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ISOLATION, CHARACTERIZATION AND QUANTITATIVE PROTEOMICS ANALYSIS OF CHICKEN DENDRITIC CELLS FOLLOWING INFECTION WITH INFECTIOUS BURSAL DISEASE VIRUS

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

June 2015

DEDICATED WITH LOVE AND GRATITUDE TO:

MY LOVELY HUSBAND (MOHD HANIFF BIN ABD KADIR),

SON (MOHAMMAD YAZDAN ANIQUE) AND DAUGHTER

(NUR LUTHFIATUS SOLEHAH)

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Infectious bursal disease (IBD) is an extremely contagious and acute disease of young chicken cause by infectious bursal disease virus (IBDV). IBDV can infect B lymphocytes and macrophages. However, study on the involvement of chicken DCs during pathogen infection especially in IBDV infection has not been studied. Hypothesis of this study was chicken DCs are susceptible to IBDV infection and aimed to characterise the interaction between IBDV and chicken DCs as well as the proteomics profiles of chicken DCs during IBDV infections.

DCs were isolated from bone marrow and spleen for in vitro and ex-vivo study, respectively. The isolated DCs were characterized based on morphology, viability and immunophenotyping while IBDV detection were performed based on immunofluorescence antibody test (IFAT), quantitative real-time polymerase chain reaction (qRT-PCR) and flow cytometry. qRT-PCR was also used to detect the expression of selected cytokines from IBDV-infected DCs. Quantitative proteomics using iTRAQ coupled with tandem LC-MS/MS approach and flow cytometry analysis were performed to quantify and validate differentially regulated proteins of BM-DCs.

Morphologically, uninfected BM-DCs were rounded in shape whilst BM-DCs treated with LPS and vvIBDV showed stellate shapes. Both LPS-treated and vvIBDV-infected BM-DCs expressed high levels of CD86 and MHC class II antigens (>20 %) ($p<0.05$). In addition, vvIBDV-infected BM-DCs showed significantly higher numbers of apoptotic cells compared to LPS. Replication of vvIBDV was detected in the infected BM-DCs as evidenced by the increased in the expression of VP3 and VP4 antigens based on flow cytometry, qRT-PCR and IFAT. LPS was far more potent than vvIBDV in inducing the expression of IL-1β and IL-18, while the expressions of Th1-like cytokines, IFN-γ and IL-12α were significantly increased in vvIBDV treatment group.

iTRAQ analysis coupled with LC-MS/MS analysis, detect the most abundant proteins (~40 %) with a known membranous localization. From the total of 283 proteins that were identified, 55, 47 and 32 proteins were differentially regulated at 3, 6 and 12 hpi, respectively, as a result of vvIBDV infection, with the fold difference ≥ 1.5 or ≤ 0.67 and ProtScore of more than 1.3 at 95 % confidence level. Most of the protein functions that were impaired at 3 hpi were related to signaling, stress response and immune

response, for instance integrin α and β, heat shock proteins (HSPs) especially HSP90α and HSP60. Interestingly, no proteins related to signaling were activated at this time point. These findings give an indication that vvIBDV able to disrupt several important protein functions in order to infect BM-DCs at the early stage.

Control and infected splenic DCs were distinct as infected DCs showed star like shape. In addition, infected splenic DCs in both vaccine strain and vvIBDV strain expressed higher CD86 and MHCII antigens of more than 30 % at day 5 pi. Meanwhile, VP3 and VP4 proteins of IBDV were readily detected in splenic DCs starting from day 3 pi in both vaccine and vvIBDV-infected groups via IFAT, flow cytometry and qRT-PCR, where the expression of these antigens were significantly higher in vvIBDV ($p < 0.05$). Splenic DCs infected with vaccine and vvIBDV strains also expressed elevated levels of pro-inflammatory cytokines and chemokines such as IL-1β and CXCLi2 as well as Th1-like cytokines such as IL-12α and IFNγ after day 3 onwards.

In conclusion, chicken BM-DCs and splenic DCs are susceptible and permissive to IBDV infection. The virus infects DCs probably via common host proteins that are also found on other cells such as B cells and macrophages.

