CONSTRUCTION AND CHARACTERIZATION OF H5N1-RECOMBINANT FOWLPOX VIRUSES CO-EXPRESSING HOST CYTOKINES

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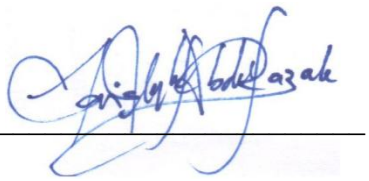
DECLARATION

I declare that the work described herein is my own except where all citations and contributions of others have been acknowledged. I declare that cDNA copies of H5, N1 and NP genes of avian influenza (AI) from strain A/Chicken/Malaysia/5858/2004 used in this study has been cloned and sequenced by Nurul Hidayah Abdullah Zawawi from Universiti Putra Malaysia, and has been exploited by four individuals in their dissertations for examination in Universiti Putra Malaysia.

I also declare that no part of this dissertation has been submitted for any other degree in the UK or overseas.



MARIATULQABTIAH ABDUL RAZAK

ABSTRACT

Possessing a large double stranded DNA genome up to 300 kb, fowlpox virus (FWPV) has been developed to express avian influenza virus (AIV) antigens since the late 1980s. A more advanced approach would be to coexpress host cytokines from such recombinants. This thesis describes the strategy to construct H5N1-recombinant FWPV (rFWPV) coexpressing chicken Interleukin 12 (IL-12) or Interleukin 15 (IL-15), and discusses the immunogenicity of the recombinants following inoculation into specific-pathogen-free (SPF) chickens.

Previously cloned and sequenced cDNAs encoding full-length H5 and N1 of influenza strain A/Chicken/Malaysia/5858/2004 genes were amplified by PCR and inserted into plasmid pEFL29, under the control of a copy of the vaccinia virus p7.5 early/late promoter. The expression cassettes were recombined into the genome of the FP9 strain of FWPV at the fpv002 locus. Recombinant viruses were produced by transfection of the plasmid into chicken embryo fibroblasts (CEFs) after infection with FP9, and isolated by six fold plaque purification on CEFs using X-Gal selection. Chicken IL-12 or IL-15 genes, under control of a synthetic/hybrid poxvirus promoter, were inserted into a 'transient dominant selection' recombination plasmid, pPC1.X. The cytokine expression cassettes were then recombined, at the fpPC1 (fpv030) locus, into rFWPV already carrying AIV genes. Following three rounds of passage in CEFs in the presence of mycophenolic acid (MPA), recombinant viruses carrying the *gpt* gene were isolated. These unstable recombinants were plaque-purified in the absence of MPA until they lost the *gpt* gene spontaneously, verified by their failure to replicate in the presence of MPA. Recombinant proteins were successfully detected using western blotting and indirect immunofluorescence assay (IFAT).

Parental and rFWPV (10^5 PFU) were inoculated subcutaneously into one-day-old SPF chickens. Sera from chickens immunized with rFWPV/H5 and rFWPV/H5/IL-15 demonstrated viral neutralizing activities, based on the haemagglutation inhibition (HI) test, in which reached a peak at Week 3. A competitive enzyme-linked immunosorbent (ELISA) assay detected N1-specific antibodies induced by rFWPV/N1 and rFWPV/N1/IL-12 at Weeks 4 and 5. Non-specific cellular immune responses were assessed by flow cytometric analysis to enumerate CD4+ and CD8+ T-lymphocytes in peripheral blood. Results of Experiment 2 showed chickens vaccinated with rFWPV/H5, rFWPV/H5/IL-15, rFWPV/N1 and rFWPV/N1/IL-12 demonstrated a higher increase in CD8+ than CD4+ T cell population, relative to control and chickens vaccinated with parental FWPV. Weekly weighing showed that chickens vaccinated with rFWPV/H5/IL-15 had the highest body weight compared to other groups, while the rFWPV/N1/IL-12 group showed the significantly lowest body weight.

In summary, this study showed diverse immunogenicity of H5N1-rFWPV coexpressing IL-12 or IL-15. It also demonstrated a weight sparing effect of co-expressing IL-15 in rFWPV vaccines. The results provide the basis for future homologous challenge studies, using live H5N1 virus to evaluate the protective efficacy of the rFWPV vaccines.

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ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AIV	Avian influenza virus
ANOVA	analysis of variance
ANT3	membrane adenine nucleotide translocator 3
ATP	Adenosine-5'-triphosphate
BCG	bacille Calmette-Gue´rin
β-gal	beta galactosidase
bp	base pairs
BSA	bovine serum albumin
^{o}C	degree centigrade
cDNA	complementary DNA
CEF	chicken embryonic fibroblasts
ChIL	chicken interleukin
CO_2	carbon dioxide
CPE	cytopathic effects
CSC	Clinical Science Centre
CTL	cytotoxic T lymphocyte
CVA	chorioallantois VV Ankara
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxy nucleotide triphosphates
dsDNA	double stranded DNA
dsRNA	double stranded RNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
e/l	early or late
ELISA	enzyme-linked-immunoabsorbent assay
ER	endoplasmic reticulum
eTF-2α	eukaryotic initiation factor 2α

F	forward
FWPV	Fowlpox virus
GM-CSF	granulocyte-macrophage colony-stimulating factor
gpt	xanthine-guanine phosphoribosyl tranferase
HA	haemagglutinin
Hb	haemoglobin
HIV	human immunodeficiency virus
HPAI	high pathogenic avian influenza
HPAIV	high pathogenic avian influenza virus
IBDV	infectious bursal disease virus
IFN	interferon
IFNAR	interferon-receptor
Ig	immunoglobulin
IL	interleukin
IRF-3	IFN-regulatory factor-3
ISG	IFN-stimulated genes
ISRE	IFN-stimulated response elements
ITR	inverted terminal repeat
JAK-1	Janus kinase family
kbp	kilo base-pair
kD	kilo Dalton
L	litre
LB	Luria Bertani
LPAI	low pathogenic avian influenza
LPAIV	low pathogenic avian influenza virus
M	molar
µL	microlitre
mL	millilitre
mM	millimolar
mAB	monoclonal antibodies
MCHC	mean corpuscular haemoglobin concentration

MCP-1	monocyte chemotactic protein-1
MCS	multiple cloning site
MCV	mean corpuscular volume
MDV	Marek's disease virus
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein
MOI	multiplicity of infection
MPA	mycophenolic acid
mRNA	messenger RNA
MVA	modified Vaccinia virus Ankara
Mx	myxovirus resistance protein
MXH	MPA–xanthine-hypoxanthine
n	sample size
NA	neuraminidase
NBBS	newborn bovine serum
NDV	Newcastle disease virus
NEB	New England Biolabs
NFkB	nuclear factor-kB
NK	natural killer
nm	nanometer
NP	nucleoprotein
NS	nonstructural
OAS	2'-5' oligoadenylate synthetase
OD	optical density
ORF	open reading frame
%	percentage
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PFU	plaque forming units

p.i.	post infection
PI3K	phosphatidylinositol 3-kinase
PKR	protein kinase R
PRRSV	porcine reproductive and respiratory syndrome virus
R	reverse
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBC	red blood cells
REV	reticuloendotheliosis virus
rFWPV	recombinant fowlpox virus
rFWPV/H5	recombinant fowlpox virus strain FP9 expressing H5 gene of AIV
rFWPV/H5/IL-15	recombinant fowlpox virus strain FP9 co-expressing H5 gene of AIV and chicken IL-15
rFWPV/N1	recombinant fowlpox virus strain FP9 expressing N1 gene of AIV
rFWPV/N1/IL-12	recombinant fowlpox virus strain FP9 co-expressing N1 gene of AIV and chicken IL-12
RIG-I	retinoic inducible gene-I
RNA	ribonucleic acid
RT	room temperature
RT-PCR	real-time PCR
rt-PCR	reverse transcriptase-PCR
rVV	recombinant Vaccinia virus
S	synthetic
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium duodecyl sulphate-polyacrylamide gel
SEM	standard error of the mean
SPF	specific-pathogen-free
ss	single stranded
ssRNA	single stranded RNA
STAT	signal transducers and activators of transcription
Taq	*Thermus aquaticus*

TBE	Tris-borate-EDTA
TDS	transient dominant selection
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
TYK-2	tyrosine kinase-2
U	unit
V	Volt (s)
VDAC1	voltage-dependent anion channel 1
vRNA	viral RNA
vRNP	viral ribonucleoprotein
VV	*Vaccinia virus*
WBC	white blood cells
WT	wild-type
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ACRONYMS

FAO	Food and Agriculture Organization of the United Nations
HSE	Health and Safety Executive
IACUC	Institutional Animal Care and Use Committee
IAH	Institute of Animal Health
ICTV	International Committee on Taxonomy of Viruses
ILAR	Institute for Laboratory Animal Research, a component of the National Research Council, National Academy of Sciences, Washington, US
UPM	Universiti Putra Malaysia
US	United States
VRI	Veterinary Research Institute

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To my father,

whose vision prevails the capability and excellence of his beloved family

with unconditional love and belief

CHAPTER 1

Introduction

1.1 Influenza

Influenza, which is caused by single-stranded, negative-sense RNA viruses of the family *Orthomyxoviridae* is an infectious disease of birds and mammals, including humans. Although the first relatively well-recorded influenza pandemic occurred in 1580, the most notorious was the 1918 influenza pandemic, which killed an estimated 40 million people worldwide, with approximately 675,000 in United States alone (Potter, 2001; Taubenberger, 2006).

1.1.1 Influenza classification

Influenza viruses exist in 3 major genotypes: A, B and C. Influenza B and C, which are predominantly human pathogens, have not caused pandemics. In contrast, influenza A (infecting humans, horses, swine, birds, whales, seals, cats, leopards, tigers, civets and dogs (Suarez and Schultz-Cherry, 2000a; van den Berg *et al.*, 2008)) causes annual endemics, epidemics every few years and pandemics at irregular intervals of decades. Influenza A viruses are divided into subtypes based on antigenic differences between their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), both of which are important determinants for neutralizing antibodies. To date, 16 HA subtypes and 9 NA subtypes have been identified (Fouchier *et al*., 2005). All avian influenza viruses are of type A. Historically, all influenza A virus subtypes, except H13 (Webster *et al.*, 1992), occur asymptomatically in wild birds of the Orders Anseriformes (ducks, geese, and

swans) and Charadriformes (i. e., gulls, terns, surfbirds, and sandpipers). Shedding of the virus in the faeces of these host species, particularly by wild waterfowl, contaminates the environment thus permitting consistent cycles of infection (reviewed by van den Berg *et al.*, 2008).

1.1.2 Molecular biology

The influenza A virus genome consists of eight negative strand RNA segments, the complements of which encode eleven different proteins. The HA segment (number 4) encodes the full-length HA protein (HA0, in which giving two fragments, HA1 and HA2, upon cleavage by proteases at the polybasic region), membrane attachment/fusion glycoprotein, which is synthesized on membrane-bound ribosomes. The protein binds specifically to sialic acid receptors that are linked to galactose, generally by an α2,3-linkage in avian and equine hosts, or α2,6-linkage in human host, thus allowing influenza virion to attach to the cell (Subbarao *et al.*, 2000; Neumann *et al.*, 2010). Upon successful attachment, the virion is endocytosed into the cell. Acidified-endosome activates the fusion domain of the HA protein hence releases the viral RNA into cytoplasm. NA, which is a surface glycoprotein and enzymatically active, promotes the penetration of avian influenza virus into target cells during the onset of infection, and the release of budded virus particles from the cell membrane by cleaving sialic acid residues from carbohydrate moieties on surfaces-bound receptors of infected cells, at the final stage of viral replication (Ohuchi *et al.*, 2006). The neuraminidase activity also helps to prevent virus from self-aggregating or remaining to cell membranes (Li *et al.*, 1993).

In any glycoprotein, the sugar moieties (glycans) linked to the polypeptide (by an enzymatic process termed glycosylation) play vital biological roles in the folding of the glycoproteins during biosynthesis and in stabilizing the protein conformation in the active form (Vliegenthart and Montreuil, 1995). Core glycosylation, which occurs in the lumen of the endoplasmic reticulum (ER), is essential to the creation of a functional HA protein (Roberts *et al.,* 1993). Recent studies also implicate N-glycans flanking the receptor-binding site of HA as potent regulators of influenza virus replication (Wagner *et al.*, 2000). Apart from the trimming and processing of HA glycans, other post translational modifications of HA, including its proteolytic cleavage into subunits, have also been studied extensively (Roberts *et al.,* 1993).

The M2 membrane protein acts as an ion channel, which is important for maintaining the internal pH of the virion, offering optimal conditions for uncoating of the virus after cell entry (Betakova, 2007). The surface proteins HA, NA and M2 are embedded in the viral envelope. The other proteins, PA, PB1, PB2 and NP, form ribonucleoprotein (vRNP) complexes responsible for transcription of the viral genome. The complexes are connected with the viral envelope by the matrix protein, M1, which is required to induce the nuclear export of vRNP (Bui *et al.*, 2000; Suarez and Schultz-Cherry, 2000a). Studies of fifty five sequences of nine or more amino acids of the polymerases (PB2, PB1, and PA), NP, and M1 proteins of avian and human influenza A virus isolates, show that the proteins are at least 80% conserved, despite the evolutionary variability (Heiny *et al.*, 2007). PB1-F2 protein, derived from a second open reading frame (ORF) of PB1, can induce apoptosis by interacting with the inner mitochondrial membrane adenine

nucleotide translocator 3, ANT3, and the outer mitochondrial membrane voltage-dependent anion channel 1, VDAC1 (Chen *et al.*, 2001, Zamarin *et al.*, 2005). It interacts directly with a viral polymerase PB1 protein, and localizes to both the cytoplasm and the nucleus of infected cells, suggesting a pivotal function in AIV replication (Mazur *et al.*, 2008)

The NS segment encodes NS1 and NEP proteins, using different open reading frames, from the same RNA segment. While NEP proteins mediate the nuclear export of vRNPs (Neumann *et al.*, 2000; Suarez and Schultz-Cherry, 2000a), NS1 protein antagonises the host cell antiviral interferon (IFN) system in multiple ways. It also regulates temporal viral RNA (vRNA) synthesis (Falcon *et al.*, 2004; Min *et al.*, 2007), inhibits pre-mRNA splicing (Lu *et al.*, 1994), suppresses RNA interference (Li *et al.*, 2004) and activates phosphatidylinositol 3-kinase (PI3K) to mediate anti-apoptotic response (Hale *et al.*, 2006; Ehrhardt *et al.*, 2007; Shin *et al.*, 2007).

1.2 Avian influenza virus

1.2.1 Pathogenicity

Avian influenza viruses infecting poultry can be further divided into two distinct groups (pathotypes) based on their ability to cause disease (pathogenicity). The endemic form, low pathogenicity avian influenza (LPAI), causes mild symptoms and low mortality rates; the highly pathogenic avian influenza (HPAI), defined as ‘fowl plague’ in 1878, was initially recognized in Italy as causing flock mortality as high as 100% in chickens (Capua and Alexander, 2004; Lupiani *et al.*, 2009). Avian influenza virus (AIV)

pathogenicity level is indexed by measuring the intravenous virus pathogenicity index (IVPI). The IVPI is the mean score per bird per observation over a 10-day period after AIV infection. During this period, an index of 0.00 is denoted to living birds without any clinical sign, while an index of 3.00 is denoted if all birds died within 24 hours.

HPAI viruses are only of H5 and H7 subtypes but not all viruses of these subtypes cause HPAI. The deduced amino acid sequence at the HPAI cleavage site of the precursor haemagglutinin (HA0) contains multiple basic amino acids, arginine (R) and lysine (K) (Wood *et al*., 1994). The cleavage site allows ubiquitous proteases (furin and PC6) present in many body tissues, which may be induced by a subtilisin endoprotease, to activate the haemagglutinin (Horimoto *et al.,* 1994). This will enable virus replication throughout the host, causing lethal damage to vital organs and tissues. Proteolytic activation is therefore crucial for effective virus spread and for virus pathogenicity (Rott, 1979; Rott, 1992; Stieneke-Grober *et al.,* 1992). In cell culture, a polybasic motif of –R/K-X-K/R-R (X represents a nonbasic residue) is sufficient for a complete haemagglutinin cleavage but, if a carbohydrate chain is nearby, a X-X-R-X-R/K-R motif is needed (Vey *et al.,* 1992; Wood *et al.,* 1993, Horimoto *et al.,* 1994; Senne *et al.*, 1996; Ito *et al.,* 2001). Alterations to the consensus sequence may lead to loss of pathogenicity for chickens.

In contrast, LPAI have a single arginine at the cleavage site which limits cleavage to that by host proteases such as trypsin and trypsin-like enzymes, restricting the replication of the virus to sites in the host where such enzymes are found, such as in the respiratory and

intestinal tracts (Capua and Alexander, 2004). Recent evidence (Li *et al.*, 1990; Rohm *et al.*, 1995; Banks *et al.*, 2000) clearly supports the hypothesis that HPAI may arise unpredictably from LPAI, in poultry or in the wild birds in which LPAI are endemic, by mutation at the cleavage site, probably by polymerase slippage in H5, or in other subtypes by recombination with other viral or non-viral RNAs (Garcia *et al.*, 1996; Perdue *et al.*, 1998).

1.2.2 Determinants of host range and pathogenicity

Pathogenicity determinants are found in several proteins independently or in combination. In addition to the widely-accepted HA and NA proteins of AIV as important AIV pathogenic determinants (Section 1.1.2), PB2, NS1 and PB1-F2 proteins have been recently identified to possess similar character.

1.2.2.1 PB2

In 1993, Subbarao and colleagues recognized a single amino acid substitution at position 627 of PB2 as a host range determinant, with lysine often found in mammalian isolates and glutamate being normal in avian isolates. Eight years later, Hatta *et al.* demonstrated the importance of the lysine at PB2-627 in the HPAI H5N1 strain A/Hong Kong/438/97, compared to the glutamate at the same position in the LPAI H5N1 strain A/Hong Kong/436/97, to virulence in a mouse model, indicating the first report on pathogenicity effect of polymerase gene PB2. During a H7N7 influenza virus outbreak in poultry in 2003, PB2-627K was found in the virus isolated from a lethal case, that of a Dutch veterinarian who died with pneumonia, while PB2-627E was invariably found in non-

fatal human cases (Fouchier *et al.*, 2004). It was later established that a lysine to glutamate alteration at PB2-627 of influenza A H1N1/PR/8/34 has profound effects upon the surface electric charges of PB2 3/3 domain (amino acids 535-759) and lowers its RNA-binding ability (Kuzuhara *et al.*, 2009). However, this mutation does not increase the virulence of H1N1 2009 pandemic viruses in a mouse model (Zhu *et al.*, 2010). Apart from substitutions at position 627, substitution of aspartate to asparagine at position 701 of the PB2 protein of H5N1 Hong Kong 1997 or Vietnam 2003-2008 human isolates was found to confer lethality in mammals (Hiromoto *et al.*, 2000; Li *et al.*, 2005; Le *et al.*, 2010). Salomon *et al.* (2006) also emphasize that the human H5N1 A/Vietnam/1203/04, containing a polybasic HA cleavage site, still requires adaptive changes in proteins of the polymerase complex to retain its lethal phenotype.

1.2.2.2 NS1

In 2006, Li *et al.* demonstrated that two goose-derived H5N1 virus isolates, which both possessed polybasic amino acids at their HA cleavage sites, have divergent pathogenicity profiles in chickens. They showed that an alanine to valine substitution at position 149 of the NS1 protein in A/goose/Guangdong/1/96 results in antagonism of type I IFN production, resulting in a high mortality rate, while the inverse substitution attenuates A/goose/Guangdong/2/96.

In a mouse model, substitution of proline for serine at NS1-42 increased the virulence of H5N1 A/duck/Guangxi/12/03 (Jiao *et al.*, 2008), and a serine to glycine substitution at

the same position of the RNA-binding-defective NS1 of A/WSN/33 increased its virulence (Donelan *et al.*, 2003).

Recently, the presence of a PDZ ligand domain (acronym combining the first letters of three proteins, post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1)) at the C-terminal residues (ESEV-COOH) of the NS1 protein (Obenauer *et al.*, 2006), has been demonstrated to have the ability to enhance virulence in mice (Jackson *et al.*, 2008). In chickens, 15-nucleotide deletions at position 263-277 (Long *et al.*, 2008) and 612-626 (Zhu *et al.*, 2008) of the NS1 gene were also found to increase or attenuate the H5N1 virus pathogenicity, respectively.

1.2.2.3 PB1-F2

Marjuki *et al.* (2010) reported a three amino acid changes in PB1-F2 protein of the H5N1 virus A/Vietnam/1203/2004 associates with lethality rate of mallard ducks. In a mouse model, a single amino acid substitution, PB1-F2 66 N-S, was discovered to affect the pathogenicity of H5N1 Hong Kong 1997 virus and resurrected H1N1 pandemic 1918 virus (Conenello *et al.*, 2007). This point mutation also delays induction of IFN-β, retinoic inducible gene-I, RIG-I, and several IFN-inducible genes (Conenello *et al.*, 2010). PB1-F2 66 N-S substitution from the H1N1 pandemic 2009 virus also enhances expression of the proinflammatory genes IFN-γ, IL-1β, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP-1β) and chemokine (C-C motif) ligand-5 (CCL-5, also known as RANTES [Regulated upon Activation, Normal T-cell

Expressed, and Secreted]**)** of infected mice, and dysregulates peripheral leukocyte and neutrophil levels in ferrets. The mutation is not, however, detrimental to the survival of either host (Hai *et al.*, 2010).

1.2.3 Immune responses to avian influenza virus

1.2.3.1 Innate immune responses

IFNs are secreted from most cells of vertebrate organisms. In response to viral infection, the IFN system is the key mechanism to inducing an antiviral state, which involves signalling pathways to induce the expression of a large number (Sen, 2001) of genes, the so-called IFN-stimulated genes (ISGs). The absence of IFN has even been shown to increase lethality upon viral infection of mice (Bouloy *et al.*, 2001; Ryman *et al.*, 2000) and humans (Dupuis *et al.*, 2003) with an otherwise intact adaptive immune system.

The roles and functions of many ISGs are unknown. However a number of the key antiviral effectors have been studied for many years and their roles and functions are becoming better understood. Amongst these are protein kinase R (PKR), RIG-I and Mx. PKR directly recognizes and autophosphorylates viral dsRNA, contributes to autophagy (degradation process of cytoplasmic portions) and activates transcription factor, nuclear factor-kB (NFkB). PKR upregulation by IFN-β also phosphorylates host eukaryotic initiation factor 2α, eTF-2α, to allow non-specific translation initiation inhibition of both host and viral protein synthesis (Garcia *et al.*, 2006).

Another ISG, RIG-I, is able to sense viral intracytoplasmic dsRNA, initiating a signalling cascade that results in phosphorylation and nuclear translocation of cytoplasmic IFN-regulatory factor-3, IRF-3, to induce IFN production (Grant *et al.*, 1995). A recent study established that RIG-I is probably absent from chickens (but not ducks) and, somewhat controversially, suggests that this may explain the relative vulnerability of these species to influenza viruses (Barber *et al.*, 2010).

Secreted IFN-α/β can be recognised in an autocrine or paracrine manner by the type I IFN-receptor, IFNAR, on producing and neighbouring cells, respectively. IFNAR associates with Janus kinase family, JAK-1, or tyrosine kinase-2, TYK-2, thus allowing regulation of Signal Transducers and Activators of Transcription (STAT) 1 or 2. Phosphorylated STAT1/2 forms a complex, known as ISG factor-3 (ISG3), with IRF9, which then translocates to the nucleus and binds to IFN-stimulated response elements, ISREs, to instigate ISG transcription (Haller *et al.*, 2006). Antiviral ISGs include 2'-5' oligoadenylate synthetase (OAS), which synthesises short 2'-5' oligoadenylates to activate another ISG, ribonuclease L (RNaseL), which in turn directly cleaves cellular and viral RNA.

Another antiviral ISG is myxovirus resistance protein, Mx, hypothesised to block ssRNA and dsRNA viruses by endocytosis and vesicle transportation (Sen, 2001). In a mouse model, expression of Mx protein enhances resistance to influenza viruses both *in vitro* and *in vivo* (Suarez and Schultz-Cherry, 2000b). Microarray gene expression analysis by Sarmento *et al.* (2008) indicates high level expression of IFN-α and myxovirus resistance

protein-1, Mx-1, upon HPAI H5N1 strain A/chicken/Hong Kong/220/97 virus infection of chickens. However, induction of PKR, Mx protein and IL-6 (cytokines secreted by T helper lymphocytes type 2) shows an abortive protection against HPAI H5N1 (Daviet *et al.*, 2009). The influence of Mx protein induction is arguable as it provides a different antiviral response depending on chicken breed (Ko *et al.*, 2002).

AIV NS1 protein, however, has the ability to antagonise IFN pathways in several ways, including: binding and sequestering dsRNA (Garcia-Sastre, 2001), interfering with the activation of IRF3 (Talon *et al.*, 2000), NFkB (Wang *et al.*, 2000), Jun N-terminal kinase and AP-1 transcription factors (Ludwig *et al.*, 2002). NS1 may also form an IFN-β-inhibition-complex with RIG-I and PKR (Guo *et al.*, 2007; Mibayashi *et al*., 2007; Opitz *et al*., 2007) and obstruct activation of the 2'-5' oligoadenylate synthetase/RNAseL antiviral pathway (Min and Krug, 2006). IFN pathways and their inhibition by NS1 are illustrated in Figure 1.1.

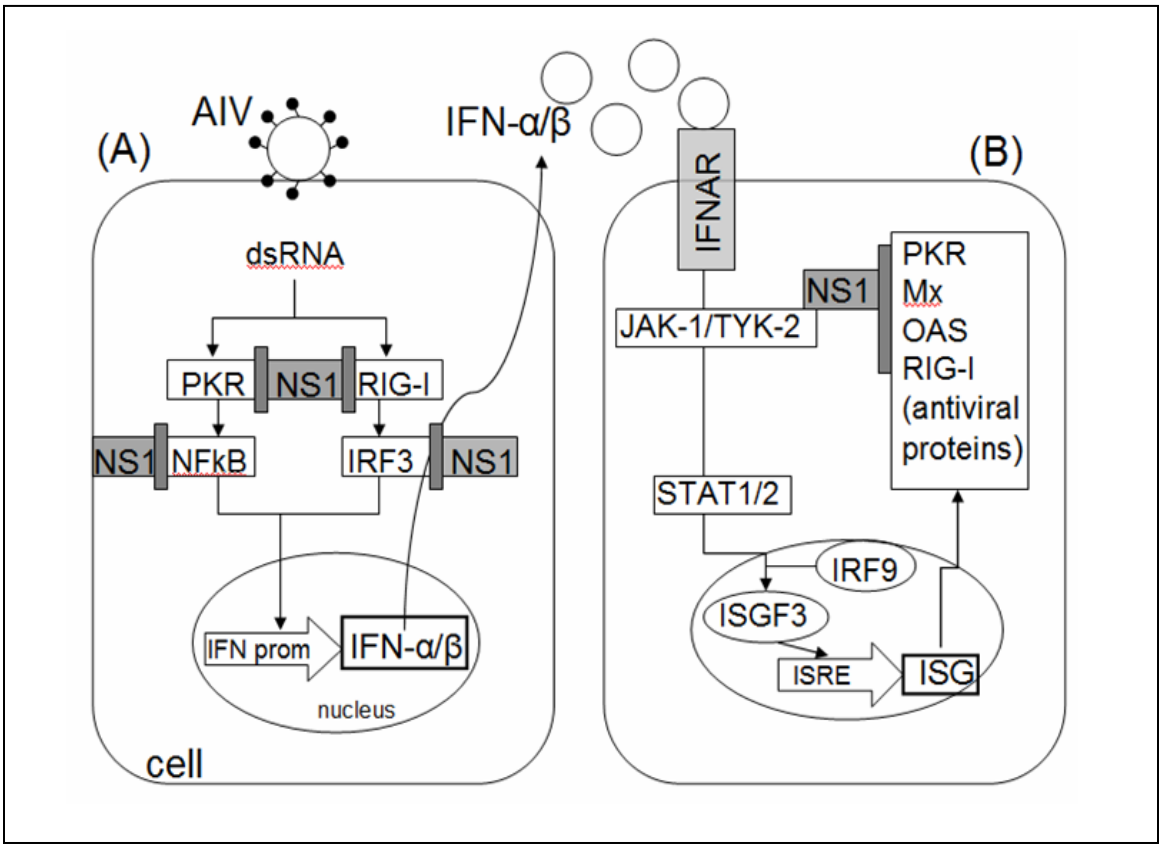

Figure 1.1. Schematic showing points of antagonism of the IFN pathways by AIV NS1 protein. (A) NS1 forms an inhibition complex with PKR, RIG-I, IRF3 or NFkB to block IFN induction. (B) NS1 directly neutralises the antiviral products of the ISGs. Modified from Haller and Webber (2007).

