UNIVERSITI PUTRA MALAYSIA

EFFECTS OF ALUM AND ADDAVAX ON IMMUNE RESPONSES AND PROTECTION INDUCED BY ECTODOMAIN OF INFLUENZA M2 PROTEIN DISPLAYED ON NODAVIRUS CAPSID

ONG HUI KIAN

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By

ONG HUI KIAN

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

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November 2021

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Multiple copies of conserved ectodomain of matrix 2 protein (M2e) of influenza A virus (IAV) were genetically fused to the end of the C-terminal region of the capsid protein of *Macrobrachium rosenbergii* nodavirus producing chimeric proteins which assemble into virus-like particle (VLPs) displaying M2e-epitopes (NvC-M2ex3). Immunisation and virus challenges in BALB/c mice suggested that NvC-M2ex3 was indeed immunogenic and protective against lethal mouse-adapted A/PR/8/34 (H1N1) and A/HK/8/68 (H3N2) challenges. Nevertheless, previous studies lack mechanistic data that explain the protective effect of NvC-M2ex3. In addition, the effects of adjuvants on the immune responses and protection induced by NvC-M2ex3 remain elusive to date. Therefore, the objective of this study was to investigate the effects of two adjuvants, Alum and AddaVax on the immune responses and protection elicited by NvC-M2ex3 in BALB/c mice. Following immunisation, NvC-M2ex3 was shown to be well tolerated in animal, particularly when adjuvants were not involved in the formulation. Nevertheless, splenomegaly was observed in animal immunised with NvC-M2ex3 in the presence of Alum. No apparent morbidity was manifested in all mice immunised with NvC-M2ex3. Immunogenicity study indicated that antibody responses induced by NvC-M2ex3 were tailored by the adjuvants. AddaVax was demonstrated to induce a helper T-cell type 1 (Th1) skewed immune responses as supported by a higher IgG2a:IgG1 ratio and stronger Th1 cytokines profile, contrary to Alum. Immunophenotyping via flow cytometry analysis indicated that NvC-M2ex3 and adjuvants induced a CD4+ T-cell dominant response, higher macrophage but lower natural killer (NK) cell populations. Gene expression analysis of the mouse spleen via quantitative polymerase chain reaction (qPCR) suggested upregulation of T-cell related genes, corresponding to helper T-cell type 2 (Th2) and Th1 immunities induced by NvC-M2ex3 adjuvanted with Alum and AddaVax, respectively. Viral challenges indicated that NvC-M2ex3 conferred 100% protection against mouse-adapted H1N1 and H3N2 infections and reduced viral loads in the lungs and oropharynx of the mice. Co-administration of NvC-M2ex3 with adjuvants was demonstrated to be critical in improving morbidity in H1N1 but not in H3N2

challenges. All mice succumbed to the infection were shown to induce a CD8 T-cell dominant response which might contribute to immunopathology. Depending on the strains of IAV and adjuvants used, NvC-M2ex3 was shown to alter the splenic NK cell and macrophage counts differently. Gene expression study on the spleen of the mice challenged with H1N1 or H3N2 indicated that lower expression of genes associated with T-cell activation and cellular cytotoxicity correspond to improved disease outcomes. As a summary, NvC-M2ex3 induced protective immunity against IAVs and adjuvants were shown to improve protection against H1N1 infection. Although different adjuvants activated distinctive immune responses, both adjuvants contributed to protection against IAV challenges and ameliorated morbidity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN ALUM DAN ADDAVAX TERHADAP TINDAK BALAS DAN PERLINDUNGAN IMUN YANG DITIMBUL OLEH ECTODOMAIN PROTEIN INFLUENZA M2 YANG DIPAPARKAN PADA NODAVIRUS CAPSID

