Short Communication

Morphological and Immunophenotypical Properties of Chicken Bone Marrow-Derived Dendritic Cells Towards Wild Type and H5-Recombinant Fowlpox Virus Infections

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Abstract | Fowlpox is a common viral disease caused by fowlpox virus (FWPV), which infects birds. Fowlpox is an important disease in commercial poultry farming, as it can affect many production-system variables, such as size, performance and viability. Studies on the interactions between chicken bone marrow-derived dendritic cells (chBM-DCs) and FWPV and its recombinant viruses are limited compared to other avian viral infectious diseases. Therefore, this study characterised the response of chBM-DCs to infection with the wild-type (WT) FP9 strain of FWPV compared to a recombinant FWPV carrying the *H5* gene of avian influenza virus (rFWPV-H5) using morphological and immunophenotypical observations. The results showed that the rFWPV-H5-infected chBM-DCs formed better dendrites and stellate cells, compared to the WT FWPV. The recombinant virus also expressed higher levels of the surface markers CD86 and MHC class II double-positive chBM-DCs 12 and 24 h post-infection. In conclusion, chBM-DCs were susceptible to WT FWPV and rFWPV-H5 infections, where the recombinant virus induced phenotypic maturation of the cells better than the WT virus. This study supports further understanding of the innate immune response after FWPV infection in chickens.

Keywords | Fowlpox, Avian influenza virus, Chicken, Dendritic cells, Haemagglutinin

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INTRODUCTION

Fowlpox is an important disease in commercial poultry farming, as it affects many production-system variables, such as size, performance and viability. The mild cutaneous form of the causative fowlpox virus (FWPV) infection from biting insects is characterised by nodular, multifocal skin lesions on un-feathered areas of the comb and wattle, the face and the legs of infected birds. The more severe diphtheritic form, which is caused by inhalation or ingestion of infectious droplets, leads to proliferative abrasions on the mucous membranes of the upper gastroin-

testinal and respiratory tracts. FWPV possesses a large double-stranded 300 kb DNA molecule that is visible as an inclusion body under a light microscope after staining. Due to this ease of observing and propagating embryonated chicken eggs and embryo fibroblast (CEF) monolayer cultures, vaccines against FWPV were among the first to be licensed (Giotis and Skinner, 2019). The attenuated, live FWPV vaccine strains, which were developed during the 1920s, have paved the way for their manipulation as recombinant vectors expressing antigens of significant avian pathogens, such as avian influenza virus (AIV; (Mariatulqabtiah et al., 2019; Majid et al., 2020) and Newcastle disease virus (Sun et al., 2008). Recombinant FWPV expressing the *H5* gene of AIV (rFWPV-H5) has been commercially used in chickens since the 1990s. In comparison to the conventional killed AIV vaccine, the rFWPV vaccine platform uses an *in vitro* culture system and offers several advantages, including a strict host range for viral replication, targeting of multiple antigens due to its large gene cloning capacity, ability to differentiate between infected and vaccinated flocks and avoids the use of adjuvants and laborious egg inoculation for a better containment from accidental release into the environment (Skinner et al., 2005). Despite a large number of studies on the immune response after rFWPV-AIV inoculation of chickens, the direct susceptibility of host innate immune cells, i.e. dendritic cells (DCs), upon FWPV and rFWPV-H5 infection is unknown. Nonetheless, the HD11 monocytic, macrophage cell line can sense WT FWPV infections but downregulates the IFN and MHC transcripts as a response, potentially due to the presence of predisposed viral suppressors for pattern-recognition receptor signalling (Oliveira et al., 2021). Similar to macrophages, DCs are professional antigen-presenting cells (APCs), which play a central role in innate immune defence before subsequent regulation of the adaptive immune response. The expression of MHC class I and II and costimulatory molecules, such as CD40, CD80 and CD86 on their surfaces, increases once DCs are stimulated, indicating that the cells are undergoing a complex process of maturation (Yasmin et al., 2015). Therefore, the present study characterised the morphological and immunophenotypic responses of chBM-DCs after WT FWPV and rFWPV-H5 infections.

The *in vitro* experiments performed in this study were conducted in a Biosafety Level 2 facility following the policies and ethical standards of the local Department of Biosafety, through the Institutional Biosafety and Biosecurity Committee of Universiti Putra Malaysia (reference no. JBK(S) 600-1/3/20(16)). Animal handling and the protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (reference no. UPM/IACUC/AUP-R065/2019).