1.2.3.2 Adaptive immune response

Host adaptive immunity against AIV can be divided into humoral and cell-mediated responses. Humoral immune responses include systemic (IgM and IgY) and mucosal (IgA) antibody production. These antibodies bind to the virus thus preventing virus attachment and entry. In comparison to internal and non-structural proteins of AIV, the surface proteins, HA and NA, are the main antigens capable of inducing neutralizing antibody, thereby acting as protective determinants in chickens (Suarez and Shultz-Cherry, 2000). However, recent findings show influenza viruses have recombined and mutated their HA and NA protein, indicating evolutionary variability of the viruses (Steinhauer and Skehel, 2002; Fiers e*t al.*, 2004). The external domain of the M2 surface protein, M2e, has also been shown to induce a response that reduces influenza virus infectivity, morbidity and mortality *in vivo*, despite its failure to elicit a strong humoral response, in mice (Neirynck *et al.*, 1999; Fan *et al.*, 2004). In chickens, immunization with recombinant *Salmonella* expressing M2e peptide conjugated with a tumor necrosis factor ligand family member, CD154, increases IgY responses significantly. The vaccine also provides protection and reduces viral shedding after LPAI, but not HPAI, viral challenge (Layton *et al.*, 2009).

Limited characterization of the chicken mucosal immune responses upon AIV infection were undertaken. Nonetheless, IgA was detected in bile ducks upon infection with different influenza isolates (Higgins *et al.*, 1987).

Cellular immunity involves: (i) activation of macrophages which can produce cytokines and engulf foreign particles through phagocytosis, (ii) antigen-specific cytotoxic T-lymphocytes and (iii) natural killer (NK) cells, which can release granzymes and perforins to induce apoptosis upon recognition of receptors on virally infected cells (Cullen and Martin, 2008). Human macrophages infected with influenza A H5N1 virus express high levels of mRNAs encoding tumor necrosis factor-α, TNFα, RANTES, MIP1α/β and MCP-1 (Cheung *et al.*, 2002).

The virus also triggers secretion of IFN-γ-stimulated protein (IP-10), interleukin (IL)-6, and RANTES in primary human bronchial and alveolar epithelial cells (Chan *et al.*, 2005). In an animal model, susceptibility to a lethal H5N1 virus infection of mice that lacked chemokine (C-C motif) ligand-2 (CCL2), IL-6 or TNFα is equivalent to those of wild-type (Salomon *et al.*, 2007). In another study in TNFα signalling cascade-disrupted mice, the mortality rate was not significantly reduced despite the decreased morbidity (Szretter *et al.*, 2007). Host adaptive immune responses observed upon AIV infection are illustrated in Figure 1.2.

Recent data suggest that a series of the chicken's major histocompatibility complex (MHC; haplotype B2, B12, B13, B19 and B21), has a different influence on resistance to HPAI viruses (Hunt *et al.*, 2010). In fact, Sarmento *et al.* (2008) suggest the possibility that AIV might have evolved a mechanism(s) to inhibit MHC Class I expression.

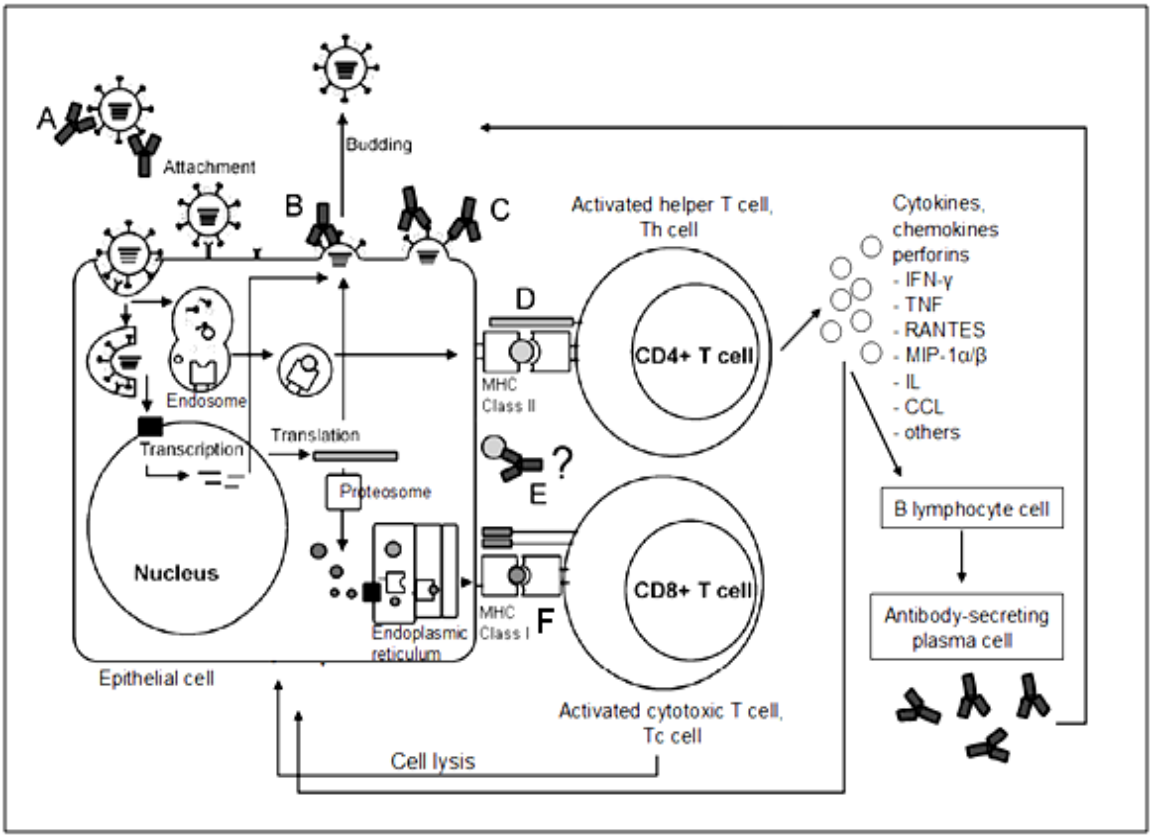

Figure 1.2. Overview of host adaptive immune response upon AIV infection. (A) HA-specific antibodies bind to the viruses' HA proteins thus prevent infection of cells. (B) M2e-specific antibodies bind to M2e on virus-infected cells and induce antibody-dependent cell-mediated cytotoxicity (ADCC). (C) NA-specific antibodies block enzymatic activity of viral NA thus prevent progeny viral release. (D) Pathogens and proteins are broken down into peptides within acidified endosomes and bind to MHC Class II. The complexes are subsequently transported to the cell surface for recognition by CD4+ T cells. (E) The role of NP-specific antibodies is poorly known. (F) The proteosome degrades influenza viral proteins into peptides in the cytosol of the infected cell. The peptides are subsequently translocated to the endoplasmic reticulum and bind to MHC Class I. The peptide-MHC complexes are transported to the cell surface for recognition by CD8+ T cells, which then induce lysis of the infected cells. Modified from Flint *et al* (2004) and Bodewes *et al.* (2010).

1.2.4 Current H5N1 panzootic and human infection

In 1997, 1.4 millions of chickens and various numbers of other domestic birds in Hong Kong were affected (either died of infection or were slaughtered) by HPAI H5N1 (Capua and Mutinelli, 2001). The HA gene of the virus derived from A/goose/Guangdong/1/96, the first H5N1 virus isolated from sick geese in Guangdong province in China a year before. In fact, most of HA genes of HPAI H5N1 viruses belong to this lineage (reviewed by Neumann *et al.*, 2010). The outbreak caused 18 cases of human infections, of which 6 were fatal. The infections represent the first documented fatal cases of influenza transmitted directly from avian species to humans (Subbarao *et al*., 1998).

There were no reported cases of H5N1 infections in poultry or human after 1997 until February 2003, when the H5N1 panzootic began in many S.E. Asian countries including Hong Kong, Japan, Laos, Malaysia, Korea, Thailand, Vietnam with still ongoing reappearance of human cases in China, Indonesia, Egypt and Cambodia (Peiris *et al*., 2004; Zhou *et al*, 2007). It is believed that the outbreaks originated in Yunnan and Guangxi provinces in 2002, have spread the virus to Indonesia and Vietnam, and other S. E. Asian countries (Wang *et al.*, 2008). HPAI H5N1 predominated in China in early 2004 when ducks, geese and chickens were being infected in 16 provinces. Then, after a major outbreak in many species of wild, migratory waterfowl and wading birds at Qinghai Lake in 2005-2006, the virus spread widely to around 60 countries including several Europe states (Gall-Recule *et al.*, 2008), Russia (Lipatov *et al.*, 2007)**,** India, Australia, New Zealand and Mongolia (Chen *et al.*, 2006). A new sub-lineage of the virus detected in

2005, China's 'Fujian-like' viruses, caused epizootics in Russia (Lvov *et al.*, 2008), Hong Kong, Laos, Malaysia and Thailand.

Since the re-emergence of H5N1 in 2003, there have been many reported cases of human infection. Until 16th March 2011, Indonesia has reported their 174th case, Egypt 130th and Vietnam 40th, with the last fatality case occurred in Indonesia on 13th February 2006. A typical case is that of a recent H5N1 human infection, identified in February 2006, where a 36-year-old man died from several complications, including respiratory failure, liver failure, renal failure and disseminated intravascular coagulopathy (Zhou *et al*., 2007). Although human to human spread has not generally been observed, infections of H5N1 HPAI in humans have been a major concern as humans also possess the ubiquitous protease, furin (Capua and Alexander, 2007). Recent reports have identified the significant levels of α2,3-linked sialic acid, which is the preferential binding site for influenza viruses in avian host, in human epithelial cells from respiratory tract (Nicholls *et al.*, 2007). However, the cooler temperature of human proximal airways (32^{o}C) compared to distal airways (37^{o}C) may restrict avian influenza virus replication, which occurs at 40 to 42^{o}C in the avian enteric tract (Scull *et al.*, 2009), thus limiting avian-to-human spread.

1.2.5 H5N1 outbreaks in poultry in Malaysia

The first report of the presence of HPAI H5N1 in Malaysian poultry was in August 2004. The outbreak occurred among a free-range chicken flock in the state of Kelantan, near the Thailand border (Figure 1.3). A few cases continued to re-emerge in September and

November 2004 (WHO, 2008). Sequence analysis of the HA gene revealed that viruses isolated from both scenes were clustered with Vietnam's and Thailand's 2003-2005 virus isolates. The group was phylogenetically most closely-related to viruses isolated from domestic and migratory birds in Hong Kong in 2002-2003 (Chen *et al.*, 2006). The outbreak was suppressed until 2006, when free-range chicken flocks in Selangor and Perak were infected in February and March, respectively. Early detection and a stringent control strategy allowed Malaysia to eradicate the virus in April 2006, shortly after the onset (Webster *et al.*, 2006a; WHO, 2008). According to phylogenetic study of the HA gene, the Malaysian 2006 virus isolates (A/Chicken/Malaysia/935/2006) formed an independent sublineage with viruses isolated from ducks in Fujian, China in 2005, and distinctly separated from the 2004 cluster (Boltz *et al*, 2006; Wu *et al.*, 2008). This evidence suggests that instead of being due to recirculating viruses of the previous strain, the 2006 outbreak represented a new introduction into Malaysia.

In June 2007, an H5N1 outbreak, which involved semi-free ranging chickens, reappeared in a village in Petaling, Selangor. In line with its response to the previous occurrence, Malaysian authorities implemented a policy to rapidly cull and safely dispose of all infected birds, and the birds within a 1 km-radius of the affected flock, and set up a 10 km-radius quarantine zone. Three months later, the World Organization of Animal Health (OIE) declared Malaysian poultry free of H5N1 viruses.

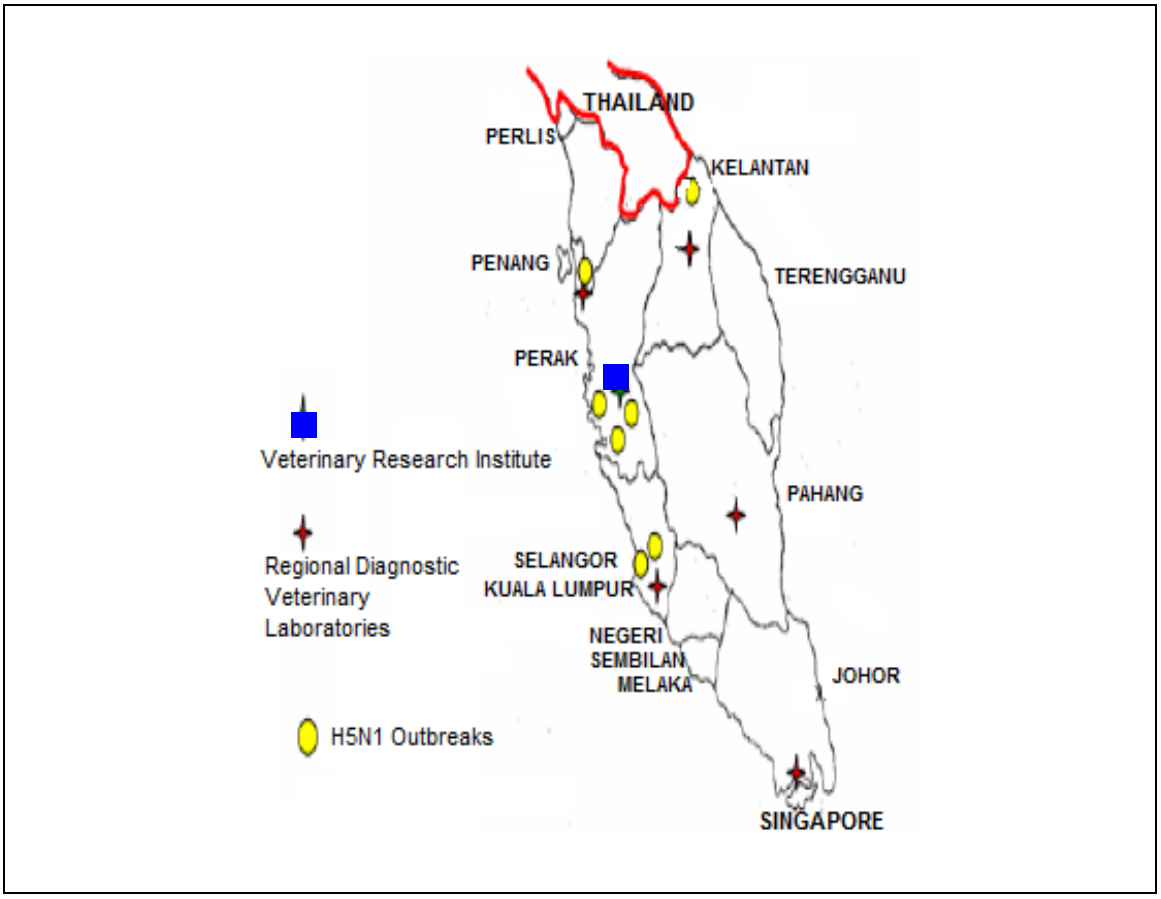

Figure 1.3. Map of Peninsular Malaysia, denoting the locations of HPAI H5N1 outbreaks in Malaysian poultry. The first outbreak was reported among a free-range chicken flock in the state of Kelantan, near the Thailand border in August 2004. In February and March 2006, free-range chicken flocks in Selangor and Perak were infected, respectively. In June 2007, the outbreak re-emerged in a village in Petaling, Selangor, infecting semi-free ranging chickens. The Veterinary Research Institute, VRI, Ipoh, Perak, is indicated by a rectangle ( ), Regional Diagnostic Veterinary Laboratories is represented by a star ( ), while the H5N1 outbreaks are denoted by a circle ( ).

1.2.6 Avian influenza vaccines

Essentially four different types of avian influenza vaccines have been developed for use in poultry, namely (i) inactivated whole influenza viruses, (ii) *in vitro* expressed AIV proteins, (iii) *in vivo* expressed AIV proteins and (iv) nucleic acid (DNA plasmid) vaccines. For the past 30 years, vaccines against HPAI have been developed based on inactivated whole-virus, by using strains of LPAI viruses (LPAIV) isolated from outbreaks in poultry or from surveillance of wild or domestic birds (Swayne *et al.,* 2008). Such vaccines were proved to work, with the notable feature of offering broad cross-protection against diverse field viruses (Swayne *et al.*, 2000). Recently, heterologous inactivated vaccines (H5N2 and H5N9-based isolates) with low homology (as low as 84%) between their HA1 amino acid sequence and the challenge strains (2003-2004 Asian H5N1 HPAI viruses), were shown to be protective against flock morbidity and mortality (Swayne *et al.*, 2006; Bublot *et al.*, 2007). Despite the effectiveness, inactivated vaccines carry several disadvantages. The foremost is biosecurity during the production of inactivated AI vaccines, the risk of virus escape and spread being high as they are cultivated in embryonated chicken eggs. Secondly, the yield of total viral protein also may be lower than expected, resulting in difficulties in large scale vaccine preparations (Lipatov *et al.*, 2004). Furthermore, it is difficult to identify infected birds compared to vaccinated birds by routine serological tests. The potency of the vaccine is also highly dependent on antigen quantity in each dose and adjuvant system (oil emulsification).

In a second approach to vaccination, ample amount of AI proteins can be expressed in an *in vitro* system, using eukaryotic cell cultures, plants (Nemchinov and Natilla, 2007),

yeast (Saelens *et al.*, 1999), bacteria (e.g. Davis *et al.*, 1983; Horthongkham *et al.*, 2007) or viral vectors, such as baculovirus (e.g. Crawford *et al.*, 1999; Hu *et al.*, 2006). The crude or purified proteins can be administered as vaccines. Although the production of this vaccine is safer compared to inactivated whole AI vaccines, it shares the same drawbacks of high cost, and dose and adjuvant system-dependence.

Thirdly, AI gene sequences can be inserted into bacterial or viral vectors producing recombinant vectored-vaccines, and their *in vivo* expression in the host (such as chickens) can be driven by live administration. In poultry, a replicative fowlpox virus-based vector expressing H5 of AIV vaccine (Webster *et al.*, 1991) and more recently, a recombinant Newcastle Disease Virus (NDV)-based vaccine (Ge *et al.*, 2007), have been licensed. These viral-vectored vaccines offer many advantages over inactivated whole virus, including co-immunisation against the vector agent, though with several limitations (discussed in Section 1.3.7 and 1.3.8).

Fourthly, vaccines based on DNA plasmids with cDNA inserts that code for AI proteins (also termed nucleic acid vaccines or DNA vaccines), have been shown to be efficacious, with HA gene inserts being the most immunogenic (van den Berg *et al*, 2008). The plasmids elicit immune response via their uptake and expression by antigen-presenting cells or myocytes (Suarez and Schultz-Cherry, 2000b). Rao and colleagues (2008) have shown that DNA vaccines using multivalent HA are able to protect chickens against HPAI H5N1 strain A/Vietnam/1203/2004. Several advantages of this type of vaccine are (i) they are readily modifiable upon identification of circulating virus strain, and (ii) large

scale production is feasible (Hoare *et al.*, 2005). The main problem of DNA vaccines is inefficient gene delivery and expression. An alternative way for its delivery is to use *Shigella flexneri* (Vecino *et al.*, 2004) or *Salmonella typhimurium* (Qu *et al.*, 2008), while the cellular uptake might be enhanced by using application of a short electrical pulse to muscle tissues (Liu *et al.*, 2008).

1.2.7. AI vaccination policy in Malaysia

In Malaysia, as in Europe, vaccination against avian influenza among poultry is considered a last resort, as the virus may circulate undetected, resulting in endemicity (Veits *et al.*, 2008). However, the Malaysian Government realizes the potential of vaccination as a complementary measure in the eradication of HPAIV.

1.3 Poxvirus vectors

The *Poxviridae* family is divided into two subfamilies, *Chordopoxvirinae*, and *Entomopoxvirinae*, which infect vertebrates and insects, respectively. The *Chordopoxvirinae* are subdivided into eight genera (Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus) and are better characterized than *Entomopoxvirinae* (in which subdivided into three genera, Entomopoxvirus A, B and C).

1.3.1 Vaccinia virus

Vaccinia virus (VV) is a type-species of Orthopoxvirus. Most genes characterized from poxviruses are from VV. In addition, studies on host innate immunity response upon

poxviruses infection are also more ascertained on VV. Four morphologies are displayed by a VV, (i) intracellular mature virus (IMV), which have one or two surrounding membranes, (ii) intracellular enveloped virus (IEV), which derived from a second membrane wrapping event from the trans golgi, (iii) extracellular enveloped virus (EEV), and (iv) cell-associated enveloped virus (CEV), which attached to the cell surface to allow induction of actin tail formation to be force away from the infected cell (Smith and Law, 2004).

1.3.2 Avipoxviruses

According to International Committee on Taxonomy of Viruses, ICTV, Avipoxvirus is the only genus in *Chordopoxvirinae* which infect birds, not mammals. More than 232 species of the approximately 9000 bird species reported by van Riper *et al*. (2007) have acquired natural poxvirus infections, including the endangered Southern giant petrel, *Macronectes giganteus*, a long-lived, large, pelagic bird found in the Antarctica (Shearn-Bochsler *et al.*, 2008).

1.3.3 Fowlpox virus

Possessing a large double stranded DNA genome up to 300 kb, Fowlpox virus (FWPV) is the best-studied and type species of the Avipoxvirus (Skinner *et al.*, 2005). Using improved microscope and histological technique, FWPV was first detected in chicken cells by Bollinger in 1873. The observed large, intra-cytoplasmic inclusion bodies were now termed Bollinger's inclusion bodies (Bolte *et al.*, 1999). To date, two FWPV sequences have been determined; a virulent US FWPV with 288 kb genome (Afonso *et*

al., 2000), and an attenuated vaccine strain, FP9 with 266 kb genome (Laidlaw and Skinner, 2004). FWPV causes a widespread, contagious disease of poultry and other galliform birds, resulting in reduced egg production, decreased growth rates in broilers, blindness and may cause death (Afonso *et al.*, 2000; Skinner *et al.*, 2005). The common form of fowlpox is cutaneous, involving spread by biting insects. It is milder than the diphtheritic form, which involves droplet or dust-borne infection of the mucous membranes resulting in higher mortality (Afonso *et al.*, 2000; Skinner *et al.*, 2005).

Replication of FWPV occurs exclusively in the cytoplasm of infected cells and the virions are nonicosahedral (Drillien *et al.*, 1987). Productive replication of FWPV is restricted to avian cells. Although FWPV enters into, and induces synthesis of proteins in, mammalian cells, the production of infectious progeny virus is abortive (Afonso *et al.*, 2000; Skinner *et al.*, 2005; Mingxiao *et al.*, 2006). Foreign proteins expressed by recombinant FWPV (rFWPV) are displayed on the cell membrane in the case of viral surface glycoproteins (Taylor and Paoletti, 1988; Taylor *et al.*, 1988).

Field isolate strains of FWPV are likely to carry a near full-length, infectious provirus of reticuloendotheliosis virus (REV), which can cause REV dissemination with implications for FWPV virulence (Hertig *et al.*, 1997). Proviral sequences have only ever been found at a single genomic location in FWPV, suggesting a single ancestral insertion (Moore *et al.*, 2000). A more recent survey, however, showed FWPV can retain viability and produce disease in wild birds and poultry even when REV inserts are absent (Davidson *et al.*, 2008). Most FWPV vaccines have lost REV coding sequences and retain only partial

REV-long terminal repeats, except the Australia vaccine strain FWPV-S, which carries a nearly full-length REV sequence, leading to its discontinued usage as a vaccine (Singh *et al.,* 2000).

The morphogenesis of Avipoxviruses appears to differ from that elucidated for the Orthopoxviruses such as VV (Boulanger *et al.,* 1998) (Section 1.3.1), in that IMV particles acquire their additional external membrane to form EEV by budding at the cell membrane rather than by wrapping with a double intracellular membrane at the trans-golgi network or endosomes with subsequent loss of the outer of these two membranes by fusion at the cell membrane.

1.3.4 Fowlpox virus strain FP9

Following embryo passage then 438 serial passages of the European virulent FWPV strain HP-1 in chicken embryonic fibroblast cells (CEF), the derived, attenuated strain HP438 (Mayr and Malicki, 1966) was passaged twice further in CEF at a low multiplicity of infection (MOI) and plaque-purified twice to obtain the laboratory-adapted, highly attenuated FWPV strain FP9 (Mockett *et al.*, 1992). The FP9 genome was mapped and compared with that of Vaccinia virus (VV), the type species of Orthopoxvirus genus (Mockett *et al.*, 1992), and later its complete sequence was compared with pathogenic FWPV strains USDA and HP-1 (Laidlaw and Skinner, 2004). Passage-specific mutations, which are probably involved in FP9 attenuation, affect no more than 46 open reading frames (ORFs), especially members of protein families such as the Ankyrin repeat (a stretch of well-conserved 33 amino acids with protein-binding role) proteins, rather than

obvious immunomodulator genes. However, the specific determinants and mechanism of attenuation remains unclear (Laidlaw and Skinner, 2004).

FP9 encodes 247 predicted ORFs, numbered as orthologues of FWPV USDA, e.g. fp9.002 (left) to fp9.259 (right), and contains 10.2 kbp inverted terminal repeats (ITRs; Laidlaw and Skinner, 2004). FWPV USDA encodes 260 putative genes and contains 9.5 kbp ITRs (Afonso *et al.*, 2000). Several genes non-essential for viral replication have been described, such as FP-SNAP, FP-CEL1 and FP-PC1, which are all homologues of cellular genes (Laidlaw *et al.*, 1998). The nature of an immunodominant 39K core protein encoded by fp9.168 and an orthologue of VV A4L has been described (Boulanger *et al.*, 1998). FP9 carries only a partial long terminal repeat of REV (Laidlaw and Skinner, 2004).

1.3.5 Immune response to fowlpox virus infection

1.3.5.1 Immune responses to abortive fowlpox virus infections in mice

Recently, Lousberg *et al.* (2010) identified the role of plasmacytoid dendritic cells, pDCs, as major inducers of Type I IFNα/β upon FWPV infection, in mice. This finding corresponds to the known behaviour of FP9, which is to activate mammalian dendritic cells (Brown *et al.*, 2000; Morse *et al.*, 2005).

VV-encoded IFN modulators have been described extensively. They include the VV E3 intracellular protein, which binds and sequesters dsRNA thus inhibiting IFN induction (Davies *et al.*, 1992) as well as preventing activation of PKR and 2', 5'-OAS; the soluble

B18 protein which binds extracellular IFN (Symons *et al.*, 1995) and a soluble 43 kD protein (B8R), which binds IFN-γ of human, cow, rabbit and rat hosts as well as that of chickens (Alcami and Smith, 1995).

Three FWPV genes were identified as encoding the major immunodominant proteins in mice. They were fpv140 (encoding the FWPV 30/35 kD orthologue of VV H3), fpv168 (encoding the FWPV 39 kD orthologue of VV A4) and fpv191 (encoding the FWPV orthologue of VV p4c) (Laidlaw and Skinner, 2004). The most highly immunogenic part of fpv168 is the central 131-amino acid repeat region (Boulanger *et al.*, 1998).

Several studies have addressed the FP9 ability to elicit CD4+ or CD8+ T-cell responses against antigens in mice, including Webster *et al.* (Webster *et al.*, 2006b), in which FP9 was shown to be immunogenic for CD8+ T cells and capable of inducing protective efficacy, with or without prime-boost combinations with pre-erythrocytic malaria antigen thrombospondin-related adhesion protein and a string of CD8+ epitopes (METRAP). FP9 also confers higher cellular immunogenicity against recombinant antigens from *Plasmodium berghei* (circumsporozoite protein) or human immunodeficiency virus type 1 (a Gag-Pol-Nef fusion protein), when given as a priming or boosting dose, compared to Webster FPV-M FWPV strain (Cottingham *et al.*, 2006). The prime-boost approach relates to initial introduction of a specific antigen to develop immune response against that antigen, followed by secondary presentation of that same antigen using a different vector in order to focus the immune response on the shared antigen rather than on vector proteins. Frequently in experimental approaches, DNA plasmid expressing the antigen is

used as a prime, whilst a viral vector, for instance FWPV, expressing the same antigen is used as a boost (though different viral vectors may be used instead of, or in addition to, the DNA prime).