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Penyambungan beberapa salinan ektodomain protein matriks 2 (M2e) virus influenza A secara genetik ke penghujung C protein kapsid *Macrobrachium rosenbergii* nodavirus dijalankan untuk menghasilkan viral partikel (VLPs) yang memaparkan M2e-epitop (NvC-M2ex3). Kajian jangkitan dan imunisasi pada tikus BALB/c menunjukkan bahawa NvC-M2ex3 adalah imunogenik dan melindungi tikus daripada dijankiti oleh A/PR/8/34 (H1N1) dan A/HK/8/68 (H3N2). Walaubagaimanapun, kajian terdahulu kekurangan data mekanistik yang menjelaskan kesan perlindungan NvC-M2ex3. Di samping itu, kesan adjuven terhadap tindak balas imun dan perlindungan yang dirangsangkan oleh NvC-M2ex3 masih sukar difahami. Oleh itu, objektif kajian ini adalah untuk mengkaji kesan dua adjuven, Alum dan AddaVax terhadap tindak balas imun dan perlindungan yang ditimbulkan oleh NvC-M2ex3 pada tikus BALB/c. Melalui kajian imunisasi, NvC-M2ex3 terbukti dapat ditoleransi dengan baik pada haiwan, terutama ketika adjuven tidak dimasukkan dalam formulasi. Walaupun begitu, splenomegali telah diperhatikan berlaku pada haiwan yang diimunisasi dengan NvC-M2ex3 dan Alum. Tidak ada morbiditi yang nyata pada semua tikus yang diimunisasi dengan NvC-M2ex3. Kajian imunogenisitas juga menunjukkan bahawa tindak balas antibodi yang ditimbulkan oleh NvC-M2ex3 dipengaruhi oleh adjuven yang digunakan. AddaVax didapati mendorong tindakbalas imun sel T-pembantu jenis 1 (Th1) yang disokong oleh nisbah IgG2a: IgG1 yang lebih tinggi dan profil sitokin Th1 yang lebih kuat, bertentangan dengan Alum. Imunofenotaip melalui analisis sitometri aliran menunjukkan bahawa NvC-M2ex3 dan adjuven mendorong tindak balas dominan sel-T CD4+, makrofag yang lebih tinggi dan populasi sel pembunuh semula jadi (NK) yang lebih rendah. Kajian ekspresi gen limpa tikus melalui tindak balas rantai polimerase kuantitatif mencadangkan pengaktifan gen berkaitan sel T, selaras dengan tindak balas imun pembantu sel-T jenis 2 (Th2) dan Th1 yang ditimbulkan oleh NvC-M2ex3 dengan Alum dan AddaVax, masing-masing. kajian cabaran virus menunjukkan bahawa NvC-M2ex3 memberikan perlindungan 100% terhadap jangkitan H1N1 dan H3N2 dan mengurangkan kandunagn virus load pada tikus orofaring dan paru-paru. Pemberian bersama NvC-M2ex3 dengan adjuven terbukti penting dalam mengurangkan tahap morbiditi pada H1N1 tetapi tidak dalam infeksi

H3N2. Semua tikus yang terkena jangkitan terbukti menyebabkan tindak balas dominan sel-T CD8+ yang mungkin menyumbang kepada imunopatologi. Bergantung pada ketegangan IAV dan adjuven yang digunakan, NvC-M2ex3 ditunjukkan untuk mengubah jumlah sel NK splenik dan makrofag secara berbeza. Kajian ekspresi gen pada limpa tikus yang dijangkiti oleh H1N1 atau H3N2 menunjukkan bahawa ekspresi gen yang berkaitan dengan pengaktifan sel-T dan sitotoksisitas sel yang lebih rendah selaras dengan penambahbaikan hasil penyakit. Kesimpulannya, NvC-M2ex3 memberikan perlindungan terhadap IAV dan adjuven meningkatkan perlindungan terhadap jangkitan H1N1. Walaupun adjuven yang berlainan mengaktifkan tindak balas imun yang tersendiri, kedua-dua pembantu ini menyumbang kepada perlindungan terhadap cabaran IAV dan penurunan morbiditi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follow:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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ργ	picogram (10^{-12} g)
µg	microgram (10^{-6} g)
µL	microlitre (10^{-6} L)
µm	micrometre (10^{-6} m)
µM	micromolar (10^{-6} M)
ADCC	antibody-dependent cell mediated cytotoxicity
ADCP	antibody-dependent cell mediated phagocytosis
ANOVA	one-way analysis of variance
APC	allophycocyanin
AS03	adjuvant system 03
Baso	basophil.
BN	band neutrophil
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
bp	basepair
BSA	bovine serum albumin
℃	degree Celsius
CD	cluster of differentiation
CDC	complement-dependent cytotoxicity
cDNA	complimentary DNA
Ct	cycle threshold
C-terminal	carboxyl terminal
CTL	cytotoxic T-cells
CV	column volume