The initial stock of attenuated, lab-adapted WT FWPV strain FP9 was obtained from Dr. Mike Skinner (GenBank Accession No. AJ581527.1), while the rFWPV-H5 was constructed previously (Mariatulqabtiah et al., 2019). Both viruses were propagated in chorioallantoic membranes (CAMs) of 10-day-old specific-pathogen-free (SPF) chicken embryos and harvested 7 days later. The control and infected CAMs were either fixed in 10% formalin for histological examination using haematoxylin and eosin staining or homogenised with a tissue raptor (Qiagen, Hilden, Germany) before centrifugation at 5,000 × g at 4°C for 20 min. The viruses from the homogenate were adapted in CEF cells during three serial passages. Viral DNA from all passages was extracted according to the manufacturer's instructions (PureLink™ Viral RNA/DNA Mini Kit, Thermo Fisher, Waltham, MA, USA). Genetic stability of the WT FWPV and rFWPV-H5 was verified by polymerase chain reaction (PCR) using the FP9orf176 primer set (5′-ACG TGT CCC TTT ACC TCC-3′ and 5′-TTC CTT GCC ATC TAC GCC-3′; GenBank Accession no. AJ581527 (Laidlaw et al., 2013) and the H5 primer set (5′-ATG GAG AAA ATA GTG CTT TTT G-3′ and 5′-TTA AAT GCA AAT TCT GCA TTG TAA CG-3′; GenBank Accession no. DQ320934 (Mariatulqabtiah et al., 2019), respectively, from the extracted viral DNA. PCR amplification was performed based on the manufacturer's instructions from the MyTaq™ Red Mix (Bioline, Taunton, MA, USA). Each PCR sample consisted of 100 ng of DNA template, 20 µM of the forward and reverse primers, 10 µL of 2× MyTaq Red Mix and distilled water, in a total reaction volume of 20 µL. The mixture was incubated in the C1000 Touch™ BioRad thermal cycler (BioRad Laboratories, Hercules, CA, USA) with 26 cycles of 95°C (0.5 min), 59°C (0.5 min) and 72°C (1.5 min).

Femurs and tibias of 4–7-week-old SPF chickens (reared at the Animal Research Centre, Institute of Tropical Agriculture and Food Security) were isolated (Yasmin et al., 2015). Approximately 30.0 × 10^6 bone marrow cells were seeded into T-25 tissue culture flasks. The cultures were maintained in RPMI complete medium (Sigma-Aldrich, Singapore) with 10% chicken serum (Gibco, Grand Island, NY, USA), 1 U/ml penicillin, 1 µg/ml streptomycin (Gibco) and 1% L-glutamine (Gibco). Recombinant chicken granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 (1/250) (Yasmin et al., 2015) were added to the media to promote the generation of chBM-DCs. The stimulated cells were maintained at 37°C in a 5% CO_2 incubator for 6 days to allow differentiation before the mock (0.1 ml 1% PBS [v/v]) or viral infections at a multiplicity of infection of 0.3, for 2 h before washing. Cellular morphological changes and contamination were routinely observed and photographed under an inverted microscope (Leica Microsystems, Singapore) at 24 h

post-infection (PI). The WT FWPV and rFWPV-H5-infected chBM-DCs (10^6 cells) were stained with specific fluorochromes (Lightning Link™ labelling kits, Innova Biosciences, Cambridge, UK) before immunofluorescence labelling; allophycocyanin-conjugated mouse anti-chicken CD86 and peridinin chlorophyll A protein-conjugated mouse anti-chicken MHC class II (both from Southern Biotech, Birmingham, AL, USA) were incubated in the dark at 4°C for 45 min (Yasmin et al., 2015). Then, the percentages (mean ± SD) of the double-stained cells at 6, 12 and 24 h PI were determined by flow cytometry using a BD LSRFortessa™ Cell Analyser with BD FACSDiva software (BD Bioscience, San Jose, CA, USA). Differences between time points were analysed with the Kruskal–Wallis and Dunn's multiple comparison tests, while a *t*-test was conducted to compare the results between two groups. Statistical analyses were performed using GraphPad software (GraphPad Software Inc., La Jolla, CA, USA) A P-value < 0.05 was considered significant.