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ISOLASI, PENCIRIAN DAN ANALISIS PROTEOMIK KUANTITATIF SEL DENDRITIK AYAM SEMASA JANGKITAN VIRUS PENYAKIT BURSA BERJANGKIT

Oleh

NOR YASMIN BINTI ABD RAHAMAN

Februari 2015

Pengerusi : Profesor Abdul Rahman Omar, PhD
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Penyakit berjangkit bursa (IBD) adalah penyakit yang sangat berjangkit dan akut kepada ayam yang disebabkan oleh virus penyakit berjangkit bursa (IBDV). IBDV boleh menjangkiti sel B dan makrofaj. Walaubagaimanapun, kajian ke atas penglibatan DCs ayam semasa jangkitan patogen termasuk jangkitan IBDV belum dikaji. Hipotesis kajian ni adalah DCs ayam juga terlibat di dalam jangkitan IBDV dan tujuan kajian ini adalah untuk mencirikan interaksi antara IBDV dan DCs serta melibatkan kajian profil proteomik DCs ayam semasa jangkitan IBDV.

DCs diambil daripada sumsum tulang dan limpa untuk kajian *in vitro* dan *ex vivo*, masing-masing. DCs yang dijangkiti dicirikan berdasarkan morfologi, peratusan sel hidup, imunofenotip manakala pengesanan IBDV dilaksanakan dengan menggunakan ujian antibodi imunopendarfluor (IFAT), reaksi rantai polymerase waktu nyata kuantitatif (qRT-PCR) dan aliran sitometri. qRT-PCR juga digunakan untuk mengesan ekspresi sitokin terpilih daripada DCs yang dijangkiti IBDV. Kajian kuantitatif proteomik yang menggunakan iTRAQ berserta pendekatan LC-MS/MS dan analisis aliran sitometri dilakukan untuk mengukur dan mengesahkan protein BM-DCs.

Secara morfologi, BM-DCs yang tidak dijangkiti menunjukkan bentuk bulat, manakala BM-DCs yang dirawat dengan LPS and vvIBDV menunjukkan bentuk bintang. Kedua-dua BM-DCs yang dirawat dengan LPS dan vvIBDV mengekpresi tahap antigen CD86 dan MHC kelas II yang tinggi (>20 %) ($p<0.05$). Tambahan pula, BM-DCs yang dijangkiti dengan vvIBDV menunjukkan secara signifikasi sel apoptotik yang tinggi berbanding LPS. Replikasi vvIBDV di dalam BM-DCs yang dijangkiti dibuktikan berdasarkan kepada peningkatan ekspresi antigen VP3 dan VP4 menerusi analisis aliran sitometri, qRT-PCR dan IFAT. LPS dilihat lebih berkesan daripada vvIBDV dalam mendorong ekspresi IL-1β dan IL-18, manakala ekspresi sitokin Th1 iaitu IFN-γ dan IL-12α meningkat secara signifikan di dalam kumpulan rawatan vvIBDV.

Analisis iTRAQ bersama LC-MS/MS mengesan kebanyakan protein (~40 %) yang diketahui terletak di membran. Daripada keseluruhan jumlah protein iaitu sebanyak 283 protein yang dikenalpasti, sebanyak 55, 47 dan 32 ekspresi protein yang diatur secara berbeza selepas 3, 6 dan 12 jam jangkitan, masing-masing, disebabkan jangkitan vvIBDV, dengan perbezaan kali ganda ≥ 1.5 atau ≤ 0.67 dan *ProtScore* lebih daripada

1.3 pada 95 % tahap keyakinan. Kebanyakan fungsi protein yang terjejas pada 3 jam selepas jangkitan adalah melibatkan fungsi pengisyaratan, respon kepada stress dan tindak balas imun, sebagai contohnya protein integrin α dan β, *heat shock proteins* (HSPs) terutama sekali HSP90α dan HSP60. Menariknya tiada ekspresi protein yang terlibat di dalam fungsi pengisyaratan yang diaktifkan pada masa ini. Penemuan ini menunjukkan yang vvIBDV mampu untuk menjejaskan beberapa fungsi protein yang penting untuk menjangkiti BM-DCs pada peringkat awal.