1.3.5.2 Immune response to fowlpox virus infection in the permissive avian host

Understanding of avian immune responses against FWPV is paradoxically still very limited in comparison to understanding of mammalian host responses. A study by Singh and Tripathy (2003) shows *ex vivo* supernatants of buffy coat cells from chickens infected by FWPV is able to promote a lymphoproliferative response lasting for 2-4 weeks post-infection. The supernatants contain elevated levels of an approximately 48–50 kD protein, able to stimulate naive, non-adherent cells of the buffy coat cultures, in a dose dependant manner, postulated to be a stimulatory cytokine such as IFN-α, IL-15 or IL-2. The proliferative effect was followed by anti-FWPV antibody production. Hghihghi *et al.* (2010) further demonstrated an early stimulation of Type I IFNs, TLRs (3 and 7), cytokines (IL-1β and IL-8) and beta-defensin genes after TROVAC-AIV H5 infection of cultured CEFs. Higher expression was also observed for IFN-γ and IL-10 in spleen cells from vaccinated chickens, compared to controls. TROVAC-AIV H5 is a commercial, recombinant fowlpox vaccine against AIV with H5 gene insert from H5N9 strain A/Turkey/Ireland/83.

1.3.6 Putative immunomodulators encoded by fowlpox virus

Several putative orthologues of cytokines and chemokines, including TGF-β (fpv080), β-nerve growth factor (fpv076) and CC-like chemokines (fpv060, fpv061, fpv116 and fpv121) of FWPV, have been identified (Afonso *et al.*, 2000; Laidlaw and Skinner, 2004). A potential NK cell receptor, C-type lectin, found in US FWPV is predicted to bind MHC Class I and modulate immune activity through intracellular signalling pathway (fpv198). IL-18 have also been predicted in both US FWPV and FP9, using *in silico* sequence analysis (Afonso *et al.*, 2000; Laidlaw and Skinner, 2004). IFN- γ is a Type II IFN which activates macrophages and upregulates MHC Class II expression on cell surface. Based on studies of IL-18-binding protein from the host and from other poxviruses (Xiang *et al.*, 1999), it was proposed that the FWPV IL-18-binding protein homologue might inhibit IL-18-dependent IFN-γ production in infected cells (Afonso *et al.*, 2000).

Banadyga *et al.* (2007) identified fpv039 as a mitochondria-localised, anti-apoptotic protein, which shares homology to the anti-apoptotic cellular protein Bcl-2. fpv039 is intact in FP9 (Laidlaw and Skinner, 2004).

1.3.7 Recombinant fowlpox vaccines for poultry

Live FWPV vaccines have been used successfully in the poultry industry since the 1920s; many such commercial vaccines are now available but in most cases there origins and extent of attenuation are not publicly known. Since the late 1980s, rFWPV based on these attenuated FWPV have been developed to express antigens from several important avian

pathogens, including: avian influenza virus (AIV) (e.g. Taylor *et al.*, 1988; Bublot *et al.*, 2006), Newcastle disease virus (NDV) (e.g. Boursnell *et al.*, 1990; Sun *et al.*, 2006) and Marek's disease virus (MDV) (e.g. Nazerian *et al.*, 1992; Lee *et al.*, 2003). rFWPV recombinants expressing H5 protein of AIV were initially marketed in Mexico against HPAI H5N2 in 1995, and now are being used in S.E. Asia against H5N1. To date, two billion doses of rFWPV-AIV H5 vaccine have been provided in Mexico alone (Swayne *et al.*, 2008). The H5 gene inserts for these rFWPV-AIV are from A/Turkey/Ireland/83 (H5N9), or A/Goose/Guangdong/96 (H5N1). rFWPV, and other viral vectored-vaccines (such as NDV-based vaccines), hold several advantages over the conventional inactivated influenza vaccines. Firstly, uninfected poultry vaccinated with recombinant vectors can be differentiated from infected animals in so-called DIVA (Differentiating Infected from Vaccinated Animals) assays using commercial anti-NP ELISA tests or agar gel precipitation (AGP), as the former do not demonstrate antibodies to NP and M (Suarez, 2005; Mingxiao *et al.*, 2006). The other obvious advantage is that the production of these vaccines in cell culture possesses less risk of accidental influenza virus release. Furthermore, NDV vectors were shown to induce a broad immunity, including humoral, cellular and mucosal responses (Ge *et al.*, 2007). Moreover, the vectored-vaccines also were proven to provide bivalent vaccination (AI and FWPV or NDV, depending on the vector), though this is only of value to the industry in those regions where the vector virus is endemic (Steel *et al.*, 2008). Finally, the recombinant vectored-vaccine is safer for humans or for the poultry and reliable as it avoids use of the adjuvant which is a component of an inactivated vaccine (Swayne *et al.*, 1997; Qiao *et al.*, 2003; Mingxiao *et al.*, 2006; Qiao *et al.* 2006). Although the purpose of adjuvants is to increase the immune

response to the vaccine, the absolute safety of adjuvants can never be guaranteed. There still can be an accidental stimulation of various mechanisms of the immune responses by the adjuvants (Edelman *et al*., 1980, Gupta *et al*., 1993).

FWPV and NDV-based recombinant AI vaccines are designed to be inoculated in 1-day-old chick at the hatchery. Advantages of hatchery vaccination are: (i) biosecurity at hatcheries are higher compared to farms, (ii) automatic or semi-automatic administration systems are possible, (iii) lower numbers of hatcheries than farms, thus vaccination controls are focused to particular area, (iv) different vaccines can be mixed simultaneously, and (v) vaccinated chicks at hatcheries are protected at a younger age compared to those vaccinated in farms (Bublot *et al.*, 2005; van den Berg *et al.*, 2008).

1.3.8 Limitations of viral-vectored vaccines against avian influenza virus

Use of rFWPV vaccines, as with other viral-vectored vaccines (especially NDV-based), is only effective in chickens without prior vaccination and exposure to field strains of FWPV or the other respective viral-vectors (Swayne *et al*., 2000). Pre-existing immunity, whether humoral (which may be maternally-derived) and/or cell-mediated, may restrict infection by, and expression from, the vaccine vector. This might be expected to limit the efficacy of the vaccine (Skinner *et al*., 2005). Nevertheless, limited field evidence suggests that the presence of maternal antibodies to FWPV and AIV or NDV, does not interfere with the immunity induction by rFWPV (Bublot *et al*., 2005; Taylor *et al*., 1996).

Vaccination with rFWPV requires introduction to poultry via scarification (Boyle and Heine, 1994). This is more labour intensive for mass vaccination than is application in drinking water or by aerosol, but is aided by semi-automatic vaccinators and is more reliable (Skinner *et al*., 2005). Moreover, it is not an additional burden in those parts of the world where FWPV vaccination is already required due to endemic fowlpox. An alternative approach for mass application is *in ovo* vaccination. A field trial for multivalent *in ovo* vaccination, which included a rFWPV expressing Newcastle disease virus F and HN genes, demonstrated that the method was successful in the protection against both FWPV and Newcastle disease virus (Sharma *et al*., 2002). NDV-based vaccines can be administered via eye drop (Jeon *et al.,* 2008) but this too is labour-intensive. The mass vaccination technique of aerosol application is more appropriate but the need to use day-old chicks means that maternal immunity becomes a major problem.

A recent study by Wambura *et al.* (2010) described the possibility of mass oral vaccination using thermostable live FWPV (strain TPV-1) coated on oiled rice, as the protective efficacy is equivalent to the conventional wing web stab route. However, the specified vaccine strain is used against field FWPV antigens; hence more study is needed to manipulate the vaccine virus genome to carry different viral antigens.

Approaches to explore ways of improving the efficacy of rFWPV, for laboratory-based studies, would include co-expression of some host-derived immunomodulators, such as cytokines (discussed in the next section). However, there are concerns about this approach. For instance, it might conceivably lead to inappropriate responses against other

infectious agents. Moreover, there is still considerable sensitivity over experiments conducted in Australia in which recombinant Ectromelia (mousepox) virus expressing IL-4 were shown to be more pathogenic, even breaking through genetic resistance and partially overcoming protective immunity (Jackson *et al.*, 2001) This is of particular concern should the immunomodulator genes transfer to wild type strains via recombination during coinfections of wild type and vaccine viruses. Therefore, it is still doubtful whether licenses will be granted for commercial rFWPV vaccines co-expressing host immunomodulator genes (Skinner *et al*, 2005).

1.4 Cytokines

Cytokines are small, soluble proteins secreted especially by cells of immune system immediately after infection or vaccination. The proteins mediate and regulate the intensity and duration of responses comprised of a multitude of effects ranging from activation and differentiation of immune or nonimmune cells, to enhancing immune function (Hilton *et al*., 2002; Degen *et al.*, 2004). In 1986, Mosmann and Coffman first divided cloned, murine helper T lymphocytes, Th, into two functional subsets based on their secreted immunomodulator cytokines. The terms Th1 and Th2 were used for IFN-γ and IL-4-secreting Th lymphocyte clones, respectively. However, the existence of a broad spectrum of cytokine secreting cells, including multiple other haematopoietic cells and even non-haematopoietic cells, has required new terminologies, Th1-like and Th2-like, which are more suitable for *in vivo* immune dysregulation diseases and conditions (Lucey *et al.*, 1996). Table 2 shows several Th1-like and Th2-like cytokines produced by primary cultures of all leukocyte types.

Table 1.1. Leukocyte sources of Th1-like and Th2-like cytokines

Leukocyte cell type	Secreted cytokine (s)	
	Th1-like	Th2-like
Lymphocyte		
CD4+ T cell	IL-2, IFN-γ, IL-12, IL-15, TNF-β	IL-4, IL-5, IL-6, IL-10, IL-13, IL-17
CD8+ T cell	IL-2, IFN-γ, IL-15	IL-4, IL-5, IL-10, IL-17
B cell	IL-12, TNF-β	IL-6, IL-10
NK cell	IFN-γ, TNF-β	
Monocyte	IL-12	IL-6, IL-10
Macrophage	IL-12, IL-18	IL-6, IL-10
Dendritic cell	IL-12, IL-18	
Neutrophil	IL-12	
Mast cell		IL-4, IL-5, IL-6
Eosinophil		IL-4, IL-5, IL-6
Basophil		IL-4

* Modified and updated from Lucey *et al.*, 1996

Th1 cytokines, for instance IL-2, IFN-γ and TNF, are principally involved in the activation of cell-mediated immunity, while Th2 cytokines (e.g. IL-4, IL-5 and IL-6) generally affected in B cells stimulation to regulate humoral immunity (Hilton *et al.*, 2002; Degen *et al.*, 2004). In general, cell-mediated immunity is postulated to be more critical in viral clearance, during primary infection, as host defence against infections are principally mediated by Th1 cells (Ada and Jones, 1986; Bender *et al.*, 1992; Doherty *et al.*, 1997).

1.4.1 Cytokines as vaccine adjuvants

Some cytokines have been proven to perform as effective immunomodulators in animal models or clinical tests, for instance IL-1 to IL-8, IL-12, IL-18, type 1 IFN, colony-stimulating factor and tumour necrosis factor, TNF (Mingxiao *et al.*, 2006). These cytokines have been used extensively in mammalian models as vaccine adjuvants,

including in tumour immunotherapy (e.g. Dredge *et al.*, 2002, Hance *et al.*, 2009), DNA vaccines (e.g. Hartoonian *et al.*, 2009, Wei *et al.*, 2009), bacterial vaccines (Kajikawa *et al.*, 2010), and live viral vaccines (e.g. Kittel *et al.*, 2005).

In contrast to extensive studies of mammalian cytokine genes, chicken cytokine gene identification, cloning and characterization has lagged considerably. Moreover, almost all of the cytokines that have been successfully cloned in chicken are Th1-like. Recent discovery and characterization of avian cytokines including IL-1β and IL-6 (Kaiser *et al.*, 2004), IL-2 (Sundick and Gill-Dixon, 1997; Zhou *et al.*, 2005), IL-10 (Rothwell et al., 2004), IL-12β (Balu and Kaiser, 2003), IL-12 (Degen *et al.*, 2004), IL-15 (Choi *et al.*, 1999; Lillehoj *et al.*, 2001), IL-18 (Schneider *et al.*, 2000) and IL-19 (Kim *et al.*, 2009), has triggered their development as vaccine co-immunostimulators and have allowed the study of their effectiveness. For instance, Yang *et al.* (2009) have demonstrated a novel method of incorporating bioactive chicken IL-2 and chicken granulocyte-macrophage colony-stimulating factor (GM-CSF) on the surface of killed influenza virus particles. In another report, cell-cultured NDV vaccine co-administered with chicken IL-18, which was expressed in either prokaryotic or eukaryotic system, was shown to augment proliferation of peripheral blood mononuclear cells (PBMC), CD8+ to CD4+ ratios and haemagglutination inhibition (HI) titres, compared to chickens vaccinated with NDV alone. Although the innate immune response of avians is not well-characterized, their immune system is closely comparable to that of mammals, with few clear differences. Unlike mammals, chickens do not have lymph nodes and the site for antigen presentation is still questionable. Chickens do have a unique organ called the bursa of Fabricius,

which filled with numerous polyhedral follicles composed of lymphatic tissue and critical for development of B cells (William *et al.*, 2006). Chickens also have a different antibody repertoire (IgM, IgA and IgY) and mechanism of generating antibody diversity, compared to mammals (Reynaud *et al.*, 1994). Moreover, chickens have a minimal essential MHC with the size and content is much reduced than that of mammals (Kaufman *et al.*, 1999).

1.4.2 IL-15

Sharing structural and functional characteristics with IL-2, IL-15 is another T cell growth factor that plays crucial roles in cell-mediated immunity, for instance in inducing B cell proliferation and promoting CTL memory (Min *et al.*, 2002; Stevceva *et al.*, 2006). While IL-2 is selectively expressed in activated T cells, IL-15 mRNA is distributed widely in many tissues and cell types (Grabstein *et al.*, 1994). IL-2 and IL-15 also elicit overlapping immune functions in activation of cytotoxic effector cells and monocytes, and B cell immunoglobulin (Ig) synthesis via co-stimulation. In synergy with IL-12, IL-2R-bound IL-15 was found to enhance the production of IFN-γ by up-regulating natural killer (NK) cells (Carson *et al.*, 1994). NK cells offer direct or indirect lytic activity against intracellular pathogens, and aid the immune system to build an adaptive immune system to eliminate the pathogen. Thus, in virus invasion, any defect in NK cells may weaken the host defence mechanism (Fawaz *et al.*, 1999; Perera *et al.*, 2001).

Chicken IL-15 was first characterized by Choi and colleagues in 1999. They demonstrated that chicken IL-15 expressed in *E. coli* and CHO cells confers growth

promoting activity. It shares highest sequence homology with bovine IL-15 (31% identity, 43% similarity) compared to other mammals. Although the homology was relatively low, the putative chicken IL-15 sequence conserved four essential cysteine residues for the biological activity of mammalian IL-15. It was later verified that the chicken IL-15 gene possesses 187 amino acid ORF encoding a predicted 22 kD protein with two potential N-linked glycosylation sites that can stimulate spleen lymphoblast cell proliferation and enhance NK cell activity *in vitro*, activities similar to those of mammalian IL-15 (Lillehoj *et al.*, 2001).

1.4.2.1 Use of IL-15 as co-immunostimulatory molecule in vaccines

In mice, recombinant Vaccinia virus (rVV) co-expressing gp160 of human immunodeficiency virus (HIV) and hIL-15 has been shown to provide a stronger and more enduring response than rVV expressing gp160 alone (Oh *et al.*, 2003). Integration of human IL-15 into rVV Wyeth strain and modified VV Ankara, MVA, also resulted in better survival rate of athymic nude mice (Perera *et al.*, 2007) and an enhanced *in vivo* viral clearance (Zielinski *et al.*, 2010) upon lethal intranasal challenge with virulent VV, or intravenous challenge with monkeypox virus strain Zaire 79, respectively. Against bacterial infection, the group illustrated highly persistent protective immune responses, induced upon vaccination with recombinant MVA co-expressing IL-15 and five *Mycobacterium tuberculosis* antigens, in a homologous prime boost regime study against tuberculosis (Kolibab *et al.*, 2010). Enhanced CD4 and CD8 T cell memory responses, along with reduction in lung mycobacterial load in lungs, were also observed in mice

infected with Bacille Calmette-Gue´rin (BCG) vaccine, supplemented with IL-7 and IL-15 recombinant proteins, but not IL-1, IL-6 and IFN-α (Singh *et al.*, 2010).

In limited avian model studies, *in ovo* plasmid DNA vaccination against an intestinal coccidial parasite parasite, *Eimeria acervulina*, using gene 3-1E coexpressed with chicken IL-15, has been shown to induce higher serum antibody response than immunization with 3-1E alone. Following challenge with the homologous parasite, chickens vaccinated with 3-1E and IL-15 showed a significant decreased in oocyst shedding and had an increased body weight, compared to chickens vaccinated with 3-1E alone (Ding *et al.*, 2004; Lillehoj *et al.*, 2005). Similar results were obtained when the construct was given subcutaneously (Min *et al.*, 2001) or intramuscularly (Ma *et al.*, 2011). The effect in poultry of co-expression of IL-15 by rFWPV has not been previously studied.

1.4.3 IL-12

Functional IL-12 (IL-12 p70) is a heterodimeric protein (70 kD in mass), consisting of two disulphide-linked subunits, p35 (or IL-12α) and p40 (or IL-12β); heterodimerization being important for the manifestation of IL-12 bioactivity. IL-12 is produced by mononuclear cells, macrophages, dendritic cells and B lymphocytes (Stevceva *et al.*, 2006). In mammals, IL-12 was showed to mediate diverse effects on T cells and NK cells, including T cell proliferation and differentiation, and induction of IFN-γ production by NK cells (Trinchieri *et al.*, 2003). Expression of mRNA for IL-10, which is a crucial T cell regulator and potential Th1 pathway modulator, was also increased in IL-12-treated

mice (Lynch *et al.*, 2003). Chicken IL-12 was first isolated and characterized by Degen and co-workers in 2004 (Degen *et al.*, 2004). Although chicken IL-12 subunits showed restricted sequence homology (21% to 41% amino acid identity) to mammalian IL-12, the study indicated they share many functional similarities. For instance, in order to stimulate chicken cell proliferation, the chicken IL-12 p40 subunit has to be combined with the p35 subunit. Expression of bioactive chicken IL-12 has been achieved in eukaryotic systems, such as baculovirus and COS cells (Degen *et al.*, 2004), *E. coli* (Thomas *et al.*, 2008) and plant systems (Medrano *et al.*, 2010).

Neonatal mice demonstrate a reduction in peripheral expression of IL-12 compared to adult mice. Upon exposure to antigens, neonates treated with IL-12 within 24 hours of birth were shown to increase IFN-γ and IL-10 mRNA expression in their spleens. Higher IgG2a and IgG2b antibody levels were observed upon adulthood challenge of treated mice, compared to untreated mice, suggesting the potential use of IL-12 to enhance early immunity and memory antibody responses against childhood pathogens (Arulanandam *et al.*, 1999).

1.4.3.1 Use of IL-12 as co-immunostimulatory molecule in vaccines

Many studies involving recombinant IL-12 have been performed in mouse models. Although IL-12 is predicted to direct the early host immune defence against Influenza A virus infection (Hama *et al.*, 2009), reports from studies of influenza virus infection offer contradictory results, as administration of recombinant IL-12 to influenza virus-infected mice either enhanced (Tsurita *et al.*, 2001) or delayed (Kostense *et al.*, 1998; van der

Sluijs *et al.*, 2006) the virus clearance. Van der Sluijs and co-workers (2005) also observed augmented virus clearance for influenza virus-infected IL-18-deficient mice. This result was in contrast to results observed by Gherardi *et al.* (2003) and Denton *et al.* (2007).

Upon bacterial infection with *Streptococcus pneumoniae*, previous coadministration of murine IL-12 as adjuvant for pneumococcal polysaccharide conjugate vaccines, enhances IFN-γ mRNA expression, IgG2a antibody levels (Buchanan *et al.*, 2001; Lynch *et al.*, 2003), IL-10 mRNA expression and opsonic activity (Lynch *et al.*, 2003; a process by which opsonins make foreign molecules more susceptible to phagocytosis). Similar observations were made when murine IL-12 was used as adjuvant for conjugate vaccines against *Neisseria meningitidis* meningococcus (Buchanan *et al.*, 2001), while coadministration of exogenous IL-12 with live, attenuated *Francisella tularensis* vaccine enhanced the protective efficacy of the latter in mice upon lethal intranasal challenge (Baron *et al.*, 2007). However, there are concerns about adverse effects in human subjects upon co-administration of recombinant human IL-12 in pneumococcal polysaccharide vaccine, indicated by injection site pain, injection site reaction, fever, headache, myalgia, general pain, asthenia, chills and increased cough (Hedlund *et al.*, 2001).

1.5 Use of host cytokines in recombinant fowlpox vaccines

A recombinant fowlpox vaccine co-expressing HA of AIV H5N1 and chicken IL-18 (Mingxiao *et al.*, 2006) has been described. The result showed that all chickens vaccinated with rFWPV-HA-IL-18 had higher levels of cellular immunity compared to

those vaccinated with rFWPV-HA alone. Similar outcomes were observed in a study to protect chickens against a birnavirus, infectious bursal disease virus (IBDV), using rFWPV co-expressing IL-18 and an IBDV structural protein (Eldaghayes, 2005). Pigs inoculated with a rFWPV co-expressing GP5/GP3 of porcine reproductive and respiratory syndrome virus (PRRSV) with swine IL-18 were also endowed with a stronger immune response and better protection compared to rFWPV-GP5/GP3 alone (Shen *et al.*, 2007). More recently, chickens vaccinated with rFWPV co-expressing the VP2 protein of IBDV and IL-12 alone, or IL-12 co-administered with mineral oil, have demonstrated enhanced levels of IFN-γ in serum and splenocyte cultured supernatant, as well as serum neutralizing antibodies against IBDV, than those vaccinated with mineral oil alone (Su *et al.*, 2011).

1.6 Project aims

The general aim of the project is to evaluate the potential adjuvant effects of chicken IL-12 and IL-15, coexpressed with AI genes in rFWPV, with the hope of possible improvements to current H5N1-rFWPV vaccines. The cytokines were chosen mainly due to their ability to induce IFN-γ, which might be postulated to enhance cell-mediated immunity, and their novelty as immunostimulatory molecules in recombinant virus vaccines. Co-expression of IL-18 was not pursued as part of this project, as such studies were already underway within the Skinner group at Imperial College London in collaboration with scientists at the Institute for Animal Health, Compton.

This present investigation may enable several key questions pertinent to the role and function of avian cytokines to be answered:

1) Do IL-15 and IL-12 confer any significantly enhanced cellular immune response in chickens, as has been extensively demonstrated in mice?

2) Does co-expression of IL-12 in rFWPV-AI vaccines stimulate adverse toxicity effects in chickens, as was shown when it was coadministered with pneumococcal polysaccharide vaccine?

3) How appropriate would the cytokines be as adjuvants in readily available rFWPV-influenza vaccines?

1.6.1 Project objectives

In order to address the general aim, the following specific objectives were envisaged:

(i) to generate rFWPV expressing avian influenza H5, N1 and NP genes

(ii) to insert into these rFWPV recombinant genes encoding chicken IL-12 or IL-15

(iii) to vaccinate poultry using these recombinants and monitor any adverse clinical signs and

(iv) to elucidate the vaccine potential of the cytokines in enhancing humoral and cellular responses in poultry, post-vaccination.

1.7 Thesis summary

Co-expression of avian cytokines IL-15 and IL-12, by recombinant rFWPV already expressing AI antigens, may add to the limited laboratory studies on cytokine effects in avian model.

Chapter 2 describes the general methodology used in this study.

Chapter 3 describes the cloning strategy and construction method to generate the recombinant vaccines.

Chapter 4 describes the results of animal studies involving vaccination with rFWPV-AI co-expressing IL-15.

Chapter 5 describes the results of animal studies involving vaccination with rFWPV-AI co-expressing IL-12.

Chapter 6 describes the effect on host body weight of chicken IL-15 or IL-12 co-expressed from recombinant fowlpox viruses.

CHAPTER 2

Materials and Methods

2.1 Plasmid vectors

2.1.1 pCR2.1-TOPO

Vector pCR2.1-TOPO is a TA cloning vector, 3931 bp in length. It contains ampicillin and kanamycin resistance genes and a multiple cloning site (MCS) downstream of the *Lac* promoter which drives expression of, a *LacZ* gene. An insertion within the MCS interrupts the coding sequence of β-galactosidase, allowing recombinant clones to be identified as white colonies compared to blue colonies containing the parental plasmid The T7 promoter permits *in vitro* RNA transcription/translation and sequencing (Figure 2.1).

2.1.2 pGEM-T Easy

Vector pGEM-T Easy (3015 bp) contains T7 and SP6 RNA polymerase promoters flanking the MCS within a *lacZ* gene. It is a TA cloning vector and encodes a β-lactamase for selection with ampicillin (Figure 2.2).

2.1.3 pCDNA3.1(-)

Vector pCDNA3.1(-) is 5427 bp in length and contains ampicillin and neomycin as selectable marker. Genes inserted in the MCS can be expressed from the T7 promoter by co-expression of T7 polymerase (Figure 2.3).

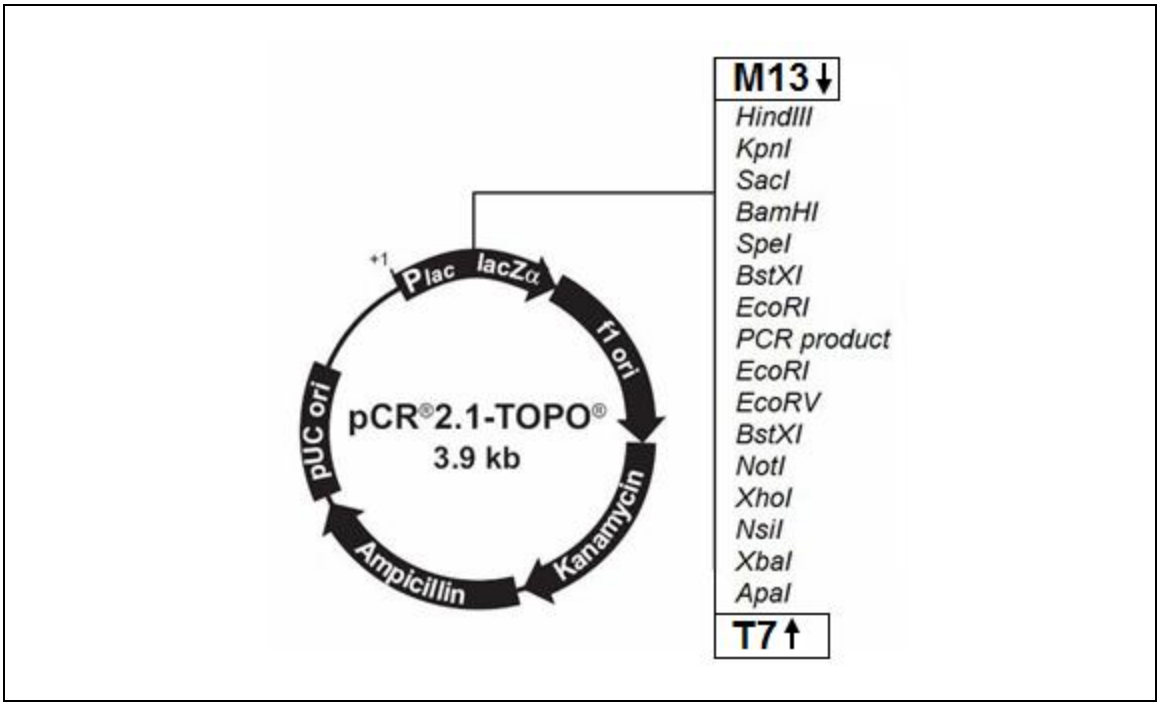

Figure 2.1. A simple map of pCR2.1-TOPO with MCS, reproduced from Invitrogen.