The WT FWPV and rFWPV-H5-infected CAM histological results revealed swollen cells with numerous eosinophilic inclusions (Bollinger bodies) distributed within the cytoplasm (data not shown). These inclusions, which are also known as viral factories or complexes, are viral replication concentrates that sequester nucleic acids, proteins and other small molecules important for viral operations, i.e. viral particle assembly and replication within infected cells. Visualisation under an electron microscope has been used to identify the internal biconcave core structure of the poxvirus particles (Borrel bodies) (Giotis and Skinner, 2019).

The presence of cytoplasmic effects during the three passages in CEFs indicated successful WT FWPV and rFWPV propagation. This method allowed the virus to adapt and replicate in cell culture. The viral DNA extracts from all passages revealed the presence of 240 bp of partial FWPV FP9 sequences from ORF 176 (Laidlaw and Skinner, 2004) and 1.7 kb of the *H5* gene from rFWPV-H5 infected CEFs (Mariatulqabtiah et al., 2019) (Figure 1). The verified viral stocks were titrated for subsequent analysis.

Generations of chBM-DCs were cultured by isolating femurs before stimulation. On day 1 post-seeding, the recombinant chicken GM-CSF and IL-4 supplemented cells were spherical and adhered to the plastic surface of the culture flask. On day 3, the cells started to become irregular in shape (Tan et al., 2010) and exhibited small and bright granules called intracytoplasmic circular granules. Such granules, which persisted until day 6 post-seeding, are a phenotypic feature of DCs derived from a monocyte precursor (Grassi et al., 1998). The cultured cells further differentiated into veiled and stellate-shaped cells, which corresponded to the typical morphologies of DCs (Tan et al., 2010) (Figure 2).

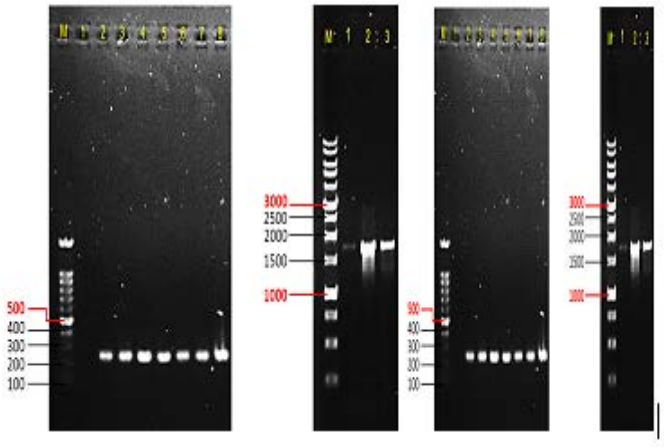

Figure 1: PCR products using (A) primer set FP9orf176 showing 240 bp expected band, whereby lanes (1) Negative control; (2) Positive control from original stock; (3) First CEF passage of WT FWPV infection; (4) Second CEF passage of WT FWPV infection; (5) Third CEF passage of WT FWPV infection; (6) First CEF passage of rFWPV-H5 infection; (7) Second CEF passage of rFWPV-H5; (8) Third CEF passage of rFWPV-H5; and (B) primer set H5 showing 1.7 kb expected band, whereby lanes (1) First CEF passage of rFWPV-H5 infection; (2) Second CEF passage of rFWPV-H5 infection; (3) Third CEF passage of rFWPV-H5 infection; M are ExactMark 100 bp or 1 kb DNA ladder marker, respectively (Base Asia). The DNA bands were observed in 1% (w/v) agarose gel.

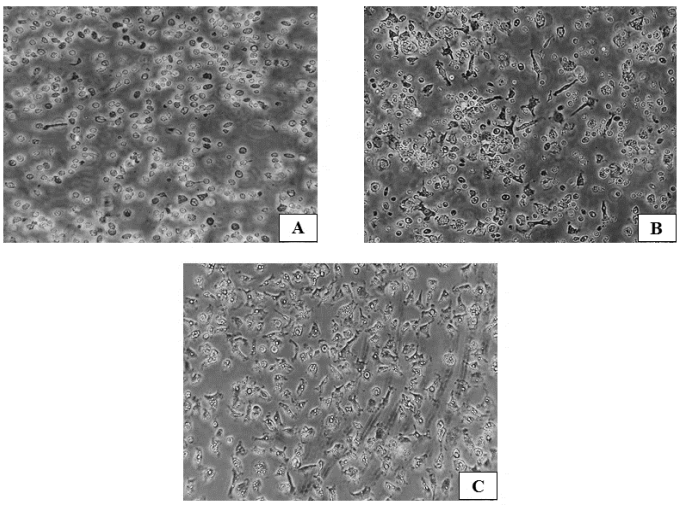

Figure 2: Morphological changes during generation and differentiation of chBM-DCs, on days 1 (A), 2 (B) and 3 (C) post seeding, using a Leica Microsystems inverted microscope. Magnification: 400X.

