MDV infection in susceptible than those of resistant chickens (Powell *et al.*, 1983). In the present study, APCs from MD resistant (6_1) and susceptible (7_2) lines were infected *in vitro* with MDV upto 3 and 5 dpi for the first time.

The overall flow cytometric results revealed that, irrespective of lines and the days-post infection, with a fixed infection ratio a higher percentage of macrophages were infected than DCs. Moreover, a higher proportion of macrophages from susceptible line (7_2) were infected compared to the resistant line (6_1) but no apparent difference was observed in the number of infected DCs between two lines. This might be an indication that macrophages play a more important role to exert resistance or susceptibility to MD than DCs. Among the immune cells, macrophages are well-known to exert resistance to herpesvirus infections. For example, a macrophage-dependent and T-cell independent resistance to systemic HSV-1 infection was demonstrated in mice where a selective reduction of macrophage function by silica treatment increased the susceptibility to HSV-1, but the reduction of thymic function by the aging process or by the combined effect of adult thymectomy and ATS (anti-mouse thymocyte serum) did not increase the susceptibility to HSV-1 (Schlabach *et al.*, 1979).

Macrophages are thought to inhibit MDV replication as they release NO (nitric oxide) through the increased activity of iNOS. NO is presumed to be crucial for inhibiting MDV replication during the cytolylic and latent phases of infection *in vivo* as an increased level of NO was observed in splenocyte cultures of MDV-infected MD-resistant chickens (Xing and Chat, 2000).

As an APC, DCs might be expected to infect at similar level to macrophages, but it was not the case in this study. Though the level of *in vitro* virus infection may vary within APCs. For example, Vatter and Brinton (2014) reported a higher number of SHFV (simian haemorragic fever virus) infected macrophages than those of DCs in macaque and baboons *in vitro*. One factor should also be considered here that the standard media for culturing DCs contained the cytokines IL-4 and GM-CSF (CSF-2) as growth promoting factors. Though no studies have been performed yet regarding MDV infection, the inhibitory role of IL-4 and GM-CSF might be crucial in MDV replication and hence low infection of DCs (Kedzierska *et al.*, 2000; Tsai *et al.*, 2013). But if only difference between the inbred lines is considered, it can be said that no apparent variation was observed between MD-resistant and susceptible lines in the context of MDV-DC infection, suggesting that, like macrophages, DCs are infected by MDV but they only act as a carrier of the virus to the lymphoid tissues and perhaps do not play a role in determining resistance to MD. However, further studies are required to clarify this.

5. CONCLUSIONS

From this study, it can be concluded that within APCs, macrophages appear to play more crucial role than DCs while conferring resistance to MD.

6. ACKNOWLEDGEMENT

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