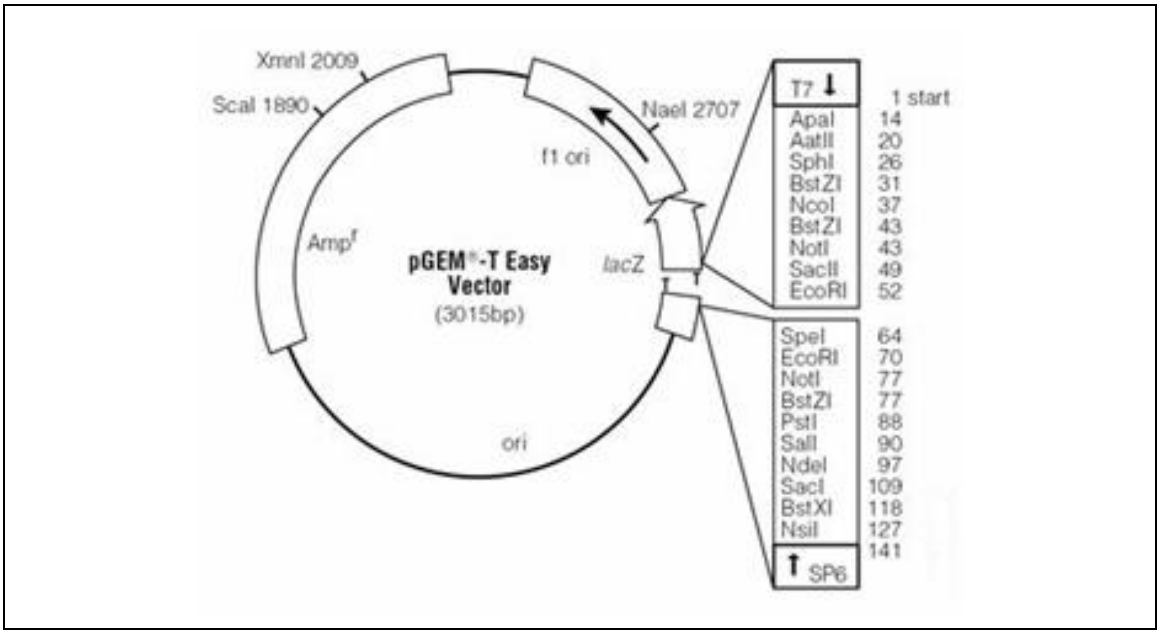

Figure 2.2. A simple map of vector pGEM-T-Easy with MCS, courtesy of Promega.

2.1.4 pEFL29

Plasmid pEFL29 (11557 bp) is a recombination plasmid for the insertion of selectable expression cassettes into FWPV. FWPV sequences in the plasmid (not shown), derived from FP9 and containing ORFs 1, 2 and 3 (equivalent to FWPV genes fpv002, 003 and 004), target the expression cassette to non-essential open reading frames in the inverted terminal repeat regions (ITRs) of FWPV. It was derived from pEFL10 by inserting the FWPV 4b promoter (Binns *et al.*, 1989) upstream of the *lacZ* gene before incorporating the VACV P7.5 promoter upstream of the P4b promoter and in the opposite orientation (Qingzhong *et al.*, 1994). The vector has a unique *SmaI* site (downstream of the p7.5 promoter and within ORF1/fpv002) used for gene insertion. A kanamycin resistance gene (Kan^R) is the bacterial selectable marker for this vector (Figure 2.4).

2.1.5 pEF*gpt*12S

Vector pEF*gpt*12S (6362 bp) contains a synthetic (S) hybrid early/late promoter upstream of an MCS. Kan^R and *E. coli gpt* genes are present as selectable markers for use in bacteria and eukaryotic cells, respectively (Figure 2.5). FWPV sequences in the plasmid (not shown) target the expression cassette to non-essential open reading frame fpv002. FWPV sequences in the plasmid (not shown), derived from FP9, target the expression cassette to non-essential open reading frame ORF 1 (equivalent to fpv002) in the FWPV ITRs. The EcoGPT cassette is embedded within the FWPV recombination sequences so that stable MPA^R rFWPV can be isolated.

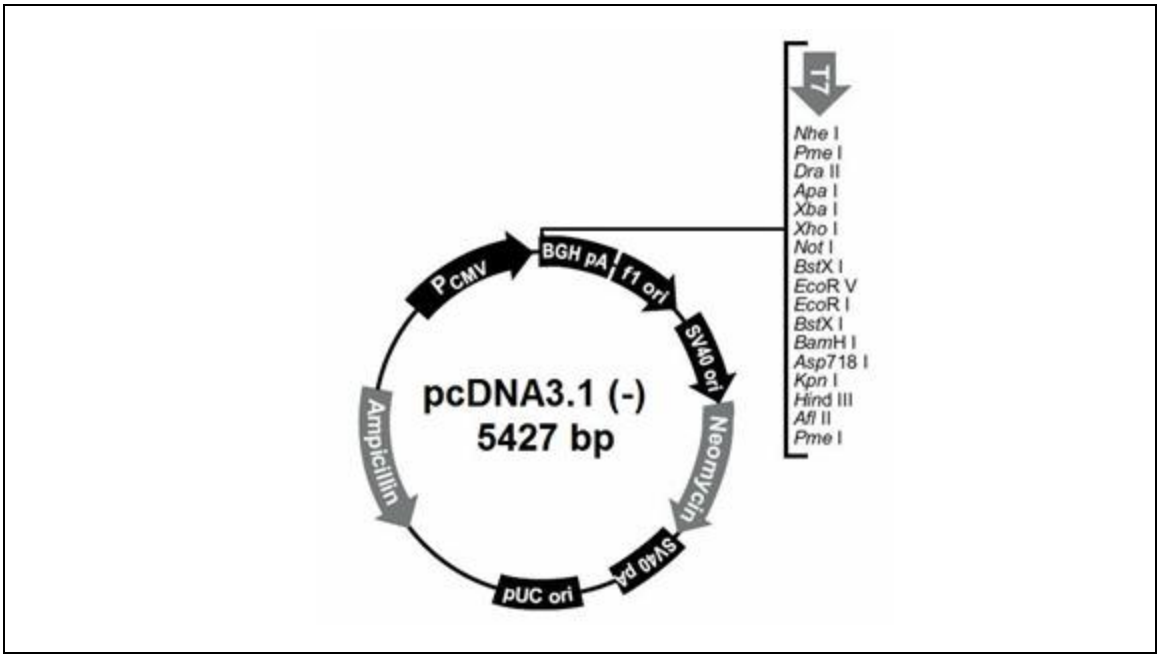

Figure 2.3. A simple map of vector pCDNA3.1 (-) with MCS, reproduced from Invitrogen.

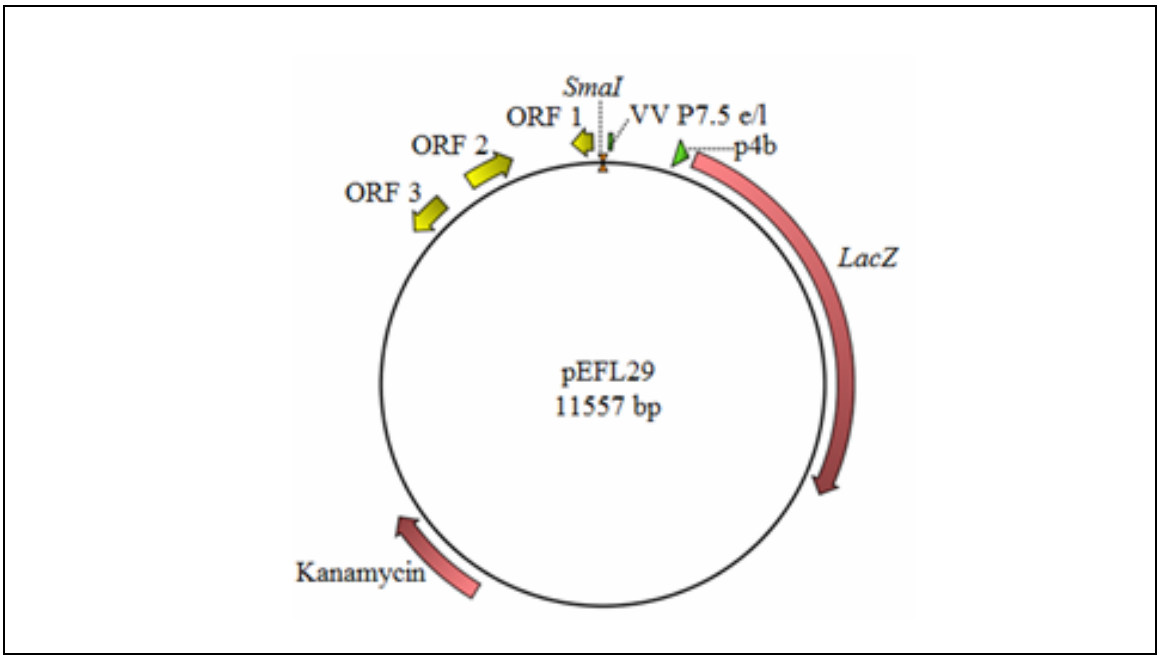

Figure 2.4. Map of vector pEFL29 with a unique SmaI (**CCC**GGG) site.

2.1.6 pPC1.X

Vector pPC1.X (5584 bp in length) was used for the construction of recombination plasmids that would insert expression cassettes for chicken interleukin genes into the FWPV genome by transdominant selection (Laidlaw *et al.*, 1998). It contains an ampicillin resistance gene and an *E. coli gpt* gene as selectable markers for use in bacteria and eukaryotic cells, respectively. The *E. coli gpt* gene is located outside the FWPV recombination sequences, allowing it to be used as a selectable marker for transdominant selection. FWPV recombination sequences are derived from the FWPV orthologue of the PC1 gene (fpv030; Laidlaw and Skinner, 2004) allowing it to be used to insert genes at this locus even when other genes are inserted at the fpv002 locus. A large part of the gene was deleted by digestion with *XbaI* followed by religation then one of the remaining *HindIII* restriction sites was deleted, leaving a unique *HindIII* site towards the C-terminus of fpv030 to facilitate cloning of inserts (Figure 2.6).

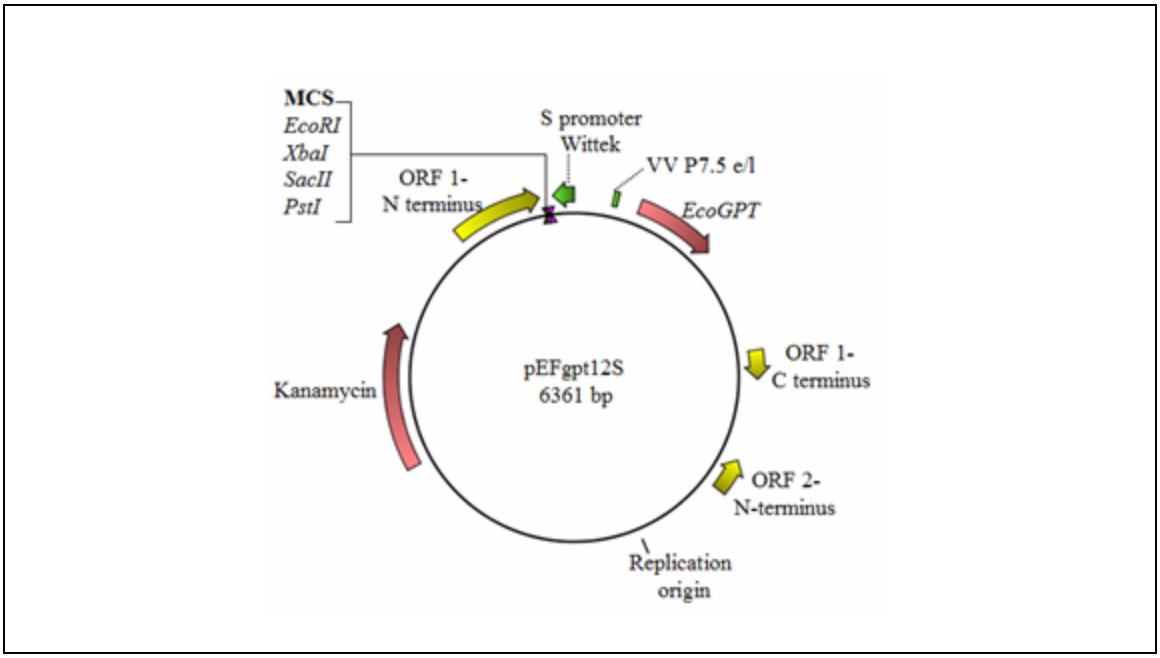

Figure 2.5. Map of vector pEF*gpt*12S, 6361 bp, with unique enzyme sites, *EcoRI* (**G**AATTC), *XbaI* (**T**CTAGA), *SacII* (**CCG**CGG) and *PstI* (**CTGCA**G).

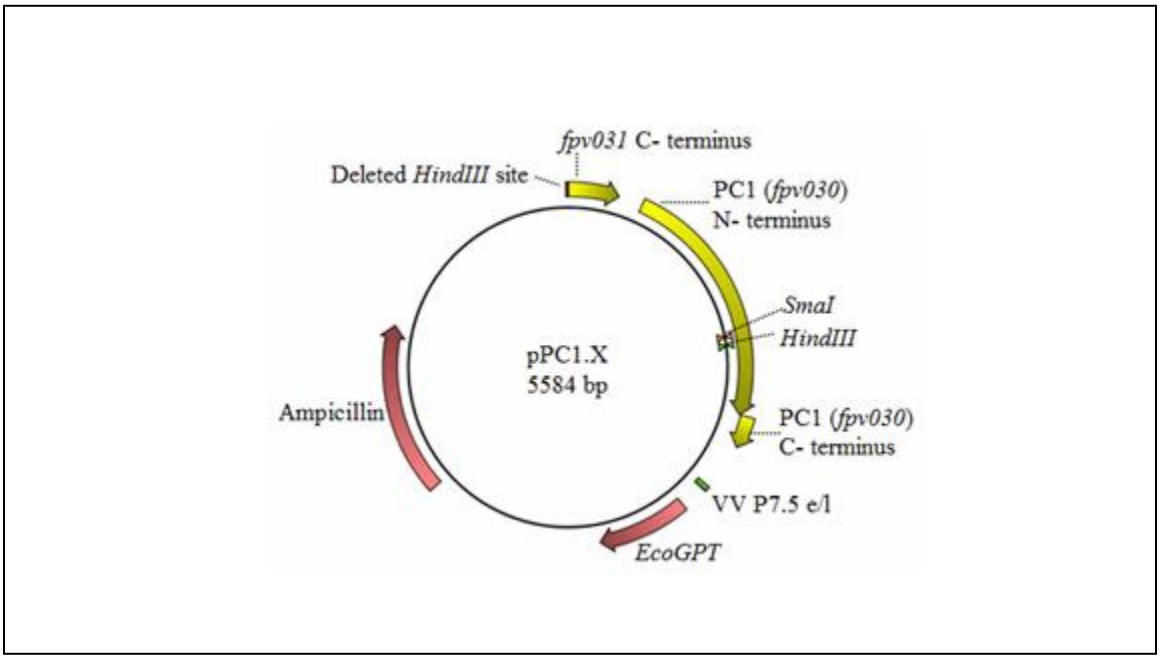

Figure 2.6. Map of vector pPC1.X, 5584 bp, with unique enzyme sites, *SmaI* (**CCC**GGG) and *HindIII* (**A**AGCTT).

2.2 Construction of recombinant plasmids containing influenza and cytokine genes

2.2.1 Cloning of AIV genes into pEFL29

cDNA copies of H5, N1 and NP genes of avian influenza (AI) from strain A/Chicken/Malaysia/5858/2004 had been cloned in pCR2.1-TOPO vector and sequenced in Malaysia by Nurul Hidayah Abdullah Zawawi from Universiti Putra Malaysia.

2.2.2 Bioinformatics analysis

The polybasic cleavage site of the H5 gene of the Malaysian HPAI virus strain A/Chicken/Malaysia/5858/2004 was identified using CLC Combined Workbench 3 software (CLC bio). The deduced H5 amino acid sequences, with and without the polybasic cleavage site, were aligned with sequences in the databases using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Restriction enzyme maps and coding sequences of genes or plasmid vectors of interest were generated using the same software to facilitate cloning strategies and primer design. Cloning strategies for AIV and cytokine genes were designed on the basis of restriction enzyme maps generated in bioinformatics analysis. For insertion into expression vectors, AIV genes were produced as PCR products (with appropriate sequences added to facilitate sub-cloning), while cytokine genes were digested from their respective plasmid vectors.

2.2.3 Preparative Polymerase Chain Reaction (PCR) of AIV genes for sub-cloning

Three primer sets were generated to amplify the full coding sequences of H5, N1 and NP gene, with either *EcoRV* or *SspI* sites incorporated at the termini (Appendix 1). Removal from the H5 gene of sequence encoding the polybasic sequence and the mutation of nucleotide 1022 from G to C, to change an arginine to a threonine, were done using mutagenic primers S(2-F) and S(1-R). Primers H5-F and S(1-R) were used to generate the first H5 fragment, while H5-R and S(2-F) were used to generated the second H5 fragment. The full length modified H5 gene, designated H5 S, was obtained through PCR overlap extension mutagenesis using 50 ng of each first round PCR product as template. The reaction mixture contained 10X Pfx buffer (5 µL; Sigma), Accuprime Pfx DNA polymerase (2 U), oligonucleotide primers (0.75 µL of each 10 µM stock) and template DNA (50 to 260 ng) in a final volume of 50 µL. PCR was performed using the protocol in Section 2.2.5, but the extension temperature was changed from 72 ^{o}C to 68 ^{o}C following guidance from the manufacturer of Accuprime Pfx DNA polymerase. Amplification of the N1 gene, using primers N1-F and N1-R, and the NP gene using primers NP-F and NP-R, was carried out with the same reaction mixture and protocol.

2.2.4 Analytical PCR reaction mixture

The reaction mixture for a small scale PCR checking contained 10X PCR buffer (2 µL; Sigma), JumpStart *Taq* DNA polymerase (0.5 U; Sigma), dNTPs (0.5 µL of 10 mM) and oligonucleotide primers (0.5 µL of each 10 µM stock), in a total volume of 20 µL.

2.2.5 PCR protocol

PCR was conducted according to the following programme:

(i) Initial denaturation at 95 ^{o}C for 3 minutes.

(ii) 4 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 50 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1.5 minutes.

(iii) 26 cycles of denaturation at 95 ^{o}C for 30 seconds, annealing at 59 ^{o}C for 30 seconds and extension at 72 ^{o}C for 1.5 minutes.

(iv) Final extension was operated at 72 ^{o}C for 10 minutes.

2.2.6 Purification of PCR products

DNA from PCR and other enzymatic reactions was purified when necessary using the QIAquick PCR Purification Kit, supplied by QIAGEN. Buffer PB (5 volumes) was first added to 1 volume of the reaction, mixed and placed in a 2 ml collection tube. The sample was then applied to QIAquick column and centrifuged at 10,000 rpm for 1 minute. Buffer PE was used to wash the column before centrifuged for 1 minute. The buffer in the collection tube was discarded before centrifuged again for 1 minute. DNA was eluted with of nuclease free water (50 μl).

2.2.7 Small-scale preparation of plasmid vectors

Small-scale isolation of plasmid vector DNA, based on adsorption of DNA onto silica in the presence of high salt after alkaline lysis of bacteria (Vogelstein and Gillespie, 1979), was performed using the QIAprep Spin Minipreparation Kit, supplied by QIAGEN. A single bacterial colony was picked from a freshly streaked selective plate and

resuspended in Luria Bertani (LB; 5 mL) broth containing the appropriate antibiotic (100 μg/mL ampicillin or 50 μg/mL kanamycin), before overnight vigorous agitation at 37 ^{o}C. Bacteria were harvested by centrifugation (13,000 rpm; 5 minutes). Buffer P1 containing 100 μg/mL RNase A (250 μL) was added and pellets were resuspended then transferred into a microcentrifuge tube. The bacteria were lysed under alkaline conditions in Buffer P2 (250 μL). After less than 5 minutes of thorough mixing, the lysate was neutralized with Buffer N3 (350 μL). Cell debris, proteins, chromosomal DNA and SDS were precipitated under the high salt concentration in the buffer. The solution was subjected to centrifugation at 13,000 rpm for 10 minutes. The supernatant was decanted to a QIAprep spin column before subsequent centrifugation (13,000 rpm, 1 minute). Adsorbed plasmid DNA was then washed with Buffer PE (750 μL) and an additional centrifugation was applied to remove residual wash buffer, before elution of DNA with DNAse free water (50 μL).

2.2.8 Restriction enzyme digestion

Restriction enzymes (restriction endonucleases) recognize and cut specific sequence in double-stranded DNA to produce sticky or blunt ends. In this study, restriction enzyme digestions were used: to produce compatible ends after PCR before cloning into plasmid vectors, to excise inserts from vector plasmids, to check the size of plasmid vectors and to determine the orientation of inserts. Reactions were allowed to proceed for 3 hours or overnight, in appropriate 10X Buffer (NEB). The requirement for BSA and the temperature for incubation depended on the enzyme. In preparation for sub-cloning, 2 to 10 μg of plasmid vectors were digested with 40 U of suitable restriction enzymes in total

volumes of 50 µL or 100 µL while for analytical purposes, 100 to 200 ng of plasmid vectors were digested with 10 to 20 U of appropriate restriction enzyme(s) in 20 µL of total volume. Restriction enyme digest products were analyzed by agarose gel electrophoresis

2.2.9 Phosphatase treatment of cut vectors

To prevent self-ligation of plasmid vector DNA linearized with single restriction enzymes, the 5' phosphate groups were removed by phosphatase treatment. Unpurified restriction enzyme digestion mixture (10 µL), which contained the plasmid vector of interest, was mixed with Antarctic phosphatase (25 U) in 10X reaction buffer in a final volume of 100 µL. The mixture was incubated at 37 ^{o}C for 30 minutes then the enzyme was inactivated by heating at 65 ^{o}C for 5 minutes. Purification of the digested vector was performed using QIAprep Spin Minipreparation Kit, before size confirmation by gel elecrophoresis.

2.2.10 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze all PCR or restriction enzyme digestion samples. Agarose (1 %) was dissolved in 1X Tris-Borate-EDTA (TBE) buffer containing 1 µg/µL ethidium bromide. Samples were mixed with equal volume of loading dye before loaded into gel wells. A DNA marker with bands ranging from 100 bp to 10 kb was always included. Gels were electrophoresed in 1X TBE at 90 V for 30 to 60 minutes or until the dye was about one third from the bottom of the gel. Gels were visualized under ultraviolet light using a GelDoc 2000 system (BioRad) for detection and recording.

2.2.11 Ligations

Ligation reactions involved 20 U of T4 DNA ligase (20 U), ATP (1 µL of each 0.05 mM stock) and 10x NEB Buffer 4 (1 µL) in a total volume of 10-15 µL. In this study, two types of ligation were used. 'Sticky-end' ligations were set up with 1:1 ratio (usually 50 ng each) between the plasmid vector and DNA insert. The ligation mixture was left at 16 ^{o}C overnight. 'Blunt-end' ligations required vector to insert ratios of 1:2 to 1:10, with an incubation temperature of 4 ^{o}C (overnight).

2.2.12 Transformation

Transformation of chemically competent bacteria with plasmid DNA was performed using the method described by Mandel and Higa (1970). Plasmid vector (1 µL) or ligation mixture (5 µL) was added to a bijou containing of 5-alpha F´I^{q} competent *E. coli* (25 µL; NEB) and incubated on ice for 30 minutes. The cells were heat-shocked at 42 °C for 90 seconds and immediately placed on ice again for 2 minutes. SOC medium (1000 µL; Invitrogen) was added and the tube was incubated at 37 °C with shaking at 200 rpm to allow expression of antibiotic resistance. After 1 hour, the mixture (50 to 200 µL) was plated onto an LB agar plate containing either ampicillin or kanamycin antibiotics (50 µg/µL). The plate was incubated at 37 ^{o}C overnight. Single colonies were picked into antibiotic-supplemented media to be grown overnight for subsequent plasmid minipreparation.

2.2.13 Selection for positive transformants

Several approaches were taken to select for positive transformants. A method using restriction enzyme digestion has been described in Section 2.2.8.

2.2.13.1 Culture PCR

Colonies that were picked after transformation were grown in LB (15 mL) supplemented with appropriate antibiotic(s) (15 µL of 50 µg/µL) at 37 ^{o}C overnight. The culture (0.5 µL) was used to provide templates for analytical PCR (Section 2.2.4).

2.2.13.2 Sequencing

Plasmid transformants which were predicted to be positive after restriction enzyme digestion or culture PCR were sent for sequencing. In a tube with 10 µL of total volume, 3.2 pmole of primer with a Tm of 50 – 55 °C were mixed with 200-500 ng (per 3 kb) of the plasmid DNA sample. The tube was then forwarded to Clinical Sciences Centre (CSC), a division of the Faculty of Medicine of Imperial College London, which is located in the Hammersmith Hospital Campus in London, United Kingdom, to be analysed. The sequencing data were assembled and evaluated using CLC Combined Workbench 3 software described in the early part of this chapter.

2.2.14 Cloning of chicken cytokine genes

Plasmid pGEMT-Easy and pCDNA3.1(-) with cytokine IL-15 and IL-12 genes incorporated, respectively, were supplied by Dr Pete Kaiser from the Institute for Animal Health, Compton. A sub-cloning strategy was designed on the basis of the restriction maps of IL-12 or IL-15 and the vectors used, pEF*gpt*12S and pPC1.X.

2.2.14.1 Cloning of chicken cytokine genes into pEF*gpt*12S

Plasmid pGEMT-Easy/IL-15 (5 µg) and pEF*gpt*12S (5 µg) were digested using *PstI* and *SacII* (40 U), separately, in 10X NEB buffer 2 (5 µL) and 100X BSA (0.5 µL), in a total volume of 50 µL. Plasmid pCDNA3.1(-)/IL-12 (10 µg) was digested using *PmeI* (40 U), while pEF*gpt*12S (4 µg) was digested using *XbaI* (40 U), in 10X NEB buffer 4 (5 µL) and 100X BSA (0.5 µL) in a total volume of 50 µL. The four individual digestion mixtures were incubated at 37 ^{o}C for 3 hours. Digested IL-15 and IL-12 gene fragments were purified by QIAquick Gel Extraction Kit after being electrophoresed, while restricted pEF*gpt*12S was purified by QIAquick PCR Purification Kit. The purified DNA samples were ligated before transformed into competent *E. coli* strain.

2.2.14.2 Cloning of chicken cytokine genes into pPC1.X

Plasmid pEF*gpt*12S/IL-15 (5 µg) was digested using *PstI* and *HindIII* (40 U), while pPC1.X (5 µg) was digested using *NsiI* and *HindIII* (40 U), in separate reaction mixtures of 10X NEB buffer 2 (5 µL) and 100X BSA (0.5 µL), in a total volume of 50 µL. Plasmid pEF*gpt*12S/IL-12 (5 µg) and pPC1.X (5 µg) was also restricted independently using *HindIII* (40 U) in 10X NEB buffer 2 (5 µL) in a total volume of 50 µL. The

digestion mixtures were incubated at 37 ^{o}C for 3 hours and purified using either QIAquick Gel Extraction Kit after being electrophoresed, or by QIAquick PCR Purification Kit. Ligations and transformations were applied to the DNA samples after the purification procedure.

2.2.14.3 End repair

Sticky ends of pEF*gpt*12S generated by digestion with *XbaI* (0.5 µg) were 'polished' using Klenow DNA Polymerase enzyme (1 U) in a reaction mixture which contained dNTP mix (10 µL of each 1mM stock) and 10X NEB buffer 2 (10 µL) in a total volume of 100 µL. The mixture was incubated at 25 ^{o}C for 15 minutes before the DNA was purified by QIAquick PCR Purification kit.

2.2.14.4 QIAquick gel extraction kit

The QIAquick gel extraction kit was used to extract and purify DNA fragments after agarose gel electrophoresis using the protocol provided by the manufacturer. The cassette of interest digested from the plasmid vector was excised from the gel using a clean scalpel under short exposure of long wave UV light. The gel slice was then weighed in a microcentrifuge tube. Three volumes of Buffer QG were added to one volume of gel (100 mg ~ 100 µL) and incubated at 50 °C for 10 minutes or until the gel slice dissolved completely. One gel volume of isopropanol was mixed with the sample and the mixture was applied onto a column with the provided 2 mL collection tube underneath, and centrifuged. Buffer QG allowed DNA to be adsorbed onto the membrane while isopropanol increased the yield. Buffer PE (750 µL) was then added to wash the column.

An additional spin was carried out after the flow-through was discarded to remove residual wash buffer. A clean 1.5 mL microcentrifuge tube was positioned under the column. Elution of DNA was with 30 to 50 μL of water. All centrifugation steps were performed at 13,000 rpm for 1 minute.