After the WT FWPV and rFWPV-H5 infections, the individual DCs were rounded and aggregated together in the culture medium. Non-infected DCs maintained veiled and

Table 1: Immunophenotyping of CD86 and MHC class II surface expressions on chBM-DCs following WT FWPV and rFWPV-H5 infections at different time points

Group	Hours post infection (PI)								
	CD86+MHC-			CD86-MHCII+			CD86+MHCII+		
	6	12	24	6	12	24	6	12	24
WT FWPV	19.00 ± 5.69^	10.07 ± 0.45**	16.00 ± 0.14*	2.37 ± 0.40*	5.37 ± 0.34	5.60 ± 0.37	0.50 ± 0.16[a]	0.47 ± 0.05[b]*	1.53 ± 0.05^[ab]**
rFWPV-H5	10.73 ± 0.70[e]	20.93 ± 0.82^[e]**	19.47 ± 0.90^*	3.53 ± 0.29^*	5.73 ± 0.09^	5.30 ± 0.28	0.30 ± 0.05[cd]	1.57 ± 0.24^[c]*	2.47 ± 0.05^[d]**

Each value represents the mean percentages of chBM-DCs sub-population ± SD, from three independent experiments (n=3), at 6, 12 and 24 hours PI by WT FWPV and rFWPV-H5. For control samples, the percentages for CD86+MC- is 1.73 ± 1.25, CD86-MHCII+ is 1.0 ± 0.37 and CD86+MHCII+ is 0.19 ± 1.02, whereby (^) indicates significant differences (P<0.05) between these values to the infected groups. Superscript values ([abcde]) represents significant differences at different time points within the same group (P<0.05). Single asterisks (*) represent significant difference (p<0.01) while double asterisks (**) represent highly significant differences (p<0.001) between different groups at the same time point.

stellate features with cytoplasmic projections. The infected chBM-DCs increased in size, compared to the control (Figure 3). Cell aggregation and the number of dendritic processes were more distinguished in the rFWPV-H5-infected cells, compared to those from the WT FWPV (Figure 3). Larger and longer pseudopodia are characteristics of mature DCs (Kim and Kim, 2019). Although we observed an enhanced size, the length of the dendrites was undetermined due to the limited capacity of the microscope. We also observed high expression levels of the CD86 surface marker on rFWPV during the immunofluorescence antibody test, which is another indication of mature DCs (data not shown). These data suggest that both viruses triggered the maturation of the chBM-DCs.

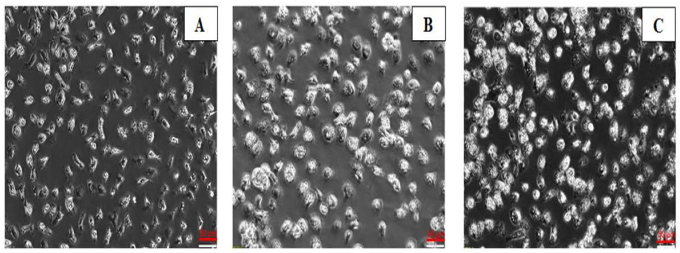

Figure 3: Morphologies of chBM-DCs for (A) non-infected control cells, (B) WT FWPV-infected cells, and (C) rFWPV-H5 infected cells, cultured in the presence of GM-CSF and IL-4, using a phase-contrast inverted microscope. Bar: 50 µm. Magnification: 200X.

Three population subsets (in percentages) were gated for immunophenotyping based on the expression of CD86 and MHC class II (CD86+MHCII–), (CD86–MHCII+) and (CD86+MHCII+) during the mock, WT FWPV and rFWPV-H5 infections (Table 1). Cell debris and lymphocyte contaminants were excluded and the gated average percent of the live cell population obtained from the analysis was >76% for all events.