DC	dendritic cells
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESCRT	endosomal sorting complex required for transport
Fc	fragment crystallisable
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
g	gram
G	gauge
p-NPP	p-nitrophenyl phosphate
HA	haemagglutinin
Hb	haemoglobin
HBV	hepatitis B virus
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPV	human papilloma virus
HRP	horseradish peroxidase
IAV	influenza A virus
IFN	interferon
Ig	immunoglobulin
IIV	inactivated influenza A vaccine
IL	interleukin
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton

kg	kilogram
L	litre
LB	Luria-Bertani
LD_{50}	median lethal dose
Lymph	lymphocyte
M1	matrix 1
M2	matrix 2
M2e	extracellular domain of matrix 2 protein
mA	milliampere
MA	mouse-adapted
MCHC	mean corpuscular
MCV	mean corpuscular volume haemoglobin concentration;
mg	milligram (10^{-3} g)
MHC	major histocompatibility complex
mL	millilitre (10^{-3} L)
mm	millimetre (10^{-3} m)
mRNA	messenger RNA
MrNV	*Macrobrachium rosenbergii* nodavirus
n	number of biological replicates
NA	neuraminidase
ng	nanogram (10^{-9} g)
NK	natural killer
nm	nanometre (10^{-9} m)
NOD	nucleotide-binding oligomerisation domain
NP	nucleoprotein

NPT	nitro-blue tetrazolium chloride
N-terminal	amino terminal
NvC	nodavirus capsid
NvC-M2ex3	nodavirus capsid fused with 3 copies of M2e
PCV	packed cell volume
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
pH	*Puissance hydrogen*
PLT	platelet
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline supplemented with Tween-20
Th	helper T-cells
Th1	helper T-cell type 1
Th2	helper T-cell type 2
TLR	toll-like receptors
TNF	tumour necrosis factor
vRNP	viral ribonucleoproteins
V	volt

v/v	volume/volume
VLPs	virus-like particles
w/v	weight/volume
WBC	white blood cell
WHO	World Health Organization
α	alpha
β	beta
γ	gamma

CHAPTER 1

INTRODUCTION

1.1 Research background

Influenza A virus (IAV) is one of the most notorious viruses in the human history. The first reliable documented influenza pandemic could be dated back to about 440 years ago in 1580. The virus first emerged in Asia and Russia, and spread to Europe and Africa followed by Americas. The actual death-toll caused by the pandemic was unknown but 8,000 deaths was reported in Rome alone. Although several studies indicated that influenza pandemics could have been emerged in the early 16th century, consensus among scholars cannot be reached (Potter, 2001). To date, a total of 10 large-scale influenza pandemics were reported. The most notorious pandemic, commonly known as the Spanish flu occurred in 1918, was accounted for the death of at least 50 million worldwide while infecting one-third of the world population (Taubenberger & Morens, 2009). The timeline of the influenza pandemics is summarised in Table 1.1. Despite the occurrence of multiple influenza pandemics since the 16th century, the causative agent of the infections remained unidentified until 1933 when Richard Shope isolated the first IAV from swine (Van Epps, 2006). Soon after successful isolation of IAV, the first inactivated influenza vaccine was produced by Thomas Francis Jr. and Jonas Salk in 1938 for use in military forces during the World War II (Barberis et al., 2016). Despite the presence of the seasonal influenza vaccines today, annual seasonal influenza epidemics remain responsible for 290,000 to 650,000 deaths globally, suggesting potential limitations of the current vaccination strategy (WHO, 2018).