DCs limpa daripada kumpulan yang tidak dijangkiti dan dijangkiti IBDV adalah berbeza kerana DCs yang dijangkiti menunjukkan bentuk bintang, Tambahan pula, DCs limpa yang dijangkiti dengan kedua-dua strain iaitu strain vaksin dan strain vvIBDV mengekspresi antigen CD86 dan MHCII dengan tinggi iaitu lebih daripada 30 % pada hari ke-5 selepas jangkitan. Sementara itu, protein IBDV iaitu VP3 dan VP4 dapat dikesan di dalam DCs limpa bermula dari hari ke -3 selepas dijangkiti strain vaksin dan vvIBDV menerusi IFAT, aliran sitometri and qPCR, dimana ekspresi antigen ini adalah lebih tinggi didalam ayam yang dijangkiti vvIBDV ($p< 0.05$). DCs limpa yang dijangkiti strain vaksin dan vvIBDV juga mengekspresi peningkatan kadar sitokin dan kemokin yang berkait rapat dengan tindak balas keradangan seperti IL-1β dan CXCLi2 serta sitokin Th1 seperti IL-12α dan IFNγ selepas 3 hari dijangkiti.

Sebagai kesimpulan, BM-DCs dan DCs limpa ayam adalah rentan kepada jangkitan IBDV. Virus ini menjangkiti DCs mungkin melalui protein perumah yang umum yang juga ditemui di dalam sel lain seperti sel B dan makrofaj.

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I certify that a Thesis Examination Committee has met on 25 June 2015 to conduct the final examination of Nor Yasmin Binti Abd Rahaman on her thesis entitled "Isolation, Characterisation and Quantitative Proteomics Analysis of Chicken Dendritic Cells following Infection with Infectious Bursal Disease Virus" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

A	Absorbance
AC-ELISA	Antigen capture-Enzyme-linked immunoabsorbent assay
AI	Avian Influenza
ANOVA	Analysis of variance
APC	Allophycocyanin
APC	Antigen presenting cells
ATP	Adenosine triphosphate
B	Base
BGM-70	Buffalo green monkey kidney
BM	Bone marrow
BM-DCs	Bone marrow derived dendritic cells
Bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
CAV	Chicken anemia virus
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CEE	Chicken embryonated eggs
CEF	Chicken embryo fibroblasts
CEK	Chicken embryo kidney
cIBDV	Classical strain of infectious bursal disease virus
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COS-7	Transformed African green monkey kidney fibroblast cells
Cq	Quantification cycle
CXCLi	Chemokine (C-X-C motif) ligand
DAMPs	Danger associated membrane proteins
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
ddH2O	Double distilled water
DIGE	Difference gel electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Dpi	Day post-infection
ds	Double stranded
EDTA	Ethylene-diamine-tetraacetic-acid
EID_{50}	50% Egg Infectious Dose
ELISA	Enzyme-linked immunoabsorbent assay
ERK	Extracellular-signal-regulated kinases
ESI	Electrospray ionization
FACS	Fluorescence activated cell sorter
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
FDC	Follicular dendritic cells

FDR	False discovery rate
FITC	Fluorescein isothiocyanate
G	Gauge
g	Gram
g	Gravitational force
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte monocyte-colony stimulating factor
GO	Gene Ontology
h	Hour
HIV	Human immunodeficiency virus
HPAI	High pathogenic avian influenza
hpi	hours post infection
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus HSP
HSP	Herpes simplex virus
IBD	Infectious bursal disease
IBD-ICX	IBD-immune complex
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
ICAT	Isotope-coded affinity tag
IDC	Interdigitating dendritic cells
IFAT	Immunoflourescent antibody test
IFN	Interferon
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide sythases
iTRAQ	Isobaric tag for relative and absolute quantitation
kDa	Kilo Dalton
KH2PO4	Potassium chloride
KH2PO4	Monopotassium phosphate
LAMP	Lysosomal-associated membrane protein
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LL	Lymphoid leucosis
LPS	Lipopolysaccharides
M	Molar
m/z	Mass-to-charge ratio
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MALDI	Matrix-assisted laser desorption/ionization
MD	Marek’s disease
MDA5	Melanoma differentiation-associated gene 5 -like receptors
MHC	Major Histocompatibility complex
MIP	Major intrinsic protein
ml	Milliliter
mM	Mili Molar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry

MYD88	Myeloid differentiation primary response 88
n	Sample size
NaoH	Sodium hydroxide
ND	Not detected
NDV	Newcastle disease virus
NF-kB	Kappa-light-chain-enhancer of activated B cells
NFQ	3' nonfluorescent quencher
ng	Nanogram
NK	Natural killer
OD	Optical density
OK	Ovine kidney
ORF	Open reading frame
P	Projection
p	Sample Proportion
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PerCp	Peridinin chlorophyll A protein
PI	Propidium iodide
pi	Post infection
PI	Propium iodide
PRRs	Pathogen recognition receptors
PSPEP	Proteomics System Performance Evaluation Pipeline
qRT-PCR	Real-time reverse transcription PCR
R^2	Coefficient of correlation
RFLP	Restriction fragment length polymorphism
RK-13	Rabbit kidney-13
RNA	Ribonucleic acid
Rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCX	Strong cation exchange liquid chromatography
SD	Standard deviation
SEM	Scanning electron microscope
sIgM	Surface immunoglobulin M
SILAC	Stable isotope labeling by amino acids in cell culture
sp	Species
SPF	Specific pathogen free
SPSS	Statistical program for social science
TAE	Tris-acetate-EDTA
Th-1	T helper-1
TLR	Toll-like receptors
Tm	Melting temperature
TNF	Tumor necrosis factor
TOF	Time of flight
tRNAs	Transfer ribonucleic acid
UK	United Kingdom
UPM	Universiti Putra Malaysia

UPR	Unfolded protein response
USA	United State of America
V	Volt
v/v	Volume per volume
Vero	African green monkey kidney
VNT	Virus neutralization test
VOPBA	Virus overlay protein blotting assay
VP	Viral protein
VRI	Veterinary Research Institute
VSV	Vesicular stomatitis virus
vv	Very virulent
vvIBDV	Very virulent strain of infectious bursal disease virus
w/v	Weight per volume
%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
1D-LCMS	One Dimensional Liquid chromatography–mass spectrometry
2-DE	Two-dimensional electrophoresis
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1

INTRODUCTION

Poultry industry in Malaysia is an important livestock sector which represents a major proportion of the industry besides aquaculture and pig productions. Malaysia has been self-sufficient in the poultry meat and eggs production since 2002 with the percentage of self-sufficient for 2011 at 132% (Mohamed *et al*., 2013). However, the industry is facing constant threat and challenges. Infectious diseases are major threat that cause significant economic losses in terms of mortality, condemnation of carcass, poor performance parameters and increased in the cost of medication (Omar, 2013). In addition, viral infections such as Marek's disease virus (MDV), retrovirus causing lymphoid leucosis (LL), chicken anemia virus (CAV) and infectious bursal disease virus (IBDV) can impair the host immune responses, hence, causing immunosuppression (Hoerr, 2010). Viral induced immuosuppression is a problematic issue that is challenging to manage in poultry flocks due to its significance impact to the health status of the chickens and can lead to vaccination failure against various diseases.

Infectious bursal disease virus (IBDV) is classified under the family *Birnaviridae* (Dobos, 1979), a bi-segmented and double-stranded RNA (dsRNA) virus with a single-shelled, non-enveloped virions (MacDonald, 1980; Müller *et al*., 1979). Serotype I IBDV strains can be grouped into different subtypes/strains namely classical strains, variant strains and very virulent strains (van den Berg, 2000). Among these strains, the vvIBDV strain have been reported in several countries and have caused serious problem in commercial poultry industry due to the inability of maternal antibody from classical IBDV vaccine in inducing complete protection (Williams & Davidson, 2005). The virus cause a disease known as infectious bursal disease (IBD) (Gumboro disease), which is difficult to control in commercial flocks since it able to resist many disinfectants and capable of causing high mortality and inducing immunosuppression in susceptible chickens (Van Den Berg *et al.,* 2004).