2.3 Construction of recombinant FWPV co-expressing influenza and cytokine genes

2.3.1 Virus and media

The initial stock of wild type FP9 was from Skinner laboratory stocks. The derivation of FP9 via 438 serial passages of the wild-type fowlpox virus HP-1, followed by plaque purification, has been described (Mockett *et al.*, 1992). All media for tissue culture works were warmed at 37 °C before used. The media constituents are described in Appendix 2.

2.3.2 Preparation of chick embryo fibroblast (CEF) primary cell cultures

CEFs for this project were either prepared from 9 to 10-day-old embryonated eggs using standard methods or provided by the Institute for Animal Health, Compton, Berkshire, UK. CEFs were cultured in either tissue culture dishes or different sizes of tissue culture flask and incubated in 10 % NBBS in DMEM media at 37 ^{o}C in 5 % CO_2 until used.

2.3.3 Fluid overlay

Growth medium in either a well or a flask was removed before an inoculum (virus stock diluted in serum free DMEM) with enough volume just to cover the cells, was added. The virus was allowed to adsorb for 1 to 2 hours at 37 ^{o}C. After aspiration, an appropriate

volume of 2 % NBBS in DMEM was added and the cells were placed again in 37 ^{o}C incubator with 5 % CO_2. When a good cytopathic effect (CPE) was observed under a microscope within 4 to 5 days (the cells changed from elongated to more circular shape), both supernatant and cells were harvested. Freeze/thawing at -80 ^{o}C was done three times to ensure intracellular virus release.

2.3.4 Agarose overlay

Growth medium was discarded then an appropriate volume of inoculum was added to the cells and left for 1 to 2 hours at 37 ^{o}C. The inoculum was then aspirated. Agarose medium (Appendix 2) was gently overlayed and left for 10 to 15 minutes at room temperature to solidify. The cells were incubated in 37 ^{o}C incubator with 5 % CO_2 again until plaques could be seen by holding the dish up to the light (4 to 6 days). To ease viewing of plaques of viruses containing *LacZ* gene, X-Gal could be added at the fourth day post infection.

2.3.5 Recombination/Transfection using lipofectin

T12.5 flasks containing 50 to 80 % confluent CEF cells were selected for infection of FP9 and transfection of recombinant plasmids. The medium from each flask was first discarded before addition of 500 µL of FP9 in pre-warmed serum free DMEM medium. The flasks were incubated at 37 °C in 5 % CO_2 for 2 hours to allow the virus to adsorb to cells, with a gentle rocking every 30 minutes. Pre-warmed 2 % NBBS DMEM media (2.5 mL) was then added to each flask and reincubated for a further 2 hours. For each transfection, two solutions were prepared in a separate bijou. One solution contained

plasmid DNA (5 µg) in serum free DMEM (50 µL), and another one contained of lipofectin reagent (10 µL) in serum free DMEM (50 µL). The two solutions were subsequently combined and incubated at room temperature for 10 to 15 minutes. In the meantime, flasks were rinsed twice with 3 mL serum free DMEM. The second rinse was left inside the flasks while awaiting the plasmid DNA-lipofectin complex. After the incubation period, 2 % NBBS DMEM media (1.5 mL) was added to each bijou with plasmid DNA-lipofectin complex and gently mixed. This solution was then added to the washed cells in the flasks after the second rinse had been discarded, and incubated at 37 °C in 5 % CO_2 overnight before replaced with 2.5 mL of 2 % NBBS DMEM. The addition of mycophenolic acid (MPA) (25 µg/mL), xanthine (250 µg/mL) and hypoxanthine (25 µg/mL) for *gpt* selection could be done at this step. The transfected cells were then incubated further for 4 to 5 days until CPE was observed. The cells and the supernatant were harvested into a bijou and freeze/thawed three times.

2.3.6 Selection of viruses

2.3.6.1 Selection of viruses containing *LacZ* gene

Agar overlay solution containing X-Gal at a final concentration of 0.4 mg/mL was pippetted onto the agar-overlaid cells at the fourth day post-infection. Successful recombination/transfection of recombinant plasmids carrying a *LacZ* gene into FP9 was demonstrated by blue plaques observed from the next day. Single plaques were picked, diluted in 500 µL serum free DMEM and freeze/thawed three times. This protocol was repeated six times to obtain plaque-purified virus.

2.3.6.2 Selection and resolution of viruses containing *gpt* gene

At the stage of recombination/transfection, the viruses were incubated in 2 % NBBS DMEM in the presence of MPA. Three serial viral passages were performed by infecting 200 µL of the virus in T12.5 flask. Virus from the third passage was sequentially diluted (10^{-3} to 10^{-6}) using serum free DMEM before being subjected to agar overlay infection in the presence of MPA. Single plaques were picked into 500 µL of serum free DMEM and freeze/thawed three times. The clones were subjected to further (three to six times) plaque purifications in non-selective *gpt* media.

2.3.7 Verification of recombinant viruses

2.3.7.1 PCR after genomic extraction

DNA was isolated from infected tissue culture cell lysates using Wizard SV Purification Kits (Promega). Using an equal volume of Wizard SV Lysis Buffer, 150 µL of harvested lysates from at least $1x10^4$ cells to a maximum of $5x10^6$ cells were lysed. Total sample lysates were then transferred onto spin columns. Centrifugation occurred at 13 000 x *g* for 3 minutes and the flow-through was decanted. To wash, 650 µL of Wizard SV Wash Solution was added into the column and centrifuged for 1 min. The liquid was discarded and the column was centrifuged for another 2 minutes to dry the binding matrix. The DNA was then eluted with 250 µL of nuclease free water. An aliquot (3 µL) from the total yield was used in small scale PCR screening.

2.3.7.2 Western blotting

For protein expression analysis, we utilized CEF infected with RG14 lysate supplied by Prof Wendy Barclay (Imperial College London), as positive control, while wild type FP9 protein lysate and lysates of CEF, DF1 and/or QT35 served as negative controls. RG14 is a reference vaccine virus strain derived from A/Vietnam/1194/2004, and was developed by NIBSC using reverse genetics technology to transfer the H5 (modified to remove the polybasic cleavage site sequence) and N1 segments into a PR8 background. The recombinant protein lysates were prepared by infecting CEFs, with the recombinant viruses at a multiplicity of infection (MOI) of 3 in a 12-well plate, for 48 hours. The cell pellet was harvested by adding 250 µL RIPA buffer (50 mM Tris pH 8.0, 0.1 % SDS, 1 % NP-40, 0.05 % w/v sodium deoxycholate and 150 mM NaCl) and squirting the pipette until total cell pellet was detached. Protease inhibitor (0.01 %) was included to minimize protein degradation.

Standard Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins expressed by the infected cells (Sambrook *et al*, 1989). A stacking gel (130 mM Tris-HCl pH 6.8, 0.1 % SDS, 0.1 % ammonium persulphate, 5.1 % acrylamide and 0.1 % TEMED) was poured on top of a resolving gel (375 mM Tris-HCl, 0.1 % SDS, 0.1 % ammonium persulphate, 9.9 to 15 % acrylamide and 0.04 % TEMED) and allowed to solidify. A 10 or 15 well comb was added prior to setting. Protein samples from either lysed cells or supernatant (10 to 30 µL) were mixed with 1X Laemmli Loading Buffer (15 % β-mercaptoethanol, 15 % SDS, 1.5 % w/v bromphenol blue and 50 % glycerol, diluted in distilled water), and heated for 1 minute at 60 to 100 °C.

Electrophoresis was conducted in SDS-PAGE running buffer (190 mM glycine, 20 mM Tris-Base and 0.1 % SDS) initially at 100 V, switching to 200 V when the samples reached the resolving gel. Protein size markers (BioRad Precision Plus Protein Standards) were run alongside. The resolved proteins and controls were transferred onto a nitrocellulose membrane, at 100 V for 1 hour at room temperature, in Transfer Buffer mix (20 % methanol, 10 % Transfer Buffer : 20 mM Tris-Base, 192 mM glycine and 0.01 % SDS, diluted in distilled water). The membrane was subsequently immersed at 4 °C overnight in 5 % skimmed milk in Phosphate Buffered Saline, PBS (200 mM phosphate and 150 mM NaCl). The solution was then removed and the membrane was incubated with primary antibody, diluted with PBS containing 2 % skimmed milk, at its respective ratios (1:500, 1:1000 or 1:2000) for 1½ hours at room temperature, with slow rotation. The membrane was then washed with PBS, five times for five minutes. Secondary antibody, conjugated with horseradish peroxidase and diluted with PBS/2 % skimmed milk, was added at an appropriate dilution, and the container was placed on a rotator for another 1.5 hours. Excess conjugate was removed and rinsed with PBS. The membrane was then developed using Amersham ECL Plus Detection Reagent (GE Healthcare). The exposure time of the membrane to autoradiograph film varied from 5 to 20 minutes.

2.3.7.3 Indirect immunofluorescence assay (IFAT)

CEF were grown in 6-well plates containing two round coverslips (1.2 cm in diameter) in each well, until the monolayer reached 80 % confluency. Each well was infected with virus stock at 0.3 MOI, while one well was left uninfected. The infection was left to proceed overnight in 2 % NBBS DMEM medium. Cells were washed twice with 5 mL

pre-warmed 1X PBS. Each coverslip was transferred into individual wells of a 12-well plate. Each was incubated with specific primary antibodies diluted in PBS, for 2 hours, with a gentle shake every 30 minutes. After three washes with 1 mL PBS, cells were incubated with secondary antibodies, conjugated with FITC, for 1 hour, before being washed again three times. All FITC procedures were conducted in dim light. Coverslips were gently placed onto glass slides, air-dried and viewed under a fluorescent microscope (model Leica DMRA II).

2.4 Characterization of immune response by *in vivo* experimental study

2.4.1 Chickens

Layer chickens of the White Leghorn breed used in all the animal experiments were imported from SPAFAS Australia Pty Ltd by Malaysian Vaccines and Pharmaceuticals Sdn Bhd (www.mvp.com.my). They were tested and declared free from specific pathogens (SPF) by Charles River Laboratories (www.criver.com).

Chickens were kept in separate cages by group, fed twice a day using commercial chicken feed (brand Gold Coin) and provided with water, *ad libitum.*

Table 2.1. Composition of chicken feed in commercial brand, Gold Coin, of Malaysia

Composition	Percentage, %
Crude protein, minimum	21.2
Crude fibre, maximum	5.0
Crude fat, minimum	5.0
Moisture, maximum	13.0
Ash, maximum	8.0
Calcium	0.8 – 1.2
Phosphorus	0.6 – 1.0

2.4.2 Animal experimental design

All animal experiments were conducted at Animal Experimental House of the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM), Malaysia, in compliance with the international Guide for the Care and Use of Laboratory Animals (ILAR, 1996), under the approval of the local Institutional Animal Care and Use Committee (IACUC) of UPM (Reference No.: UPM/FPV/PS/3.2.1.551/AUP-R72).

2.4.2.1 Animal experimental 1

Fifty four 1-day-old chicks were randomly assigned into six groups, nine in each group as follows:

(i) Control (mock-vaccinated with 100 μL PBS).

(ii) Parental FWPV FP9 (WT FP9).

(iii) rFWPV expressing H5 gene (rFWPV/H5).

(iv) rFWPV coexpressing H5 and IL-15 genes (rFWPV/H5/IL-15).

(v) rFWPV expressing N1 gene (rFWPV/N1).

(vi) rFWPV expressing N1 and IL-12 genes (rFWPV/N1/IL-12).

The viral vaccine inoculation dose was 10^5 PFU, diluted in PBS to a total volume of 100 μL. Within one hour upon preparation, the viral inoculum was inoculated subcutaneously at the scruff of the neck of each bird, using a 27-gauge needle. Clinical signs were observed twice daily post inoculation (p.i.) for two weeks.

Blood sampling (for serum) of each chicken was done on a weekly basis. At Week 2 and 5, whole blood (0.2 mL) of each chicken in each group of nine was sampled and 'pooled' into 3 groups (0.6 mL in total), for lymphocyte isolation for flow cytometry analysis (Section 2.4.7).

All chickens were weighed at Week 1 until Week 4. The chickens were left until Week 7 to distinguish their sexes before being euthanized.

2.4.2.2 Animal experiment 2

Thirty 1-day-old chicks were randomly assigned into six groups (five chickens in each group). Vaccine groups and inoculation design were similar to Section 2.4.2.1. Blood sampling (for serum) of each chicken was done on a weekly basis. At Week 2 and 4, whole blood (0.5 mL) of each chicken in each group of five was sampled for lymphocyte isolation for flow cytometry analysis (Section 2.4.7). The chickens were left until Week 7 before being euthanized.

2.4.3 Serum collection

Collected whole blood (0.2 to 1 mL, according to chicken size) was left in the syringe, horizontally, at room temperature, for five hours (no longer than six hours) to allow clotting. The transparent, yellowish liquid (serum) was gently transferred into 1.5 mL tubes and centrifuged at 1200 rpm for 10 minutes. The supernatant was collected into new clean tubes and kept in -20 ^{o}C until further used.

2.4.4 Red blood cell isolation

Chicken whole blood, 1.5 to 2 mL, was collected into vacutainers containing EDTA as anti-coagulant and 500 μL PBS. The blood was transferred into 50 mL tubes containing 30 mL cold PBS and mixed gently. The tube was centrifuged at 2000 rpm, 4 ^{o}C for 10 minutes. Supernatant was inspected to be completely yellowish, without reddish particulates (lysed red blood cells, RBC), and slowly discarded. Cold PBS, 20 mL, was added to the tube which was centrifuged again at 2000 rpm, 4 ^{o}C for 10 minutes. The clearer yellowish colour of the supernatant was noted before being discarded. Cold PBS (20 mL) was added and this step was repeated further until the supernatant was colourless. Packed RBC at the bottom of the tube were pipetted gently into a clean tube, labelled as 100 % concentrated, and kept at 4 ^{o}C no longer than two days.

2.4.5 Haemagglutination test

PBS (25 μL) was aliquoted into 12 wells (one row) of U-bottomed 96-well plates. Inactivated, low pathogenic H5 AIV (50 μL; A/Malaysia/Duck/8443/04 H5N2), provided by the Veterinary Research Institute, VRI, Ipoh, Malaysia, was added into the first well. The virus was serially diluted (twofold) into the second well until the eleventh well. The remaining 25 μL was discarded. Freshly prepared RBC (50 μL, 100 % concentration) were diluted to 0.8 % in PBS and aliquotted into all 12 wells. The minimum virus concentration that caused complete agglutination of RBC was taken as the virus titre.

2.4.6 Haemagglutinin inhibition assay

Using a U-bottomed 96-well plate, 25 µL PBS was aliquotted into the second to twelfth wells. Sample serum (50 µL) was added into the first well and half of it was serially diluted (twofold) until the eleventh well. The remaining 25 µL was discarded. H5N2 virus strain A/Malaysia/Duck/8443/04, titrated to 4 HA units, was added into the diluted sera and gently mixed. The plate was incubated at room temperature for one hour. Washed chicken RBC (25 µL of 0.8 %) was added to each of the wells and incubated for 30 minutes before viewing. HI titers were determined as the reciprocal of the highest serum dilution that completely inhibited haemagglutination.

2.4.7 Isolation of lymphocytes

Fresh, non-coagulated whole blood was diluted to 1 mL using cold PBS and was carefully layered on BD Biosciences Ficoll-Paque PLUS (2 mL) and centrifuged using a swing rotor, at 400 x g for 30-40 minutes at 4 °C. The lymphocyte layer was removed into a clean centrifuge tube and washed with 2 mL of PBS before re-centrifuging under the same conditions for 10 minutes. The supernatant was discarded. If RBC particulates were observed at the bottom of the tube, 2 mL of lysis buffer (7.56 g ammonium chloride and 2.42 g tris in total 1 L water) was added and the tube was kept in 4 ^{o}C for 30 minutes. Centrifugation was carried out as above and the step was repeated until no RBC were observed. PBS (2 mL 1X) was added to the pellet and the tube was re-centrifuged. The washing step was repeated twice. The lymphocytes pellet was resuspended in 0.2 to 0.5 mL PBS for cell counting and transferred into amber tubes. The lymphocytes were kept at 4 ^{o}C and used within one day.

2.4.8 Preparation of paraformaldehyde solution

Paraformaldehyde (2 % w/v) was prepared as a stock by adding 2 g of the powder to 100 mL 1X PBS and heating at 56 ^{o}C in a waterbath until fully dissolved. The solution was allowed to cool to room temperature. The pH was adjusted to 7.4 using 0.1 M sodium hydroxide or 0.1 M hydrochloride acid. The solution was stored at 4 ^{o}C and used no later than two weeks. A 0.5 % working solution of paraformaldehyde was prepared by adding 10 mL of 2 % stock into 30 mL 1X PBS. The solution was kept at 4 ^{o}C and was stable for one week.

2.4.9 Staining and fixing of lymphocytes

Mouse anti-chicken CD8a-PerCP-Cy5-conjugated (0.1 mg, working concentration was 1 µg/mL), mouse anti-chicken CD3-PE-conjugated (0.1 mg, working concentration was 0.5 µg/mL) and mouse anti-chicken CD4-FITC-conjugated (0.5 mg, working concentration was 0.5 µg/mL) monoclonal antibodies (all from Southern Biotech) were each added into an amber tube containing approximately 10^6 cells. The cells were resuspended and incubated at 4 ^{o}C in a 100 rpm shaker for 3 hours, with tapping every hour. The antibodies were then washed three times with 1 mL cold 1X PBS, centrifuging at 250 x g for 10 minutes at 4 °C. Supernatants were discarded each time. Cold paraformaldehyde (0.3 mL of 0.5 %) was added to the stained cell pellets, which were vortexed immediately. The stained and fixed cells were kept at 4 ^{o}C in the dark no longer than three days. The cells were transferred into Flow tubes for analysis using a BD FACSCalibur flow cytometer.

2.4.10 ELISA assays

ID Screen Influenza N1 Antibody Competition kits, purchased from ID VET, France, were used for specific detection of anti-influenza N1 antibodies in chicken sera. The kits, comprised of flat-bottom, 96-well microplates coated with N1 antigen (inactivated, whole H1N1 virus of 2009 pandemic, isolated from Brescia, Italy), 10X concentrated conjugate (anti-N1-peroxidase), positive and negative controls, dilution buffers, 20X wash concentrate, TMB as substrate solution and sulphuric acid as stop solution were stored at 4 ^{o}C. Reagents were allowed to cool to room temperature and homogenized by vortexing before use. Dilution buffer 8 (50 µL) was loaded into each well of the microplates. Positive control (50 µL) was added into wells A1 and B1, with negative control into wells C1 and D1. Samples (50 µL each) were added to the remaining wells and the plate was incubated for 1.5 h at 37 ^{o}C. Using an automatic ELISA plate washer, each well was emptied and washed three times using 300 µL of wash solution. Drying of the wells between washings was avoided. Conjugate (100 µL of 1X) prepared by diluting the 10X concentrated conjugate in dilution buffer 3, was added to each well and the plate was incubated for a further 30 minutes. Using an automated microplate washer (BioRad model 1575), each well was emptied and washed three times using 300 µL of wash solution. Substrate solution (100 µL) was added to each well before incubation for 10 minutes at 21 ^{o}C in the dark. Stop solution (100 µL) was added before the plate was read using a microplate reader (BioRad model 680) at 450 nm optical density, OD. The test was validated if the mean value of the negative control OD was greater than 0.700 and the mean value of the positive control was less than 30 % of the negative control OD.

Once the test was validated, competition percentage of antibodies from the samples was calculated using the equation below:

$$\frac{\text{OD of sample}}{\text{OD of negative control}} \times 100$$

N1 antibodies were considered to be present in the sample if the competition percentage of the sample was less than 60 %, and absent if the percentage was equal to or more than 60 %.

In principle, N1 antibodies if present in the serum samples formed an antibody-antigen complex with N1 antigen coated on the microplates. Subsequent addition of anti-N1-peroxidase conjugate bound to the remaining free epitopes of N1 antigen, forming an antigen-antibody-peroxidase complex. Addition of substrate solution, TMB, after elimination of the excess conjugate, later resulted in colouration based on the quantitity of specific antibodies present in the serum; no colour changes in the presence of antibodies, or blue colour which turned to yellow after addition of stop solution indicated the absence of antibodies.

2.4.11 Statistical analysis

Differences between groups of chicken were analyzed by One-Way ANOVA (if more than two groups) or Paired-samples T test (if two groups were compared) using SPSS Version 15 software. Results were expressed as the mean ± standard error of the mean (SEM). *P* values less than 0.05 were considered statistically significant in all cases.

CHAPTER 3

Construction of recombinant FWPV coexpressing influenza virus and cytokine genes

3.1 Introduction

All of the sub-cloning of AIV and chicken cytokine genes into FWPV recombination vectors as well as the generation, characterisation and initial bulk preparation of recombinant FWPV (rFWPV) was conducted during the first half of the project in the laboratory of Dr M. A. Skinner at Imperial College London. Construction and use of the rFWPV in London was conducted according to HSE guidelines, under the approval of the local Genetic Modification Safety Committee, at Category 1 (elsewhere known as biosafety level, BSL, 1).

The cloning strategy to construct recombinant FWPV, rFWPV, coexpressing AIV genes and chicken cytokine genes is summarized in Figure 3.1.

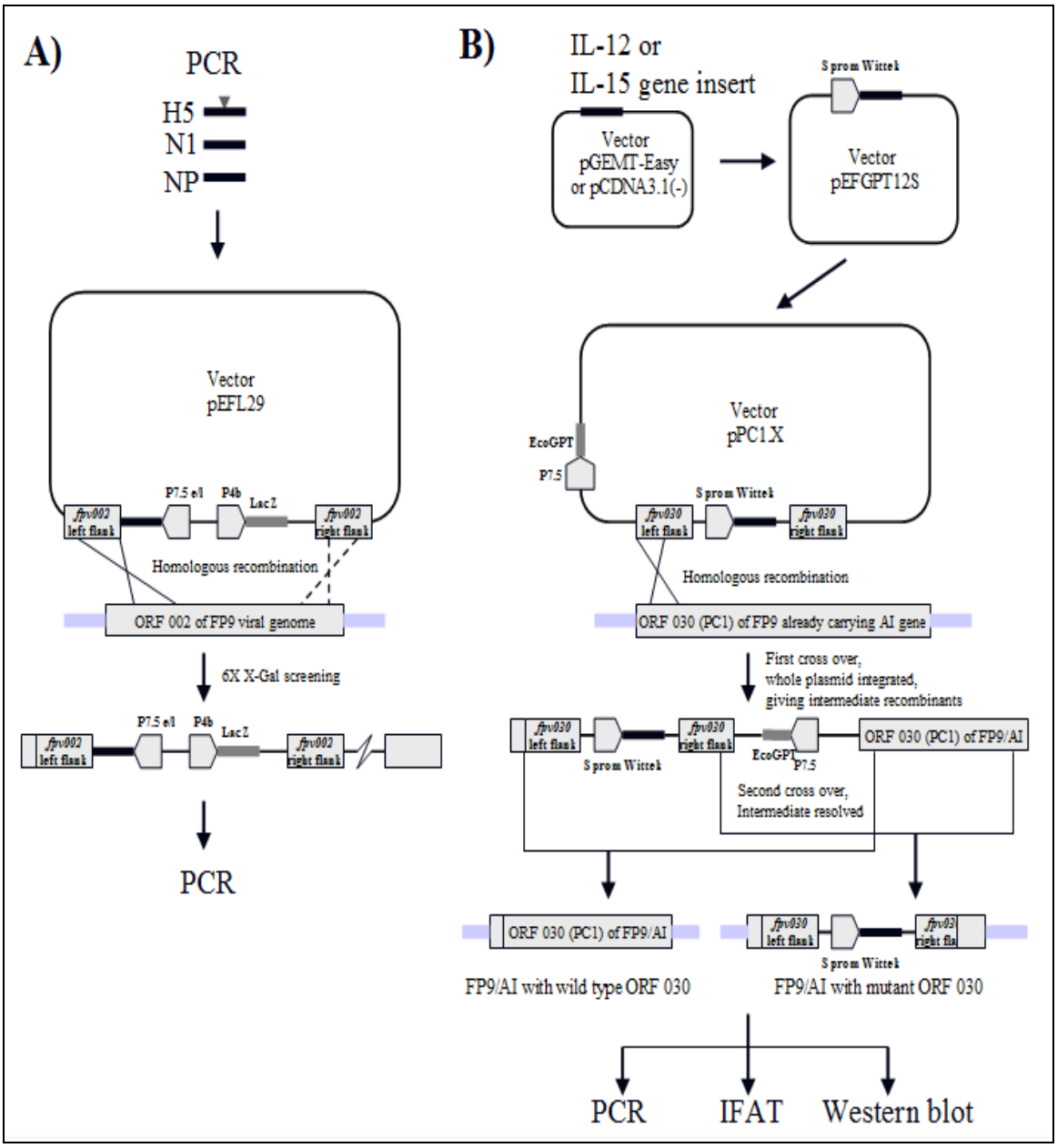

Figure 3.1. Overview of cloning strategy of AIV and cytokine genes. A) AIV genes (H5, with deleted polybasic sequence, N1 and NP) were amplified by PCR and inserted into recombination vector pEFL29 at a unique *SmaI* site. The first homologous recombination occurred at ORF 002 of FWPV and clones were isolated by 6 fold plaque purification with X-gal screening and PCR. B) Cytokine genes IL-15 and IL-12 were excised from pCDNA3.1(-) and pGEMT-Easy, respectively, into vector pEF*gpt*12S carrying the S promoter. Expression cassettes were sub-cloned into recombination vector pPC1.X. The second homologous recombination occurred into rFWPV already carrying AIV genes. The Eco*gpt* gene external to the recombination cassette in pPC1.X allowed trans-dominant selection of unstable intermediate viral clones subsequently allowed to resolve into stable AIV-rFWPV either having or not having cytokine genes. Final the presence of the inserted genes was verified by PCR, western blotting and IFAT.

3.2 Construction of recombinant FWPV expressing influenza virus genes

3.2.1 PCR amplification of AIV genes (including primer design and removal of polybasic sequence)

Three primer sets to be used to generate the full length H5, N1 and NP sequences were designed with either *EcoRV* or *SspI* restriction enzyme sites incorporated (Appendix 1). The restriction enzymes were chosen to create blunt-end PCR products.

The HPAIV haemagglutinin (HA) gene of the H5 virus contains multiple basic amino acids, arginine and lysine, that allow cleavage by ubiquitous proteases (furin and PC6) (Wood *et al*., 1994; Horimoto *et al*., 1994). To maintain compatibility with recombinant, killed H5N1 influenza vaccines (personal communication, Dr John Wood at NIBSC, Potters Bar, UK), and to reduce any potential biosafety issues, the polybasic region was removed. The sequence of the polybasic region is S-P-Q-R-E-R-**R-R-K-K**-R. Based on the sequence of the cleavage site of a low pathogenic Mexican lineage H5N2 AIV isolate (Garcia *et al*., 1996; Ito *et al*., 2001; Lee *et al.*, 2004), it was decided to delete the tetrabasic peptide motif and also to replace one of the remaining arginines (R) with threonine (T), resulting in S-P-Q-R-E-**T**-R. BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) were undertaken to compare the newly deduced amino acid sequence of the H5 with other influenza virus sequences. The most significant BLAST score is a modified haemagglutinin sequence of Influenza A virus (A/Vietnam/1203/2004) which shares 97% amino acid identity.

Deletion of the R-R-K-K motif and introduction of a point mutation at nucleotide position 1022, changing the arginine codon (AGA) to a threonine codon (ACA) were made using mutagenic primers S(2-F) and S(1-R). Use of primer pair H5-F and S(1-R) led to successful generation of the first H5 fragment (1036 bp), while use of primers H5-R and S(2-F) allowed generation of the second H5 fragment (684 bp). Full length mutated H5 gene, designated H5 S (1695 bp in length), was obtained through PCR overlap extension mutagenesis using 50 ng of each DNA fragment as templates (Figure 3.2). Later sequencing confirmed the presence of the mutated sequence: ATGLRNSPQRE**T**RGLFGAIAG.

PCR amplification of the N1 gene using N1-F and N1-R primers created a product of approximately 1353 bp, which corresponds to the expected N1 size of 1350 bp. Primers NP-F and NP-R were used to produce the NP gene. The product ran as a band between 1353 bp and 2027 bp, which matched the expected size (1497 bp) of the NP gene (Figure 3.2).