According to previous studies, immature chBM-DCs exhibit moderate levels of MHC class II surface expression molecules and a low number of costimulatory surface molecules, such as CD80 and CD86, while higher levels are displayed at maturation (Liang et al., 2015; Yasmin et al., 2015). Our data show that the WT FWPV and rFWPV-H5 infections increased the CD86+MHC– population, beginning 6 h PI, in which the recombinants expressed significantly higher levels of CD86 at 12 and 24 h PI, compared to the WT. The percentage of chBM-DCs expressing the MHC class II marker ranged from two to three-fold lower than that of the CD86+ cells. The rFWPV-H5 infection increased the MHC class II expression levels significantly as early as 6 h PI. However, the WT and rFWPV had similar CD86-MHCII+ chBM-DC populations at 12 and 24 h PI. The substantial levels of CD86 at 12 and 24 h PI, and the expedited response of MHC class II molecules at 6 h PI after the rFWPV-H5 infection may have been due to the presence of the H5 antigen. The CD86+MHCII+ chBM-DCs were significantly higher at 12 and 24 h PI due to the recombinants compared to the WT FWPV. As a surface glycoprotein that plays a critical role in determining the pathogenicity of AIV, co-expression of haemagglutinin H5 in rFWPV increased the vaccine-induced immune response compared to the control. Unfortunately, the levels of chemokines, cytokines and Toll-like receptors were not measured during the experiment, which would further support this postulation.

DCs are classified as APCs that help induce adaptive immunity at maturation, differentiation and activation. Environmental and microbial stimuli trigger the maturation and function of this immune cell. Previous studies involving mammals (Summerfield et al., 2015) and chickens (Vervelde et al., 2013) have shown that DC maturation occurs in response to different pathogens. Nevertheless, research on DCs in non-mammalian hosts is relatively more challenging due to the lack of effective reagents (e.g. poor sensitivity and low cross-reactivity between samples and

commercial antibodies) and less established immortalised DC cell lines, which accelerates *in vitro* studies. Several surface markers, such as MHC class II, DEC-205, CD11, CD11c, CD40, CD83 and CD86, are expressed on chBM-DCs (Zanna et al., 2021). After stimulation by the CCR6 receptor, the immature DCs migrate to the site of infection and are stimulated due to antigen detection. This activation is accompanied by functional and phenotypic maturation, i.e. the development of surface markers is followed by the ability to present antigens to T cells (Zanna et al., 2021). The maturation process of chicken DCs is influenced by up-regulation of CCR7 and down-regulation of CCR6 (Wu et al., 2011).

chBM-DCs had higher levels of MHC class II and CD86 after stimulation by LPS, compared to stimulation by live and inactivated very virulent infectious bursal disease virus (vvIBDV). It is interesting to note that inactivated vvIBDV induces relatively lower CD86+MHCII+ than live vvIBDV 3, 6 and 24 h PI from infected chBM-DCs (Yasmin et al. 2015). This is because the CD40 and CD86 surface expression levels are higher in chBM-DCs infected with inactivated IBDV, compared to the live strain (Liang et al., 2015). This discrepancy may be due to different strains of the virus. An experiment comprising both strains would more likely reveal the mechanism of DCs during an IBDV infection. In mammalian studies, the rFWPV strain FP9 expressing the *LacZ* gene up-regulates the expression of MHC class I, class II, costimulatory molecules B7.1, B7.2, ICAM-1 adhesion molecule and the endocytosis mediator DEC-205, after infection into immortalised DCs, called DC2.4 cells (Brown et al., 2000). Our data using freshly prepared BM-DCs isolated from the chicken femur show an increased level of CD86 and MHC class II following the WT FWPV and rFWPV-H5 infections, which was higher than the controls. Although the data were biased towards rFWPV carrying the additional AIV *haemagglutinin* gene, we conclude that chBM-DCs are susceptible to WT FWPV and rFWPV, as well as can process and respond morphologically and immunophenotypically after detecting the viral antigens. An expansion of chicken DC surface markers in future experiments may further demonstrate the regulation of CDs in the context of FWPV.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

NOVELTY STATEMENT

The present study showed that chBM-DCs were susceptible to WT FWPV and rFWPV-H5 infections, where the recombinant virus induced phenotypic maturation of the cells better than the WT virus. The recombinant also expressed higher levels of surface markers CD86 and MHC class II double positive chBM-DCs at 12 and 24 hours post infection. To our knowledge, this is the first study that describes the effect of FWPV infections on freshly prepared chicken DCs, isolated from chicken femur.

AUTHORS CONTRIBUTION

ASMA and SY: designed the experiment, conducted the experiment, analysed the data, drafted the manuscript. ARY, ARO: supervised the project, provided resources and assistance for animal procedure. HKL, WST and ARM: supervised the project, reviewed and edited the manuscript. ARM: acquired the funding.

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