Infectious bursal disease virus (IBDV) is a lymphotropic virus which known to target IgM+ B cells (Withers *et al*., 2006; Rodenberg *et al*., 1994) and macrophage (Palmquist *et al*., 2006; Khatri *et al*., 2005). Moreover, IBDV infection promotes infiltration of T cells in infected organ such as bursa of Fabricus (Rautenschlein *et al*., 2002; Kim *et al*., 2000). However, T cells are refractory to IBDV infection (Mahgoub, 2012). Hence, chicken infected with the virus develop immunosuppression due to the depletion of IgM bearing B-lymphocytes and disturbance in the innate and cell-mediated immunity responses due to direct activation of macrophage and indirect activation of T cells which subsequently lead to massive production of proinflammatory cytokines (Ingrao *et al*., 2013). In contrary, the involvement of other immune cells such as dendritic cells (DCs) during IBDV infection has not been characterized.

In mammals, dendritic cells (DCs) are well known as professional antigen presenting cells (APC) linking the innate and acquired immunity during combating infectious diseases (Steinman *et al*., 2003). DCs progenitors are originated from bone marrow and further differentiated into circulating immature DCs with high ability to capture antigen (Granucci *et al*., 2003). Once exposed to pathogens, immature DCs migrated to T cell regions of different lymphoid organs and undergo maturation with high antigen presenting capabilities particularly to CD4 helper T cells for the activation of immune responses (Liu, 2001). The activated helper T cells play an important role in activating other cells such as natural killer cells (NK), eosinophils and macrophages as well as antigen specific cells such as B cells and CD8 cytotoxic T cells. As a result, these cells are recruited to migrate to the damage or infected site in order to prevent the infection from continue to harm the host (Rescigno & Borrow, 2001).

Currently, majority of the studies on the interaction between viruses and DCs are on human virus infection. DCs infected with respiratory syncytial virus (RSV) associated with virus replication and DCs maturation (González *et al*., 2008). Meanwhile, viruses such as dengue virus, influenza virus and herpesvirus are able to replicate inside DCs but impede the maturation process (Boonnak *et al*., 2008; Fernandez-Sesma *et al*., 2006; Novak and Peng, 2005). On the other hand, viruses such as human papilloma virus able to present antigen without replicating inside DCs (García-Piñeres *et al.*, 2006). Hence, characterisation of the interplay between viruses with chicken DCs will provide valuable information in the role of DCs during infection and immunity.

In addition, chicken lack of lymph node as a defense mechanism against infection, yet, chicken also exposed to various kind of pathogen akin to mammals (Wu & Kaiser, 2011). Hence, the involvement of APC are utmost crucial in immune system of chicken. In chicken, DCs progenitor from bone marrow; follicular DCs from secondary lymphoid organs namely spleen, Harderian glands, Payer's patches and cecal tonsils; as well as Langerhans cells have been studied (Ly *et al.*, 2010; Wu *et al*., 2010; del Cacho *et al*., 2008; Igyarto *et al*., 2006). However, the involvements of chicken DCs during viral infection and immune responses are not well characterized. Thus far, no studies have investigated the role of chicken DCs during IBDV infection. Fundamental study on the interaction of DCs and IBDV will provide valuable information in understanding the role of professional APCs in chickens and their molecular interactions during IBDV infection and vaccination. Since, B cells and macrophages are also APC and are the target of IBDV infection, the hypotheses of this study are:

a) chicken DCs are susceptible to IBDV infection
b) IBDV infected DCs will secrete cytokines resemble other APC such as B cells and macrophages
c) proteomics profiling of IBDV infected DCs will identify differentially regulated DCs proteins that are important during IBDV infection

Hence, in order to address these hypotheses, the specific objectives of this study were:
a) to isolate and compare the bone marrow derived dendritic cells (BM-DCs) response following *in vitro* stimulation with vvIBDV and *lipopolysaccharides* (LPS)
b) to determine the expression levels of IBDV, DC activation markers and cytokines production of BM-DCs following *in vitro* vvIBDV infection
c) to identify the proteome of BM-DCs following *in vitro* vvIBDV infection based on iTRAQ and LC/MS-MS analysis
d) to characterize *in silico* and validate the expressions of differentially regulated proteins of BM-DCs following *in vitro* vvIBDV infection
e) to isolate and compare the splenic DCs responses based on expression levels of IBDV, DC activation markers and cytokines production following vaccine strain and very virulent strain of IBDV inoculation in SPF chickens

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