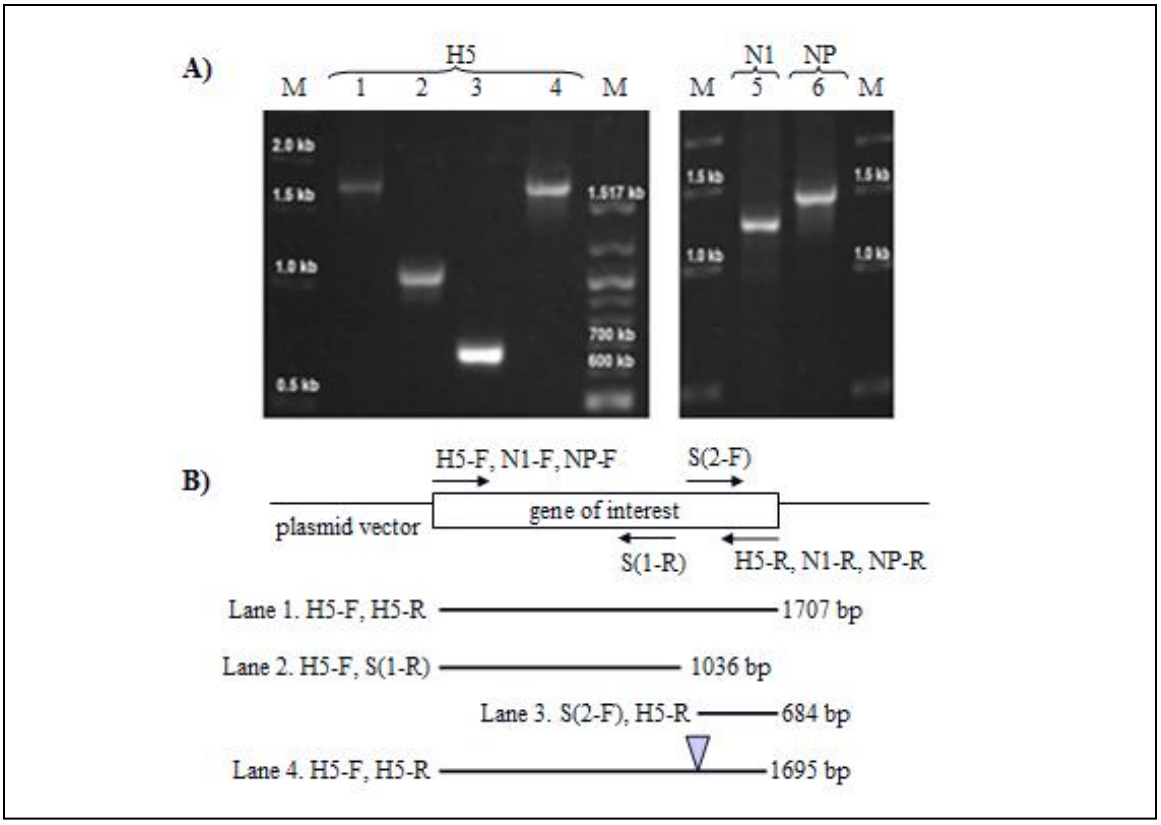

Figure 3.2. Amplification of AIV genes using PCR, including removal of multibasic sequence from H5 gene. A) Full length HA H5 gene, 1707 bp, is shown in Lane 1. Fragment 1, 1036 bp, and Fragment 2, 684 bp, of H5 were generated using mutagenic primers (Lanes 2 and 3, respectively). Re-assembled full length H5 sequence, lacking the polybasic region, 1695 bp, was obtained using PCR overlap extension mutagenesis (Lane 4). Lanes 5 and 6 show N1 (1350 bp) and NP (1497 bp) gene products, respectively. M is a ladder marker. B) Diagram showing the positions of primers used to amplify the genes and the predicted size of PCR products.

3.2.2 Introduction of influenza gene expression cassettes into the FWPV genome

In this thesis, the term 'positive transformants' is used to refer to bacterial clones carrying plasmid vectors with the desired DNA correctly inserted in the appropriate orientation. To select for positive transformants carrying pEFL29 with inserted AIV genes, several single colonies were picked from an LB plate after the transformations and grown overnight. Culture PCR using a flanking primer (annealing to the vector) and an internal primer (annealing to the inserted gene) was generally used to confirm the presence and orientation of the insert (Figure 3.3a, Figure 3.3b).

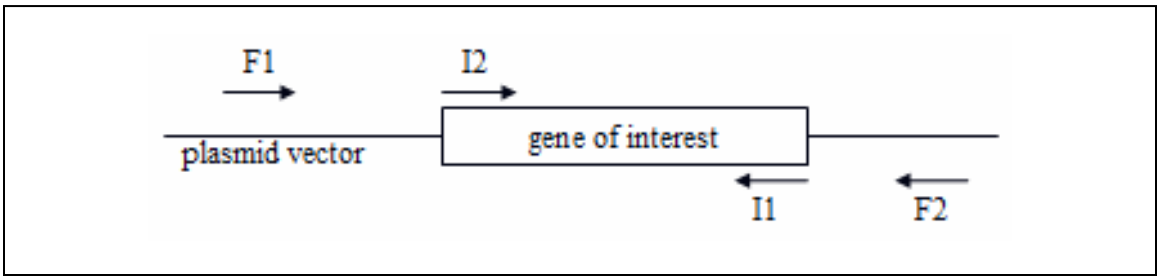

Figure 3.3a. Primer locations for PCR analysis to confirm the presence of the insert and its orientation. Two sets of primers were used, flanking primers F1 and F2, annealing to the parental vector, and internal primers I1 and I2, annealing to the 5' and 3' ends, respectively, of the inserted gene.

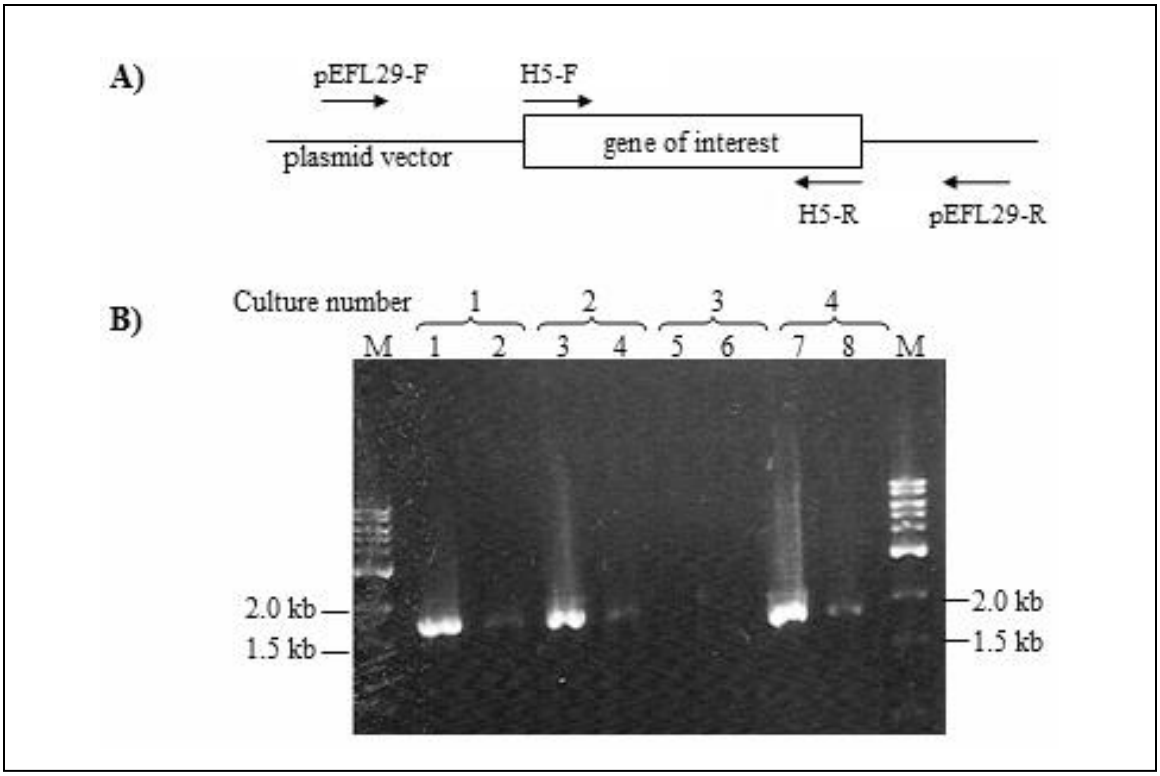

Figure 3.3b. Typical culture PCR analysis to confirm the presence of the insert and its orientation. A) Two sets of primers were used, flanking primers pEFL29-F and pEFL29-R, annealing to the parental vector, and internal primers H5-F and H5-R, annealing to the 5' and 3' ends, respectively, of the inserted gene. B) Example of culture PCR products. In this figure, pEFL29 and H5 primer sets were used to confirm the presence of H5 gene insert in pEFL29, in four cultures of single colonies. The size of the expected product is 1800 bp for both sets. Products of pEFL29-F and H5-R primers are shown in Lanes 1, 3, 5, and 7. Products of pEFL29-R and H5-F primers were indicated at Lane 2, 4, 6 and 8. Lane 1 and 2 represent culture number 1, Lane 3 and 4, culture number 2, Lane 5 and 6, culture number 3, Lane 7 and 8, culture number 4. M is a ladder marker.

Cultures that yielded positive PCR products (Figure 3.3) were subjected to plasmid DNA isolation using QIAGEN Minipreparation Kit. The extracted plasmid DNAs were digested using restriction enzymes *SphI* or *NcoI* for pEFL29 predicted to have H5/NP or N1 gene inserts, respectively (Figure 3.4). Plasmid DNA from positive transformants was then sent for sequencing (Figure 3.5a,b,c).

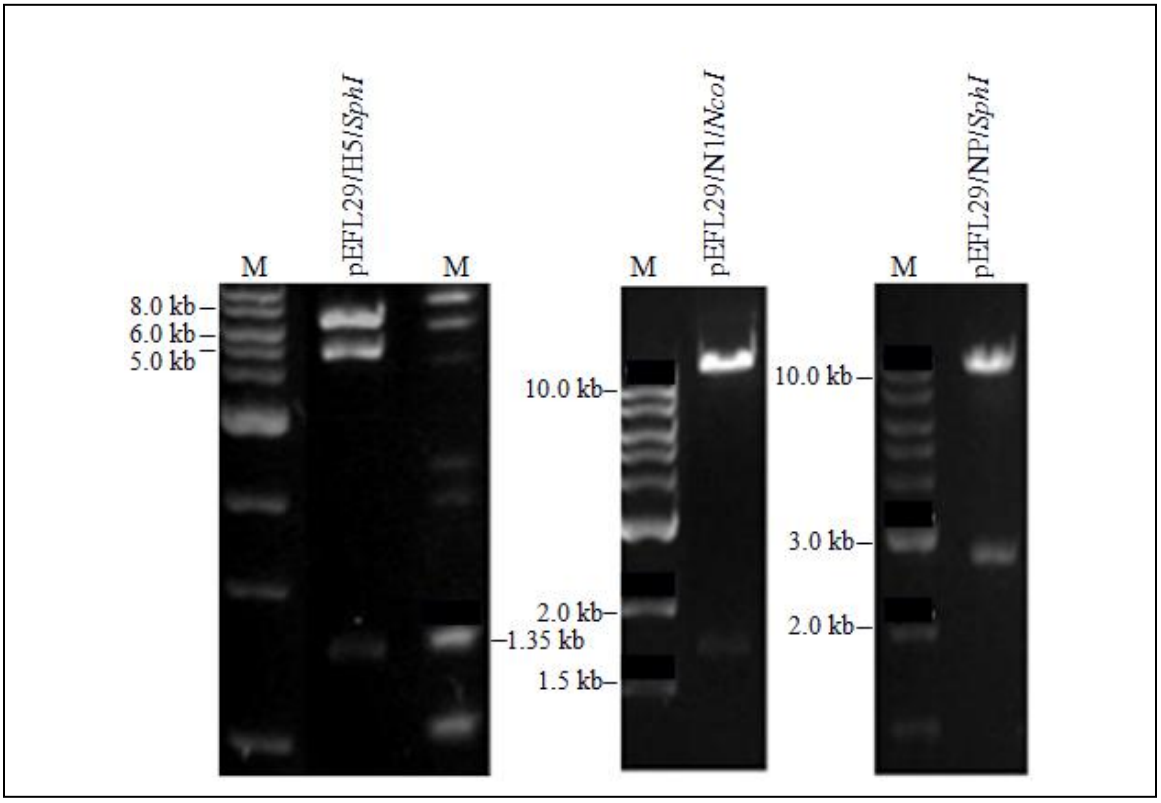

Figure 3.4. Agarose gel showing the size of plasmid pEFL29 carrying AIV genes after restriction enzyme digestion. pEFL/H5 was digested with *SphI* to give 1296 bp, 4962 bp and 7000 bp fragments. pEFL29/N1 was digested with *NcoI* to give 1743 bp and 11170 bp fragments. pEFL29/NP was digested with *SphI* to give 2740 bp and 10314 bp. M is a ladder marker.

GAATT**CCC***ATC*

ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGCATTG
GTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGT
GAAGCCTCTGATTTTGAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGA
CGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAATCCAGTCAATGACCT
CTGTTACCCAGGGGATTTCAATGCCTATGAAGAATTGAAACACCTATTGAGCAGAATAAACCA
TTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCAGTCATGAAGCCTCATTAGGGGT
GAGCTCAGCATGTCCATACCAGGGAAAGTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAA
AAAGAACAGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAACCAAGAAGATCTTT
TGGTACTGTGGGGGATTCACCATCCTAATGATGCGGCAGAGCAGACAAAGCTCTATCAAAAC
CCAACCACCTATATTTCCGTTGGGACATCAACACTAAACCAGAGATTGGTACCAAGAATAGCT
ACTAGATCCAAAGTAAACGGGCAAAGTGGGAGGATGGAGTTCTTCTGGACAATTTTAAAACC
GAATGATGCAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATACAAAAT
TGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTGGAATATGGTAACTGCAACACCA
AGTGTCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAATATACACCCTCTCG
CCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGA
AATAGCCCTCAAAGAGAG**ACA**AGAGGATTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGG
ATGGCAGGGAATGGTAGATGGTAGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGT
ACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCG
ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAAAG
GAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGTCTGGACTTATAATG
CTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAATGTCAAGA
ACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTT
TCGAGTTCTATCATAAACGTGATAATGAATGTATGGAAAGTGTAAGTAACGGAACGTATGACT
ACCCGCAGTATTCAGAAGAAGCAAGACTAAAAAGAGAGGAAATAAGTGGAGTAAAATTGGA
ATCAATAGGAATTTACCAAATACTGTCAATTTATTCTACAGTGGCGAGTTCCCTAGCACTGGC
AATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGGTCGTTACAATGCAGAATTTG
CATTTAA*

Figure 3.5a. Confirmed sequence of the H5 gene as inserted in vector pEFL29. Half of the unique *SmaI* cloning site is shown in bold. Half of an *EcoRV* site (italic), inserted to facilitate blunt-end ligation, is also shown. The sequence flanking the point mutation at nucleotide position 1022, which changes arginine (AGA) to threonine (ACA), is underlined and in bold. The sequencing primer positions are highlighted, while their orientations are indicated by arrows. P7.5 early/late promoter is not shown.

GAATT**CCC***ATT*

ATGAATCCAAATAAGAAGATAATAACCATCGGATCAATCTGTATGGTAACTGGAATGGTTAG
CTTAATGTTACAAATTGGGAACTTGATCTCAATATGGGTCAGTCATTCAATTCACACAGGGAA
TCAACACAAAGCTGAACCAATCAGCAATACTAATCTTCTTACTGAGAAAGCTGTGGCTTCAGT
AAAATTAGCGGGCAATTCATCTCTTTGCCCCATTAATGGATGGGCTGTATACAGTAAGGACAA
CAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAGAGCCATTCATCTCATGCTC
CCACTTGGAATGCAGAACTTTCTTTTTGACTCAGGGAGCCTTGCTGAATGACAAGCACTCCAA
TGGGACTGTCAAAGACAGAAGTCCTCACAGAACATTAATGAGTTGTCCTGTGGGTGAGGCTCC
CTCCCCATACAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCATGATGGCAA
CAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAATGGGGCTGTGGCTGTATTGAAATACAA
TGGCATAATAACAGACACTATCAAGAGTTGGAGGAATAACATACTGAGAACTCAAGAGTCTG
AATGTGCATGTGTAAATGGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGATCAGG
CATCACATAAGATCTTCAAAATGGAAAAAGGAAAAGTGGTTAAATCAGTCGAATTGGATGCT
CCCAATTATCACTATGAGGAATGCTCCTGTTATCCTGATGCCGGCGAAATCGCATGTGTGTGC
AGGGATAATTGGCATGGCTCAAATCGGCCATGGGTATCTTTCAATCAAAATTTGGAGTATCAA
ATAGGATATATATGCAGTGGAGTTTTCGGAGACAATCCACGCCCCAATGATGGAGCAGGTAG
TTGTGGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAA
TGGTGTCTGGATCGGGAGAACAAAAAGCACTAATCCCAGGAGCGGCTTTGAAATGATTTGGG
ATCCAAATGGGTGGACTGAAACGGACAGTAGCTTTTCAGTGAAACAAGATATCGTAGCAATA
ACTGATTGGTCAGGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGACTAGATTGC
ATAAGACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAGCACAATTTGGACT
AGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGGGTTGGTCTTGGCCAGAC
GGTGCTGAGTTGCCATTCACCATTGACAAGTAG*

Figure 3.5b. Sequencing of N1 gene in vector pEFL29. Unique *SmaI* site is shown in bold. *SspI* site (italic) was incorporated to facilitate blunt-end ligation. The sequencing primer positions are highlighted, while their orientations are indicated by arrows. P7.5 early/late promoter is not shown.

GAATT**CCC***ATC*

ATGGCGTCTCAAGGCACCAAACGATCTTATGAACAGATGGAAACTGGTGGGGAACGCCAGAA
TGCTACTGAGATCAGGGCATCTGTTGGAAGAATGGTTAGTGGCATTGGGAGGTTCTACATACA
GATGTGCACAGAACTCAAACTCAGTGACTATGAAGGGAGGCTGATCCAGAACAGCATAACAA
TAGAGAGAATGGTACTCTCTGCATTTGATGAAAGAAGGAACAGATACCTGGAAGAACACCCC
AGTGCGGGAAAGGACCCGAAGAAAACTGGAGGTCCAATTTATCGGAGGAGAGATGGGAAAT
GGGTGAGAGAGCTAATTCTGTACGACAAAGAGGAGACCAGGAGGATTTGGCGTCAAGCGAAC
AATGGAGAGGACGCAACTGCTGGTCTCACCCACCTGATGATATGGCATTCCAATCTAAATGAT
GCCACATATCAGAGAACGAGAGCTCTCGTGCGTACTGGAATGGACCCAAGGATGTGCTCTCTG
ATGCAAGGGTCAACTCTCCCGAGGAGATCTGGAGCTGCCGGTGCAGCAGTAAAGGGGGTAGG
GACAATGGTGATGGAGCTGATTCGGATGATAAAACGAGGAATCAACGACCGGAATTTCTGGA
GAGGCGAAAATGGAAGAAGAACAAGGATTGCATATGAGAGAATGTGCAACATCCTCAAAGG
GAAATTCCAAACAGCAGCACAAAGAGCAATGATGGATCAAGTGCGAGAGAGCAGAAATCCT
GGGAATGCTGAAATTGAAGATCTCATTTTTCTGGCACGGTCTGCACTCATCCTGAGAGGATCA
GTGGCCCATAAGTCCTGCTTGCCTGCTTGTGTGTACGGACTTGCGGTGGCCAGTGGATATGAC
TTTGAGAGAGAAGGGTACTCTCTGGTTGGAATAGATCCTTTCCGCCTGCTTCAAAACAGCCAG
GTCTTTAGTCTCATTAGACCAAATGAGAATCCAGCACATAAGAGTCAATTAGTGTGGATGGCA
TGCCACTCTGCAGCATTTGAGGACCTTAGAGTCTCAAGTTTCATCAGAGGGACAAGAGTGGTC
CCAAGAGGACAGCTATCCACCAGAGGGGTTCAAATTGCTTCAAATGAGAACATGGAGGCAAT
GGACTCCAACACTCTTGAACTGAGAAGCAGATATTGGGCTATAAGAACCAGAAGCGGAGGAA
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AGAAACCTTCCCTTCGAAAGAGCGACCATTATGGCAGCATTTACAGGAAATACTGAGGGCAG
AACGTCTGACATGAGGACTGAAATCATAAGAATGATAGAAAGTGCCAGACCAGAAGATGTGT
CATTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCAACGAACCCGATCGTGCCT
TCCTTTGACATGAATAATGAAGGATCTTATTTCTTCGGAGACAATGCAGAGGAATATGACAAT
TGA*

Figure 3.5c. Sequencing of NP gene in vector pEFL29. Unique *SmaI* site is shown in bold. *EcoRV* site (italic) was inserted to facilitate blunt-end ligation. The sequencing primer positions are highlighted, while their orientations are indicated by arrows. P7.5 early/late promoter is not shown.

Clones of recombinant viruses were produced by transfection of 80% confluent primary chicken embryo fibroblasts (CEFs), infected with FWPV, with pEFL29/inserts using lipofectin. The completely sequenced, highly attenuated FP9 strain of FWPV (Laidlaw and Skinner, 2004) was used as the recipient vector. The expression cassette was inserted into the viral genome by recombination at the fpv002 locus. Expression of the gene was driven by a copy of the Vaccinia virus p7.5 early/late promoter. A copy of the *LacZ* gene, transcribed from the fowlpox P4b late promoter in the opposite direction, served as a marker for selection of recombinant viruses. After isolations by six fold plaque purification on CEFs using X-Gal selection (blue plaque selection), recombinants were checked for presence of the inserted genes by PCR of extracted FWPV genomic DNA (Figure 3.6).

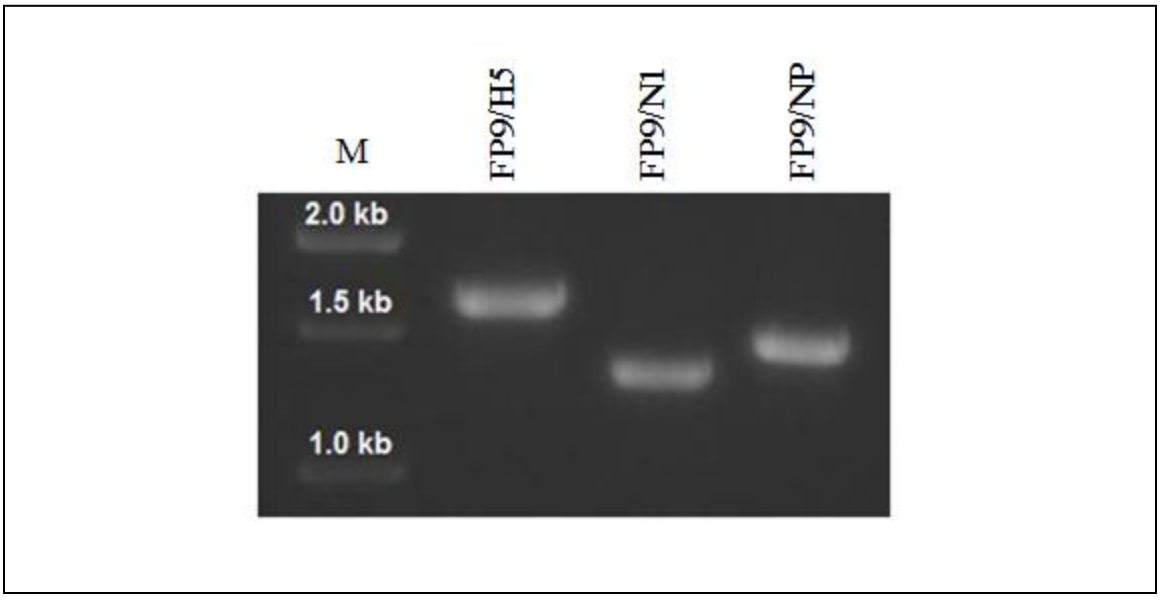


Figure 3.6. Agarose gel showing the presence of AIV genes incorporated into FWPV FP9 genome confirmed by PCR, using internal primers, after genomic DNA extraction. Recombinant FP9 carrying the H5 gene gave a 1695 bp PCR product, FP9 carrying the N1 gene gave a 1350 bp product, while FP9carrying the NP gene gave a 1497 bp product. M is a ladder marker.

3.3 Construction of recombinant FWPV co-expressing influenza and cytokine genes

3.3.1 Deletion of one *HindIII* site of pPC1.X vector

Vector pPC1.X has two *HindIII* sites located 1274 bp apart from one another. The *HindIII* site located external to the PC-1 (fpv030) open reading frame (ORF) was removed so as to obtain a unique *HindIII* site for cloning inserts within the PC-1 ORF. This was achieved by partial digestion of pPC1.X. Firstly, as a trial, 1 μg of pPC1.X was digested with 1 U of *HindIII*, sampling at sequential time intervals (from 5 to 85 minutes) prior to agarose gel electrophoresis. Analysis of the products of partial digestion (Figure 3.7) indicated that 75 minutes offered the optimal yield of singly-cut, linearised product so a further 2 μg of pPC1.X was digested for 75 min using 2 U of *HindIII* in a total volume of 50 μL.

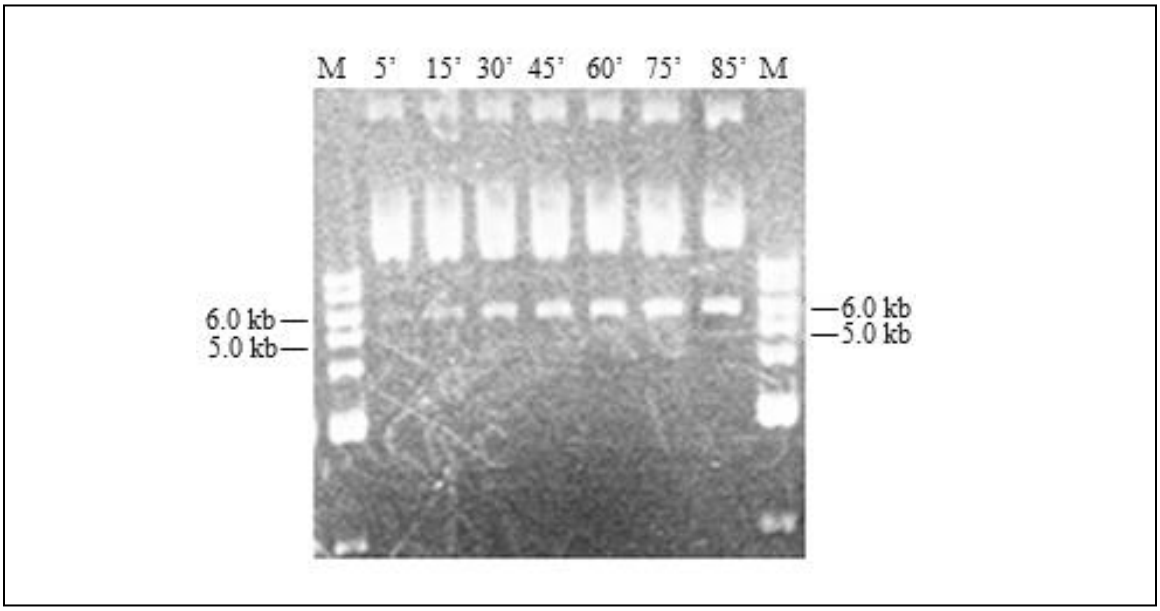


Figure 3.7. Pattern of vector pPC1.X after partial digestion with *HindIII* at time intervals. The band size of interest is 5.6 kbp. M is a ladder marker. Lanes correspond to pPC1.X pattern at specifies time intervals (in minutes).

After gel electrophoresis, a 5.6 kbp band, which corresponded to the required product, was extracted (Figure 3.8). The product was subjected to end repair using Klenow polymerase before blunt-end religation to eliminate one *HindIII* site. Twelve colonies were picked after transformation of the product; plasmid DNA was isolated and doubly digested with *HindIII* and *NsiI* to enable selection of clones with the correct *HindIII* site knocked-out (Figure 3.9). Correct knock-outs displayed 789 bp and 4802 bp products on gel electrophoresis, while incorrect knock-outs gave 502 bp and 5098 bp products. Based on the figure, recovery of clones digested at the desired site predominated (ten out of twelve gave positive knock-outs).