Table 1.1: Timeline of the influenza pandemics from 1580 to present

Name	Date	Origin	Virus	Mortality	References
-	1580	Asia	-	-	(Potter, 2001)
-	1729-1733	Russia	-	-	(Potter, 2001; Taubenberger & Morens, 2009)
-	1761-1762	Americas	-	-	(Taubenberger & Morens, 2009)
-	1780-1782	Southeast Asia	-	-	(Potter, 2001; Taubenberger & Morens, 2009)
-	1830-1837	Southeast Asia	-	-	(Potter, 2001; Taubenberger & Morens, 2009)
Russian flu	1889-1893	Asia	-	-	(Taubenberger & Morens, 2009)
Spanish flu	1918-1919	United States	H1N1	>50 million	(Taubenberger & Morens, 2009)
Asian flu	1957-1958	Singapore	H2N2	1.1 million	(Centre of Disease Control, 2019)
Hong Kong flu	1968-1969	Hong Kong	H3N2	1 million	(Centre of Disease Control, 2019)
Swine flu	2009-2010	United States	H1N1	0.15-0.57 million	(Centre of Disease Control, 2019)

1.2 Problem statement and justification

In general, influenza vaccines confer protection to the immunised individuals via the induction of the hemagglutinin (HA) and neuraminidase (NA)-specific immune responses. HA and NA are the most prominent, immunogenic surface glycoproteins of IAV. Nevertheless, these glycoproteins are highly mutative via antigenic drift or shift. Therefore, seasonal influenza vaccines have to be reconstituted annually based on the surveillance findings of the previous season to incorporate the most probable mutations of IAVs circulating in the following season (Barberis et al., 2016). Notably, these vaccines are produced based on the "smart guess", thus the vaccine effectiveness varies significantly depending on the sequence homology of the vaccine strains recruited in vaccine formulation and that of the actual circulating strains. In the cases of vaccine mismatch, the effectiveness of the vaccine is dramatically reduced (Lewnard & Cobey, 2018). In 2017, seasonal influenza epidemic in Australia hit a record-breaking hospitalisation and mortality rates due to vaccine mismatch, and the vaccine effectiveness was indicated to be an unprecedented low of 10% (Sullivan et al., 2017). In addition, current influenza vaccines are not effective against new influenza pandemic caused by new virulent strains of IAV emerged from antigenic shift, as evidenced in the "Swine flu" pandemic in 2009 (Zhang et al., 2014). Variation in vaccine effectiveness from season to season, and the necessity of annual vaccine reformulation remains challenges to be overcome.

Centre of Disease Control recommends annual seasonal influenza vaccination for maximum protection against seasonal flu. Nevertheless, recent reports indicated that vaccine effectiveness of the seasonal influenza vaccines could be dampened following consecutive influenza vaccinations. It was demonstrated that antibody avidity and antibody half-life were significantly blunted following consecutive vaccinations, possibly due to impaired T-cell response, which is also hampered following consecutive immunisations (Khurana et al., 2019; Richards et al., 2020; Zelner et al., 2019). In addition, one study also suggested that high pre-existing antigen-specific antibody titre might induce antigen clearance and epitope masking, thereby inhibiting T-cell-dependent B-cell activation in subsequent vaccination (Stacey & Miller, 2020). Nevertheless, further elucidation is required to explain this scenario from the immunological perspective.

Attributed to the limitations of current seasonal influenza vaccines, major research focus is shifting to the development of universal influenza vaccines. The ectodomain of matrix 2 protein (M2e) of IAV represents an attractive target for the development of universal influenza vaccines due to its extreme conservativity among human IAVs (Fiers et al., 2009). Nevertheless, M2e is poorly immunogenic under natural infection or active immunisation, due to its small size and low copy number on the virion surface (Feng et al., 2006; Hutchinson et al., 2014). Different virus-like particles (VLPs) were previously recruited to enhance the immunogenicity of M2e including the VLPs of hepatitis B virus (HBV) (Ravin et al., 2015), human papilloma virus (HPV) (Ionescu et al., 2006), bacteriophage (Bessa et al., 2008), papaya mosaic virus (Carignan et al., 2015), and norovirus (Tan & Jiang, 2012). These VLPs-based vaccines were demonstrated to be immunogenic, and some were shown to be protective against influenza infections in animal models. VLPs composed of repetitive viral capsid proteins mimic the natural infection pathway of the native virus, priming the host immunity without inducing severe clinical symptoms (Rhee, 2020). VLPs are devoid of viral genome, thus they are not replicative and not infectious, demonstrating superior safety profile compared to live-attenuated vaccines. In addition, some VLPs contain pathogen-associated molecular patterns (PAMPs) for innate immune sensing, suggesting potential self-adjuvanticity (Ong et al., 2017).

Capsid protein of nodavirus expressed in bacteria was shown to assemble into VLPs of approximately 30 nm in diameter (Goh et al., 2011). Genetic fusion of multiple copies of M2e-epitopes to the C-terminal end of the capsid protein of nodavirus produced chimeric proteins which assemble into VLPs displaying M2e (NvC-M2ex3) (Yong et al., 2015). Immunisation study and virus challenges in BALB/c mice demonstrated that NvC-M2ex3 was indeed immunogenic and protective against lethal mouse-adapted A/PR/8/34 (H1N1) and A/HK/8/68 (H3N2) challenges (Ong et al., 2019; Yong et al., 2015). Although antibody response was evaluated in the past, isotypes of M2e-specific antibodies were not evaluated and T-cell responses were not studied to a great extent. Furthermore, numerous studies have indicated that adjuvants improved vaccine protective efficacy in several M2e-based vaccine (Zhu et al., 2021), thus it would be valuable to evaluate the potential synergistic effects of the adjuvants on NvC-M2ex3 against IAVs. Therefore, the general objective of this study was to investigate the effects of two adjuvants, Alum and AddaVax on the immune responses and protection elicited by NvC-M2ex3 in BALB/c mice. The specific objectives of the study include:

1. To assess the general toxicity of NvC-M2ex3 in BALB/c mice.
2. To evaluate the effects of different adjuvants on the B-cell and T-cell responses induced by NvC-M2ex3 in BALB/c mice.
3. To determine the population of innate immune cells in mice immunised with NvC-M2ex3.
4. To assess the protective effects of NvC-M2ex3 supplemented with different adjuvants against H1N1 and H3N2
5. To elucidate the gene expression profiles of the splenocytes of mice immunised with NvC-M2ex3

1.3 Research hypothesis

NvC-M2ex3 is speculated to be well tolerated in BALB/c mice following immunisations. Inclusion of adjuvants in vaccine formulation is hypothesised to enhance the reactogenicity of NvC-M2ex3 which may cause splenomegaly in mice. NvC-M2ex3 is hypothesised to induce a high level of M2e-specific antibody in BALB/c mice following immunisation. Supplementation of the adjuvants to NvC-M2ex3 is speculated to further enhance the antibody response. Different adjuvants supplemented to NvC-M2ex3 are hypothesised to induce distinct innate immune responses and T-cell profiles. NvC-M2ex3 with or without adjuvants was anticipated to elicit protective response against H1N1 and H3N2. Nevertheless, co-administration of NvC-M2ex3 with adjuvants is hypothesised to further improve the protective immunity.

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