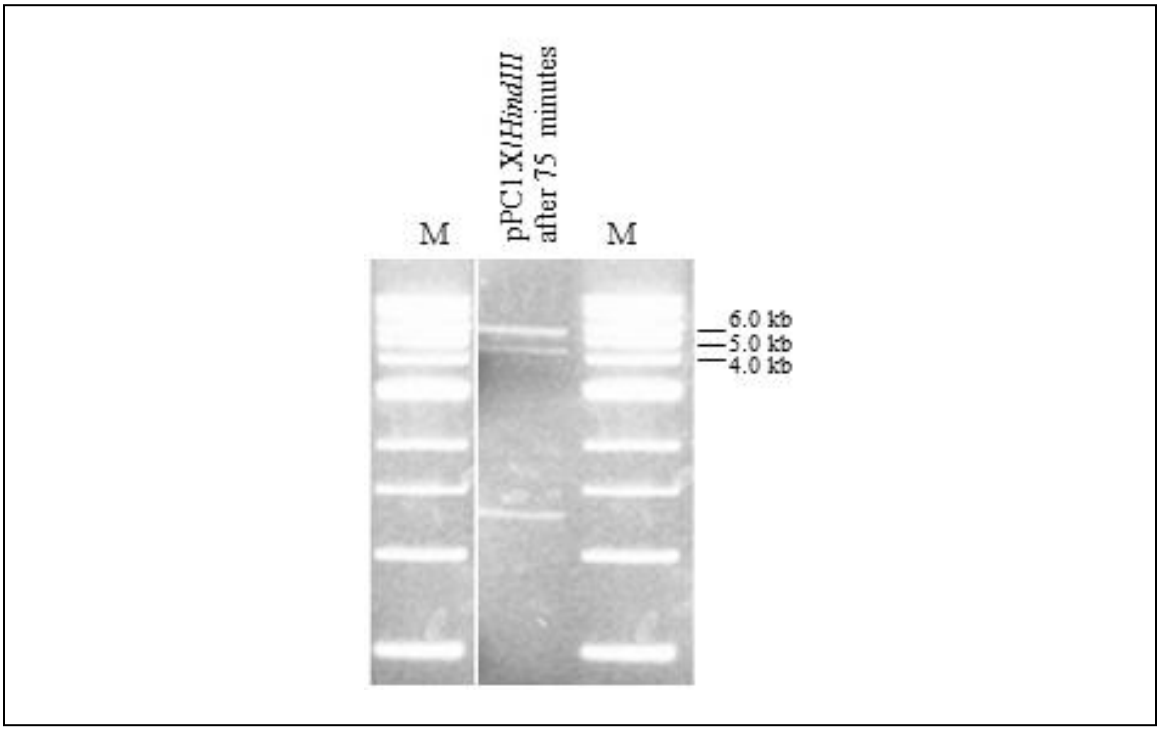


Figure 3.8. Pattern of vector pPC1.X after incomplete digestion with *HindIII* for 75 minutes. Band size of interest is 5.6 kb. M is a ladder marker.

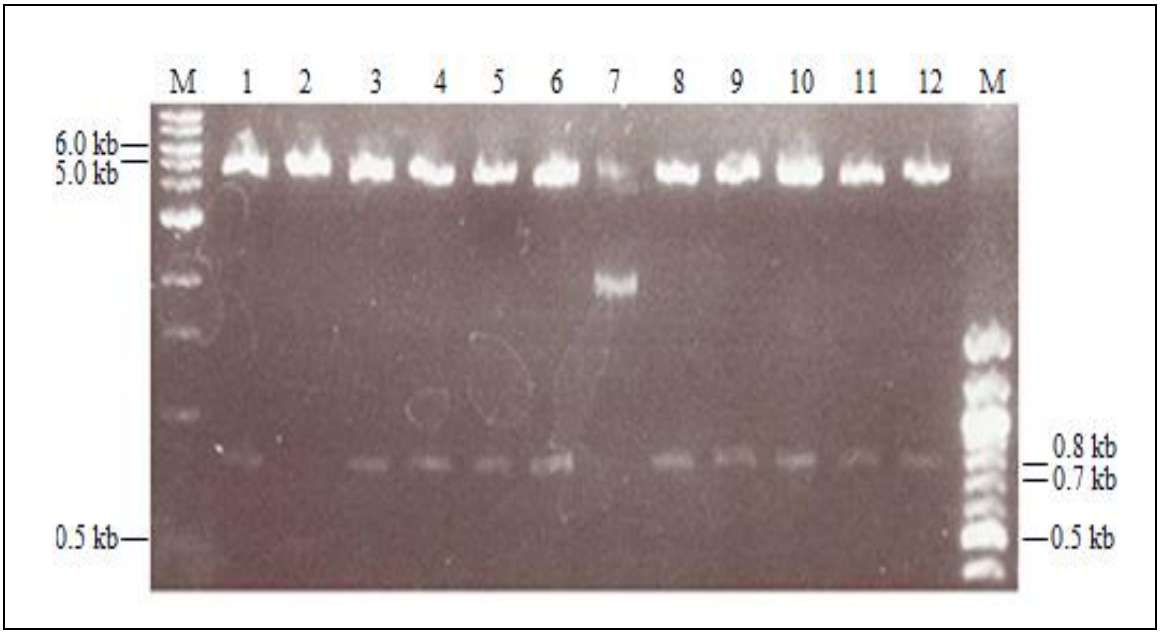


Figure 3.9. Screening of vector pPC1.X for the desired *HindIII* knockout by restriction enzyme digestion using *HindIII* and *NsiI*. Lanes 1 to 12 are from partially-digested and religated pPC1.X clones. The desired knockout gives fragments of 789 bp and 4802 bp in size, while knockout of the other site results in 502 and 5098 bp products. M corresponds to a ladder marker.

3.3.2 Transdominant selection for recombinant FWPV carrying cytokine genes

A procedure for eliminating the selectable marker, in this case the Eco*gpt* gene (*E. coli* guanine phosphoribosyltransferase), from the final recombinant virus was established by Falkner and Moss in 1990. It was termed 'transient dominant selection' (TDS). They showed that, under nonselective condition, ten out of ten vaccinia virus plaques became *gpt*- after only three cycles of plaque picking, and five of these were the required recombinants. The procedure has been used successfully to knockout many FP9 genes (Boulanger *et al.*, 1998; Laidlaw *et al.*, 1998; S. Laidlaw and M. A. Skinner, personal communication) and to introduce genes for expression (Jeshtadi *et al.*, 2005; M. A. Skinner, personal communication). In brief, recombinant viruses containing Eco*gpt* gene were selected in the presence of mycophenolic acid (MPA). In this study, the gene marker was incorporated into FP9 genome by homologous recombination with derivatives of vector pPC1.X contains inserted IL-15 or IL-12 expression cassettes to the FWPV PC1 gene corresponds to ORF 030 of the viral genome (Laidlaw *et al.*, 1998; Laidlaw and Skinner, 2004). MPA inhibits inosine monophosphate dehydrogenase, leading to arrest of the *de novo* purine metabolism pathway, and thus wild type FP9 replication. However, in recombinant FP9 containing Eco*gpt*, the expression of this gene (in the presence of exogenous xanthine and hypoxanthine) permits purine metabolism via the salvage pathway, allowing replication. Removal of the *gpt* marker is desirable, and it is achieved spontaneously when MPA selection is lifted, resulting in the production of stable viruses, half of which will carry the cytokine genes (S. Laidlaw and M. A. Skinner, personal communication). The TDS strategy used in this study is illustrated in Figure 3.10.

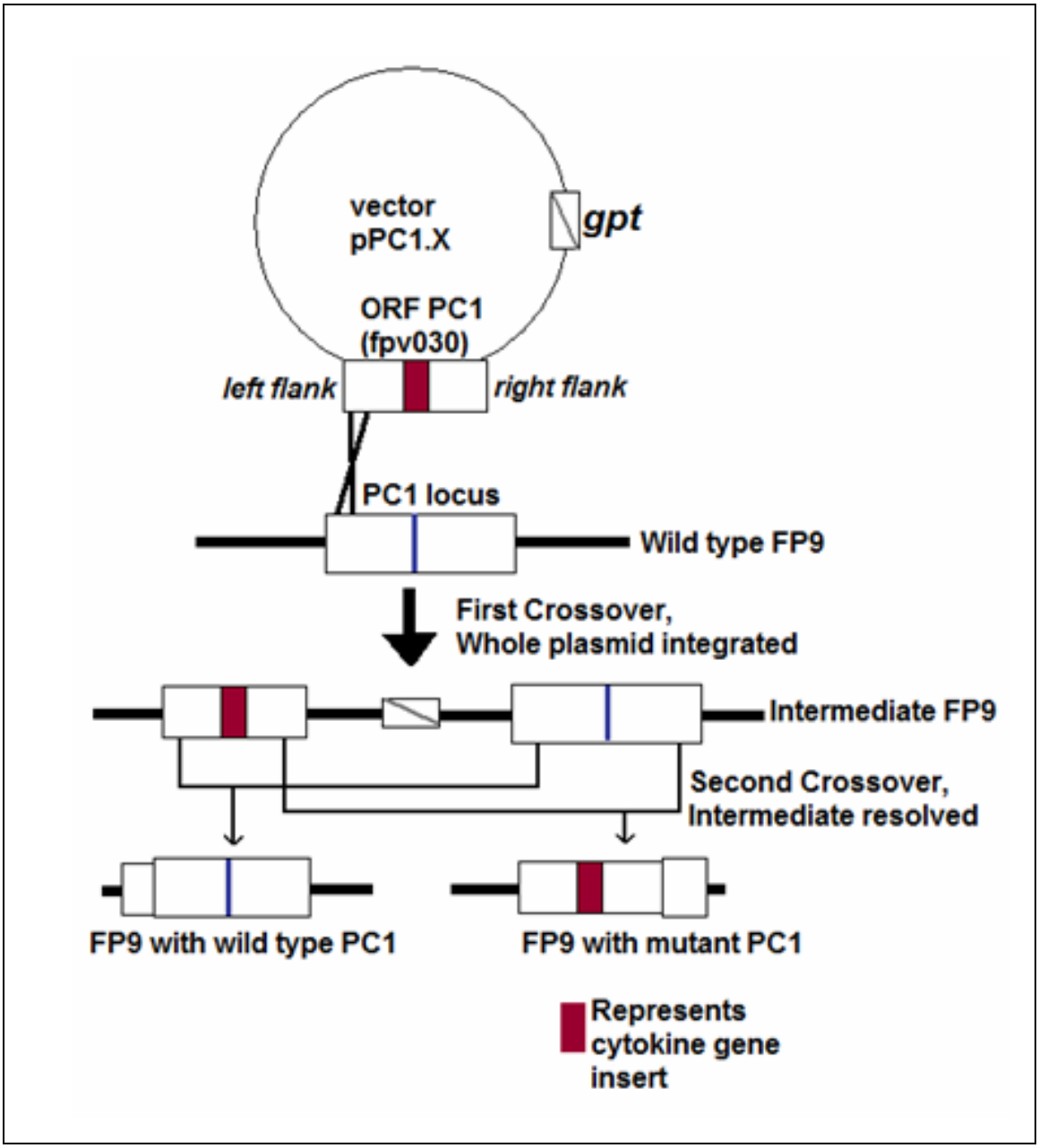


Figure 3.10. Transdominant selection strategy for recombinant FWPV. A first recombination occurs between the flanking sequence of the FWPV PC1 (fpv030) ORF encoded by vector pPC1.X and those in parental FP9. MPA was used to select the unstable intermediate viruses containing *gpt* gene. Removal of MPA selection led to resolution of the recombinants with loss of the *gpt* gene by a second crossover event. The final result was viruses containing either wild type PC1 or mutant PC1 with a cytokine gene insert.

3.3.3 Generation and selection of cytokine gene-positive transformants

The overall strategy of cytokine gene cloning is illustrated in Figure 3.11a, 3.11b and 3.11c.

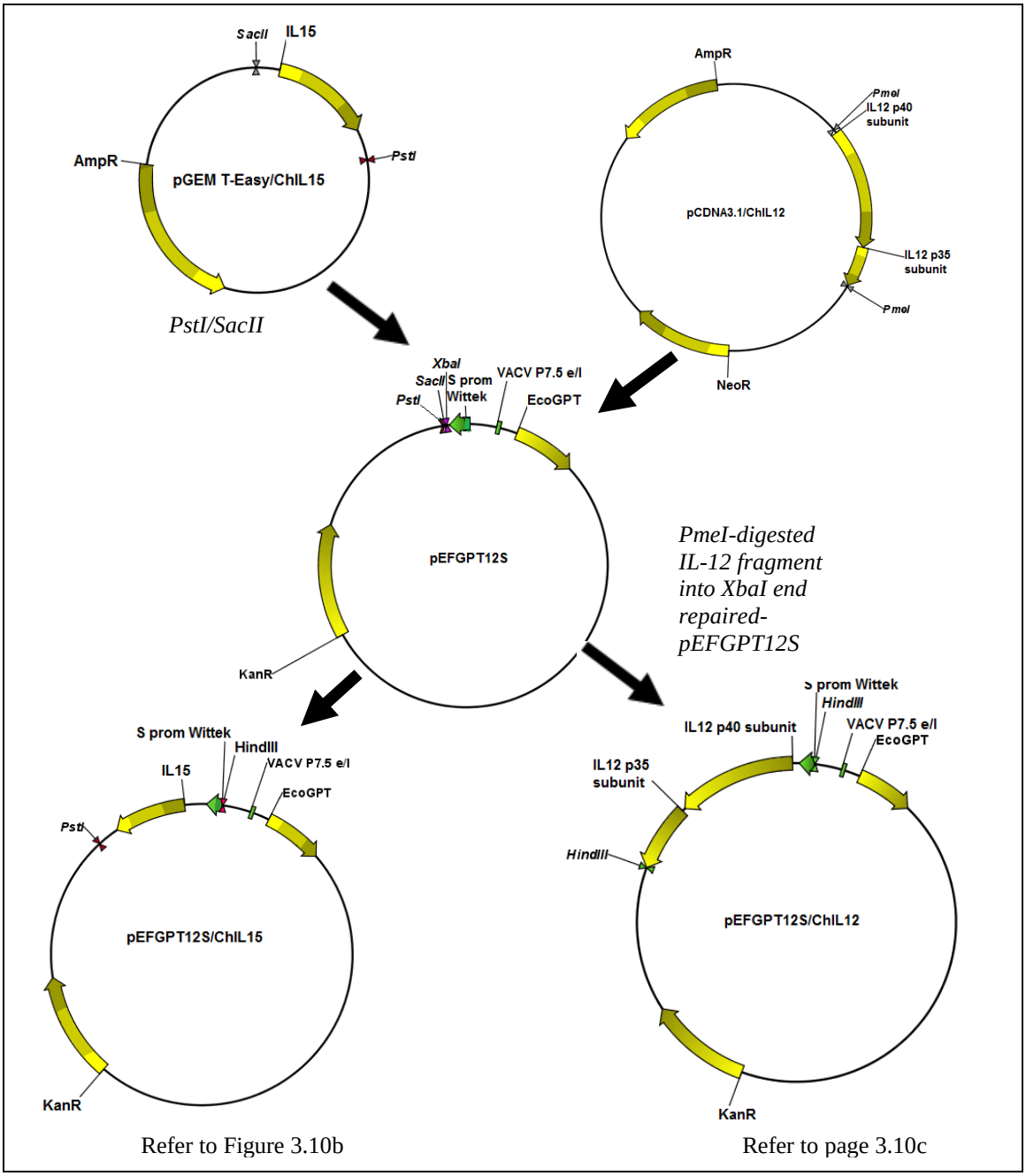
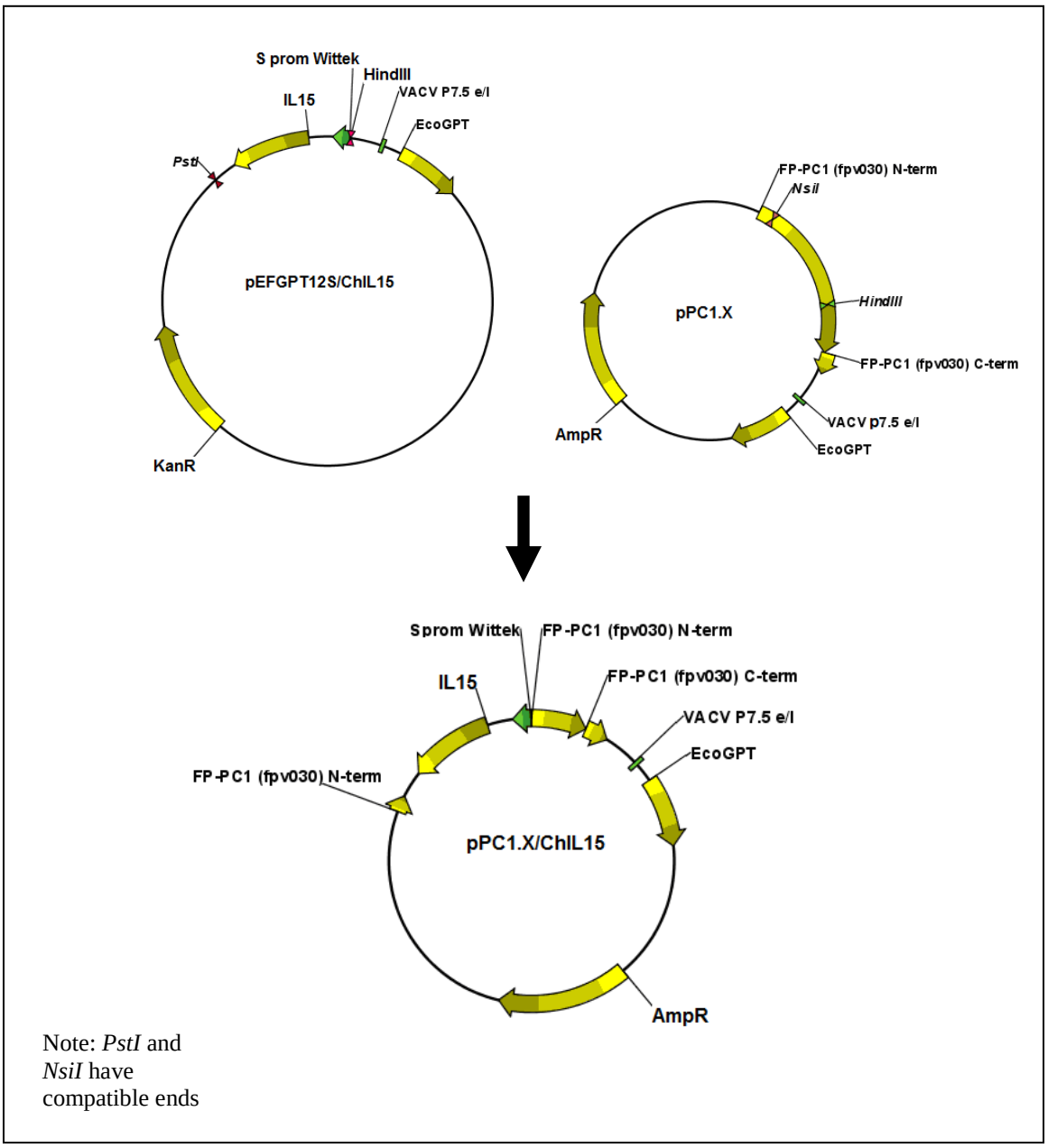
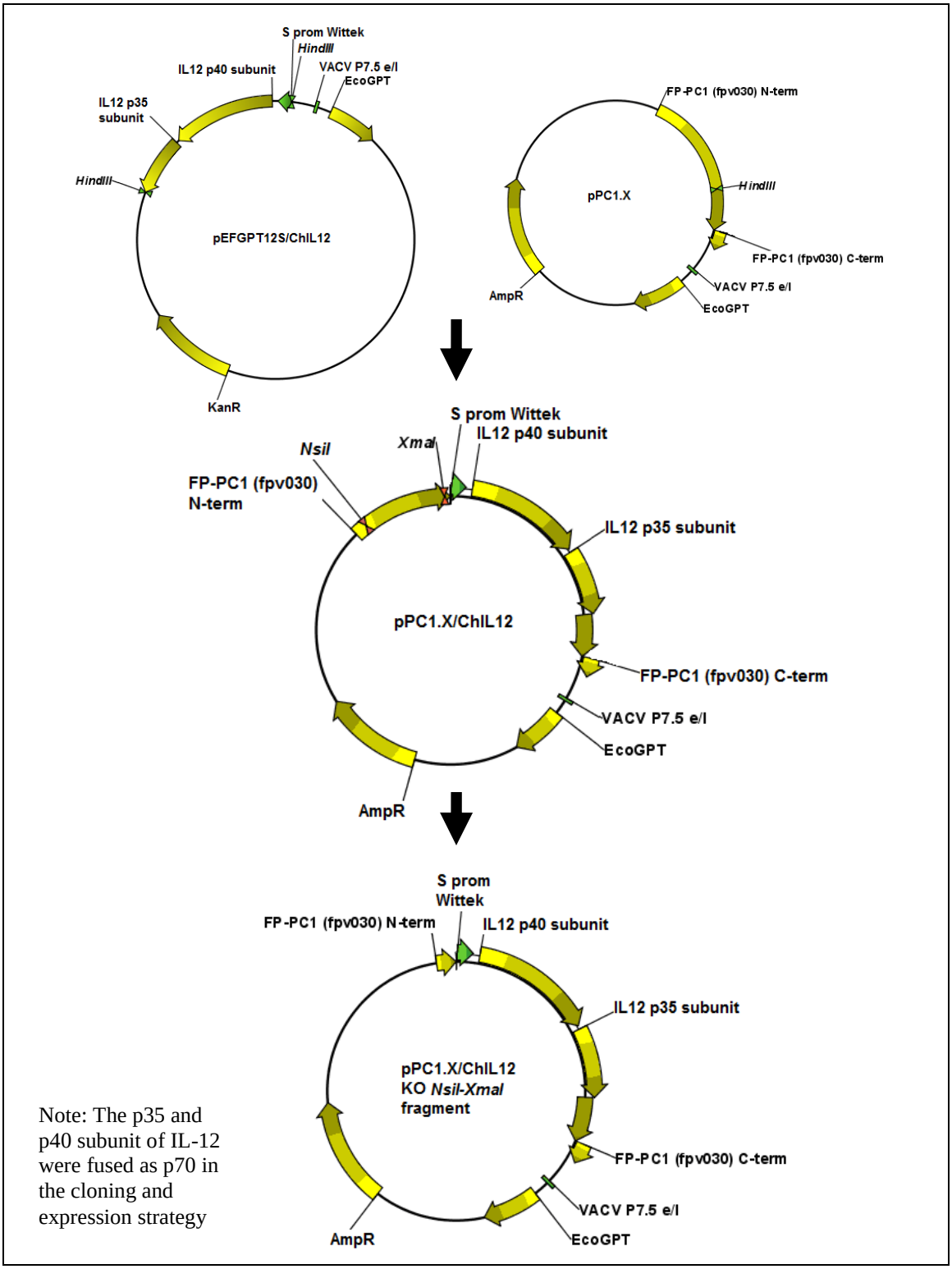


Figure 3.11a. Cloning of chIL-15 and chIL-12 into vector pEF*gpt*12S. Vector pGEMT-Easy/ChIL-15 possessed ampicillin resistant gene, AmpR, pCDNA3.1/ChIL-12 contained ampicillin and neomycin resistance genes, AmpR and NeoR, respectively, while pEF*gpt*12S contains kanamycin resistance gene, KanR. IL-15 and IL-12 inserts were cloned upstream of a synthetic promoter (S prom) Wittek. VAC P7.5 e/l served as a promoter for the expression of a selectable marker, Eco*gpt* gene.



Note: *PstI* and *NsiI* have compatible ends

Figure 3.11b. Cloning of chIL-15 with S promoter into vector pPC1.X. Vector pEF*gpt*12S/ChIL-15 possessed kanamycin resistant gene, KanR, while pPC1.X contained ampicillin resistant gene, AmpR. IL-15 and a synthetic promoter (S prom) Wittek were cloned into vector pPC1.X which contained recombination sequences of FWPV PC1 gene (fpv030). VAC P7.5 e/l served as a promoter for the expression of a selectable marker, Eco*gpt* gene.



Note: The p35 and p40 subunit of IL-12 were fused as p70 in the cloning and expression strategy

Figure 3.11c. Cloning of chIL-12 with S promoter into vector pPC1.X. Vector pEF*gpt*12S/ChIL-12 possessed kanamycin resistant gene, KanR, while pPC1.X contained ampicillin resistant gene, AmpR. IL-12 and a synthetic promoter (S prom) Wittek were cloned into vector pPC1.X which contained recombination sequences of FWPV PC1 gene (fpv030). VAC P7.5 e/l served as a promoter for the expression of a selectable marker, Eco*gpt* gene.

Chicken cytokine genes IL-15 and IL-12 were first individually inserted downstream of a synthetic/hybrid promoter (a kind gift of the late Dr Rico Wittek) in vector pEF*gpt*12S, by sticky-end or blunt-end cloning, respectively. The promoter–gene cassettes were subcloned into recombination vector pPC1.X (see Chapter 2). A 737 bp *NsiI* to *XmaI* fragment from pPC1.X/IL-12 was excised to achieve balanced lengths for the left and right flanks of the PC-1 homology region (data not shown). This was to ensure equivalent efficiencies of recombination between both flanks of the insert and the FWPV genome, so that progeny of the trans-dominant selection method had equal chances of bearing the cytokine expression cassette or not, with no bias for the parental phenotype. All positive transformants were confirmed by gel electrophoresis after restriction digests (Figure 3.12) before being sent for sequencing (Figure 3.13a and 3.13b).

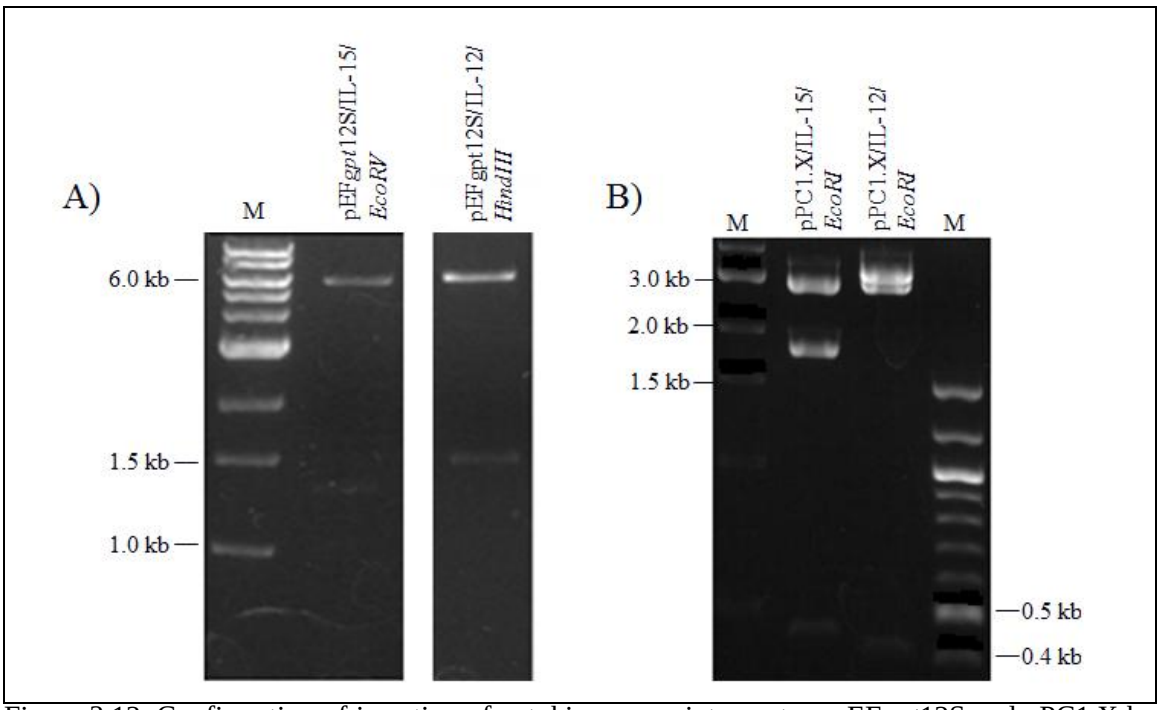


Figure 3.12. Confirmation of insertion of cytokine genes into vectors pEF*gpt*12S and pPC1.X by restriction enzyme digestion. A) pEF*gpt*12S carrying IL-15 was digested with *EcoRV*, giving 1407 bp and 5794 bp fragments. pEF*gpt*12S carrying IL-12 was digested with *HindIII*, giving 1598 bp and 6312 bp fragments. B) pPC1.X carrying IL-15 and IL-12 were digested with *EcoRI*. IL-15-positive recombinant clones of pPC1.X were identified by products of 460 bp, 1810 bp and 2825 bp; IL-12-positive clones by 418 bp, 2925 bp and 3195 bp fragments. A 737 bp fragment from pPC1.X/IL-12 had been removed previously. M is a ladder marker.

AGCTTCCCCGATAAAAATATAGTAGAATTTCATTTTGTTTTTTTCTATGCTATAAATAGGATCC
GATAAAGTGAAAAATAATTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAATCGA
ATTGCGGCCGCTCCAGAATTCTAGAGCGGCCGCCACCGCGGGAATTCGATT
ATGCTGGGGATGGCACAGCCAACACAAAACTCTGCCGGAGCACGGAGAAGGCCGGAGAGTC
AGAAAACACATGTGAAAAGTATTTGTCTCCAGTACCAACTGTATCTACTTTTGAACAGCCATT
TCTTTTGCCTTTTAAAGAATAAGACTGGACTAACCATCTTCTTCCTATGTGCTTATGTACCAAA
GACAGAAGCAAATCACTGTAAGTGGTCAGACGTTCTGAAAGATTTGGAGCTGATCAAGACAT
CTGAAGACATTGATGTCAGTTTATATACTGCAAACACATACGAGGATATAGAATGCCAGGAA
CCTGTAATGAGATGTTTTTTTTTAGAGATGAAAGTGATTCTTCACGAATGTGATATCAAAAAA
TGTAGTAGGAAGCATGATGTACGGAACATATGGAAAAATGGAAATGCAAGATTTGCAACTTA
CCAGTTGAATTCCACAACAGCAAAAAAATGCAAAGAATGTGAAGAGTATGAAGAAAAAAATT
TTACAGAATTTATACAGAGTTTTGTAAAGGTTATACAGAGGGAATGCAAAAAATACGCTAACT
AA*AATCACTAGTGAATTCGCGGCCGCCTGCATTCTTTAGATTCTTCTTTTACAGGATACAATG
TATAATTATCACTGTGTATAGACATATCTTCATCGGACTGATATCCATGATCCATAGTGGTACT
ATTTTTCATAGACATTCGTATAAACTAATTACTTAATCCATCATTTTTATTATATATTATTGTTT
GAAAGAAAAAAATACGCGATAAAATAACAATTATTATACTTGATACGAGTTTGAATTCTTATT
TTTCAACAATAT***CACGT***

Figure 3.13a. Sequencing of the IL-15 gene inserted into a derivative of vector pPC1.X. *HindIII* (AGCTT) and *NsiI* (CACGT) partial restriction sites used for cloning are shown in bold and italic. The sequencing primer positions are highlighted, while their orientations are indicated by arrows. Forward and reverse primers complementary to pPC1.X are not shown. S promoter sequences are highlighted in a darker shade. The AUG start codon is indicated by double underlining; the UAA stop codon by an asterisk (*).

AGCTTCCCCGATAAAAATATAGTAGAATTTCATTTGTTTTTTTCTATGCTATAAATAGGATCCG
ATAAAGTGAAAAATAATTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAATCGAA
TTGCGGCCGCTCCAGAATTCTAGAAACGGGCCCTCTAGACTCGAGCGGCCGCC
ATGTCTCACCTGCTATTTGCCTTACTTTCATTACTTTCCTTTGCTGCCCTTCTGGAAGCACAGTG
GAAACTTAGAGAGAATGTGTATGTCATAGAATCTGAGTGGAACGATGAGACACCAGCTAAAA
AAGTGAAGCTCACCTGTGACACATCTGATGAAGCACTGCCAGTTTACTGGAAAAAGGGAACA
GAACTGAAAGGAACTGGAAAGACTCTGACCACCGAAGTGAAGGAGTTCCCAGATGCTGGCAA
CTACACCTGCCTGTCTGCTAAGACCCACGAGATTATCAGCTACAGTTTCTTTCTCATAACTAAA
GTAGACTCCAATGGGCAAATGATACGGTCAATTCTGAAAAGCTATAAAGAGCCAAGCAAGAC
GTTCTTAAAATGTGAGGCAAAGAACTACTCTGGAATTTTCACATGTTCATGGATGACAGAAAA
TGAGAGTCCAAGTGTGAAGTTCACAATTAGGAGCCTAAAAGGCTCTCAAGGAGATGTAACCT
GCAGCAGCCCTGTGGCTCGCACTGATAAATCTGTGACTGAATACACTGCCCAGTGCCAGAAGG
AAAACTACTGTCCATTTGCCGAAGAGCACCAGCCGACTGAGATGTTCCTGGAGGTCATTGATG
AGGTGGAATATGAGAACTACACTAGTAGCTTCTTCATCAGAGATATCATAAAGCCAGACCCAC
CTCAATGTCAGTATGCAAGCACAAATGGAACTGTGACCTGGACATATCCCAAGACCTGGAGC
ACACCGAAGTCCTACTTCCCTTTGACTTTCAGGGTCAAAGTTGAAAGCACAAAGAAATACAAA
AGCAAGGTTTATGATGCTGATGAGCAGTCTATTCAGATTCCAAAGACTGGGCCAAAAGACAA
GATCTCTGTGCAGGCCAGGGATCGCTATTACAACTCATCCTGGAGTGAGTGGTCCACGCTTTG
CAGA *GGTGGCGGTGGCTCGGGCGGTGGTG*
GATCCGGTGGCGGCGGATCTCTGCCACTTCCTGCCCACAACCTGGCCAAGGGACTCAACTGCT
CCAGGGCGCTGCTGGCCGCTGCAAACGAGGCACTCCTGAAGGTGCAGAAGCAGAGGACGCTG
GGGTTTGAGTGCACCCTTGAAGAGGTCGATCTTGAAGACGTCACCAACAGTCAGAGCAACAC
AATAAAGTCCTGCACGTCTCACGATCCGGGGCCTGGAAACTGCCCCGTACTGGAAAGTTCTAC
TTTAGATATGAGCAAATGCCTGCAGGGGATCTACGAAGACCTGAAAACCTACAAGGCAGAGC
TGGGGAACCTCAAGGATCTGAGGGTGCTGACATCCATTGATGACATGATGCAAGCCCTGCAG
CCCCGCAGCCCAGCCATGCCGCAGCCCTCGCCCAGCACCACCCTTGGCTCCTTCCAGGGCCGC
ATGCGGCTCTGCGGGGTCCTGCACGCCTTCTGCCTGCGCGCAGTCACCATCGGCAGGATGCTG
GGCTACCTGAGTGCCCTCACTGCAGAGATGTAA*A***AGCT***

Figure 3.13b. Sequencing of the fusion IL-12 (p70) gene inserted into a derivative of vector pPC1.X. IL-12β (p40) subunit is separated from IL-12α (p35) by 25 nucleotides, displayed in italic. *HindIII* (AGCTT) and *NsiI* (CACGT) partial restriction sites used for cloning are shown in bold and italic. The sequencing primer positions are highlighted, while their orientations are indicated by arrows. Forward and reverse primers complementary to pPC1.X are not shown. S promoter sequences are highlighted in a darker shade. The AUG start codon is indicated by double underlining; the UAA stop codon by an asterisk (*).

3.3.4 Introduction of cytokine expression cassettes into rFWPV

The cytokine expression cassettes, driven by the S promoter obtained from the pEF*gpt*12S vector, were inserted into rFWPV already carrying avian influenza genes by recombination at a second non-essential site, the PC1 locus (fpv030; Laidlaw *et al.*, 1998; Skinner, unpublished). Following three rounds of passage in CEFs, in the presence of mycophenolic acid (MPA), recombinant viruses carrying the *gpt* gene (driven by a copy of the Vaccinia virus p7.5 early/late promoter) were isolated. Dilutions of viruses from the third passage were plaqued onto 60 mm dishes in the absence of MPA. Ten plaques were picked into serum free DMEM (500 μL). Plaque-purification, using ten plaques each time, was repeated several times until the originally *gpt*+ recombinant clones had lost the *gpt* gene spontaneously. This was verified by failure of the virus (at high concentration) to replicate in the presence of MPA.

When intermediate *gpt*+ recombinant viruses lose the *gpt* gene, they resolve either to the desired recombinant virus or revert back to parental virus (losing the cytokine inserts) so, PCR analysis of their viral DNA genomes was carried out after every stage of plaque-purification, to assay for retention of the cytokine gene. A flanking primer set was applied to screen IL-12-recombinant clones. Recombinant FP9/AIV genes showed a 984 bp PCR product, while introduction of IL-12 into the genome generated a 1934 bp product (Figure 3.14).

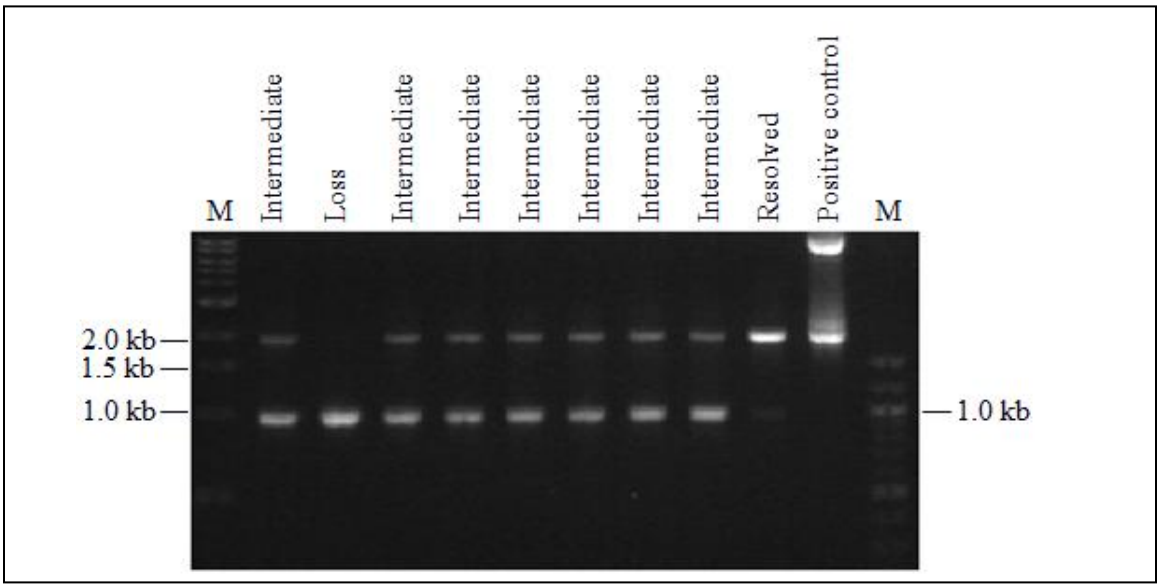


Figure 3.14. Screening extracted FP9 virus genome for the incorporated IL-12 gene using PCR. Primers used were pPC1.X-F and pPC1.X-R. Intermediate viruses demonstrate two fragments, 984 bp (without IL-12) and 1934 bp (IL-12 integrated). Recombinant FP9 viruses which lost the IL-12 insert was indicated by a single 984 bp fragment. Resolved recombinant FP9 to mutant carrying IL-12 was illustrated by a single 1934 bp fragment, alongside the IL-12 positive control product. M is a ladder marker.

In contrast to the IL-12 screening, the use of flanking primer sets was inappropriate for IL-15-clone screening, as the PCR product size for IL-15-positive clones was not sufficiently different to that for clones without inserts (Figure 3.15). Therefore, the screening of IL-15-carrying clones used primers external (pPC1.X-F) and internal (IL-15-R) to the inserted genes (the latter resulting in PCR products of 700 bp, exclusively for recombinant clones).

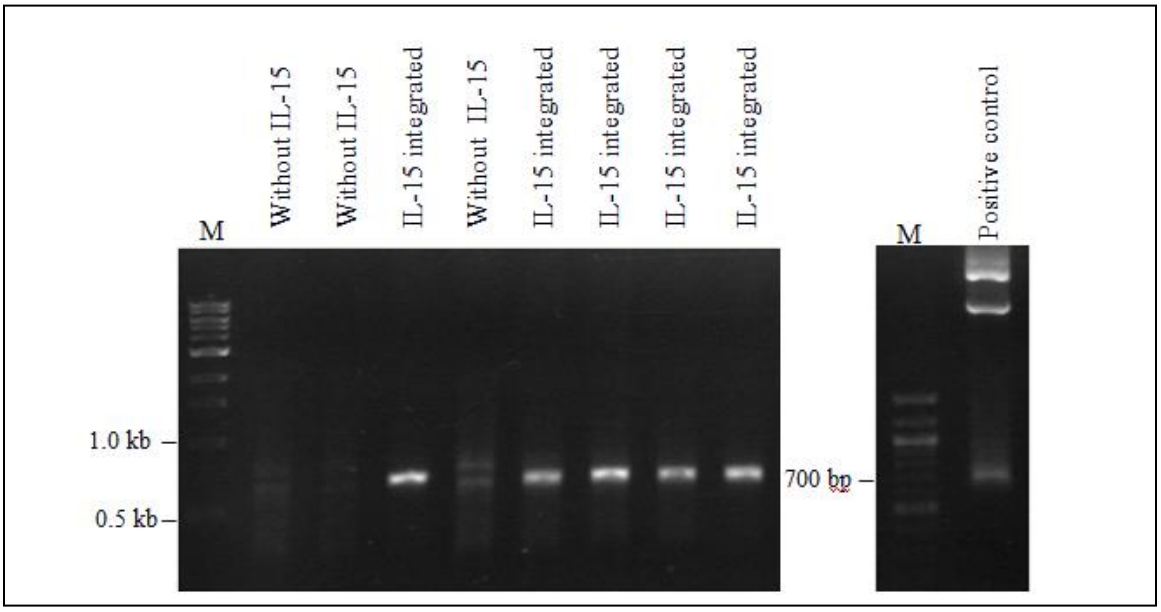


Figure 3.15. Screening of extracted FP9 virus genome for the incorporated IL-15 gene using PCR. Primers used were pPC1.X-F and IL-15-R. No PCR product was observed for recombinant FP9 clones lacking the insert. Recombinant FP9 clones with IL-15 gene integrated showed a 700 bp PCR fragment, correspond to the IL-15 positive control product, obtained using plasmid template. M is a ladder marker.

Despite the rapid loss of *gpt* as described by Falkner and Moss (1990) (Section 3.3.2), only two of the putative recombinant viruses, FP9/H5/IL-15 and FP9/N1/IL-12, successfully resolved after five and three plaque purifications, respectively. The other putative recombinant viruses (FP9/H5/IL-12, FP9/N1/IL-15, FP9/NP/IL-15 and FP9/NP/IL-12) were still in their intermediate forms even after the sixth plaque purification. The reasons for this were not further explored. It is possible that the original insertion was aberrant, occurring via non-homologous recombination at a non-specific site. Consequently, the wild-type insertion site would always remain; the intermediate recombinant can never be resolved by the normal second round of homologous recombination shown in Figure 3.10. The disrupted insertion site (bearing the cytokine expression cassette), carried on the aberrantly inserted recombination plasmid, could either be lost at the same time as *gpt* was lost, or (as appears to be the case) might remain, especially if the aberrant insertion was at a non-essential site. Screening the initial intermediate recombinants to demonstrate *bona fide* insertion at the correct site before progressing to isolate resolvants might have precluded this problem.

Time constraints precluded restarting the selection process so, as there were indications that the unresolved clones were stable, they were allowed to grow in the presence or absence of MPA (+MPA or –MPA) so that their stability could be assessed. Stocks of the recombinant viruses were prepared and titrated. The final virus titres are shown in Table 3.1.

Table 3.1. Titration of constructed recombinant viruses

Virus	Final concentration (PFU/mL)
WT FP9	1.50 x 10E6
FP9/H5	1.48 x 10E6
FP9/N1	1.45 x 10E7
FP9/NP	3.45 x 10E6
FP9/H5/IL-15	1.58 x 10E6
FP9/H5/IL-12*gpt* +MPA	8.80 x 10E5
FP9/H5/IL-12*gpt* –MPA	4.60 x 10E5
FP9/N1/IL-15*gpt* +MPA	3.03 x 10E6
FP9/N1/IL-15*gpt* –MPA	3.50 x 10E5
FP9/N1/IL-12	3.46 x 10E6
FP9/NP/IL-15*gpt* +MPA	2.07 x 10E6
FP9/NP/IL-15*gpt* –MPA	6.20 x 10E5
FP9/NP/IL-12*gpt* +MPA	6.20 x 10E6
FP9/NP/IL-12*gpt* –MPA	9.70 x 10E5

3.3.5 Expression of avian influenza (AIV) and cytokine gene inserts by rFWPV

In order to analyze expression of the AIV and cytokine genes, SDS-PAGE and western blotting were carried out for lysates of CEFs infected with the recombinant viruses. Additional indirect immunofluorescence test, IFAT, was performed to ascertain AIV H5 and N1 expressions. One polyclonal antiserum for H5, one polyclonal antiserum for N1 and three antibodies for NP were used as probes for the proteins in western blot, while one polyclonal antiserum each was used for H5 and N1 in IFAT. The list of antibodies is in Table 3.2.

Table 3.2. Polyclonal or monoclonal antibodies used in this study

Name	Analysis	Description	Dilution	Antigen	Source
ab62587	Western blot	Goat polyclonal	1:1000	Avian Influenza A haemagglutinin 5 (synthetic peptide corresponding to 12 amino acids near the amino terminus)	Abcam
ab70077	Western blot, IFAT	Rabbit polyclonal	1:1000	Avian Influenza A haemagglutinin 5 (synthetic peptide corresponding to 14 amino acids near the centre of H5N1 strain, 100% homology to A/China/GD01/2006)	Abcam
ab36566	Western blot	Rabbit polyclonal	1:1000	Avian Influenza A neuraminidase 1 (synthetic peptide, corresponding to 15 amino acids at the C terminal)	Abcam
ab70759	Western blot, IFAT	Rabbit polyclonal	1:1000	Avian Influenza A neuraminidase of A/H5N1/Vietnam/1203/2004	Abcam
ab25921	Western blot	Rabbit polyclonal	1:500	Avian Influenza nucleoprotein (synthetic peptide corresponding to amino acids 58-77)	Abcam
2F6C9	Western blot	Mouse monoclonal	1:2000	Avian Influenza nucleoprotein	Barclay, Imperial College
5D8	Western blot	Mouse monoclonal	1:500	Avian Influenza nucleoprotein	Santa Cruz
HC8	Western blot	Mouse monoclonal	1:1000	Avian interleukin 12	Kaiser, IAH
β-galactosidase	Western blot	Mouse monoclonal	1:5000	*E. coli* β-galactosidase near the C terminal	Promega

3.3.5.1 Analysis of nucleoprotein expression by rFWPV.

Nucleoprotein (NP) accumulates in the nucleus upon AIV infection. Therefore, a strong buffer, RIPA (which contains three types of detergent), was used for cell lysis so as to lyse the nuclear membrane to release nuclear proteins. Initially 2F6C9, a monoclonal antibody provided by Prof Barclay, was used as a primary antibody at a dilution of 1:1000. A band of ~56 kD (as expected based on the predicted molecular weight of native NP) was observed for all the samples, including the negative controls (uninfected and parental FP9-infected CEFs). Indeed it was faintest in the positive control (RG14 recombinant influenza virus). No such band was observed in the Marker lanes (Figure 3.16). The same outcome was observed when 5D8 antibody, purchased from Santa Cruz Biotechnology, was used at dilution of 1:500 (Figure 3.17).

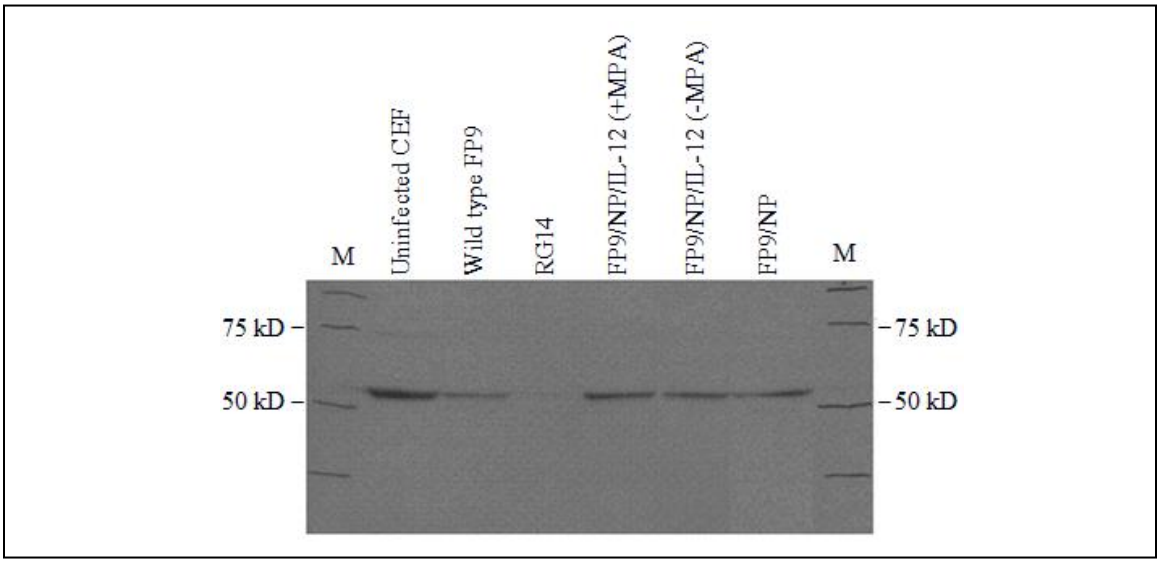


Figure 3.16. Western blot analysis of a mouse anti-nucleoprotein monoclonal antibody, 2F6C9 (kindly supplied by Prof Barclay). Lanes showed lysates of uninfected CEF, or CEF infected with different viruses. M is a protein marker.

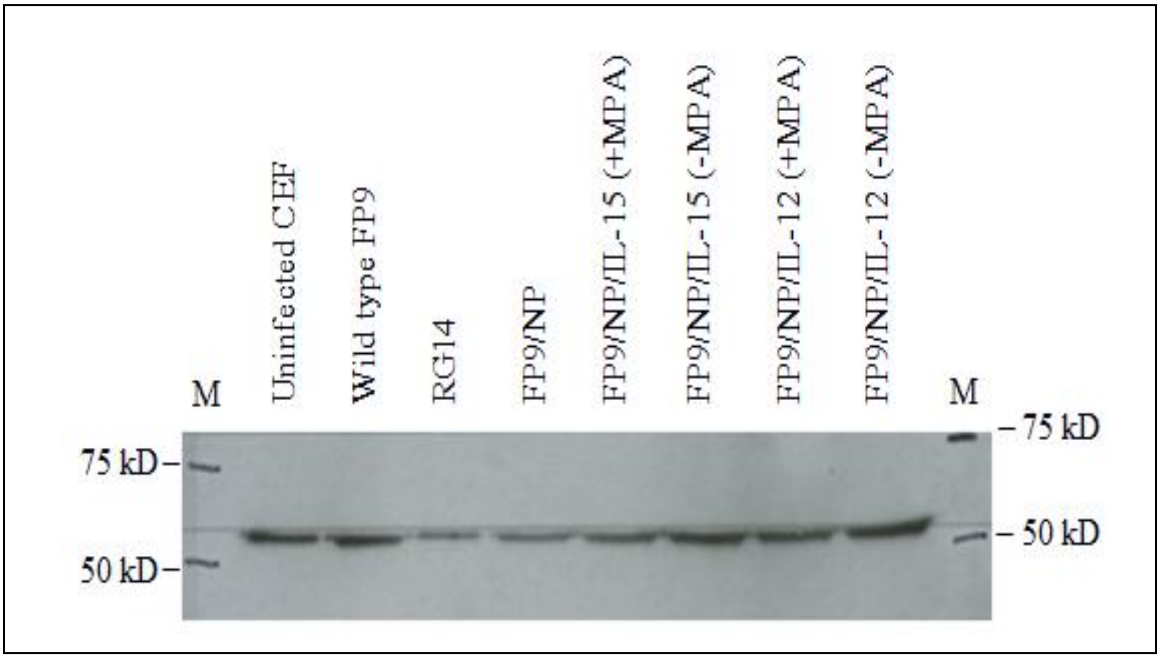


Figure 3.17. Western blot analysis of a mouse anti-nucleoprotein monoclonal antibody, 5D8 (Santa Cruz Biotechnology). Lanes showed lysates of uninfected CEF, or CEF infected with different viruses. M is a protein marker.

As the observed band would seem to be a cross reacting chicken protein, the specificity of the 5D8 monoclonal antibody was investigated further. Three different avian cell lines, CEF, DF1 and QT-35, were cultivated for 48 hours without any infection. DF1 is an immortal chicken embryo fibroblast cell line developed using conventional passage techniques by Douglas Foster in 1998 (Schaefer-Klein *et al.*, 1998), while QT-35 is a continuous cell line of Japanese quail origin. Three different types of sera, new-born bovine serum (GIBCO), fetal bovine serum (Autogen BioClear) and fetal bovine serum (BioSera), were included in the cell medium (DMEM), separately. The supplemented cells were compared with cells grown in DMEM without any serum. The aim of this experiment was to evaluate the band pattern of other uninfected avian cells treated with the same primary antibody, and to investigate whether a serum plays a role in blocking detection of the band by the antibody. All of these uninfected samples generated a ~56

kD protein band, although two minor bands (possibly degradation products) with sizes of less than 50 kD were evident with QT-35 lysates. The constant level of labelling suggests that the nature of the cultivation sera does not affect detection of the band by each antibody (Figure 3.18), suggesting that the protein is unlikely to be a serum protein and is more likely to be a cellular protein. As yet, the comparison has not been extended to non-avian cell lines.

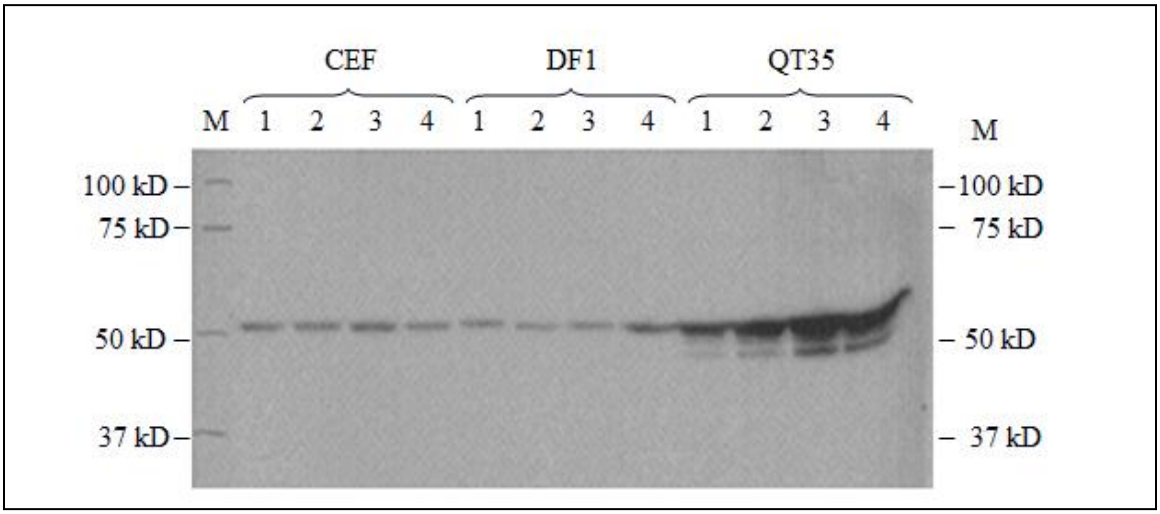


Figure 3.18. Western blot analysis of a mouse anti-nucleoprotein monoclonal antibody, 5D8 (Santa Cruz Biotechnology). Lanes 1 showed particular cells grown in DMEM without serum. Lanes 2 showed particular cells grown in DMEM with NBBS (GIBCO). Lanes 3 showed particular cells grown in DMEM supplemented with FBS of Autogen Bioclear. Lanes 4 showed particular cells grown in DMEM with FBS from BioSera. M is a protein marker.

A further experiment was performed using ab25921 (diluted at final concentration of 1 μg/mL), acquired from Abcam. Immunogen for this antibody is a synthetic peptide corresponding to amino acids 58-77 of AIV NP. However, the result was a relatively consistent band pattern, including several strong bands (at ~95 kD & ~63 kD) observed across the recombinants as well as positive or negative controls. No band unique to the recombinants was observed, though there was a band at ~60 kD common only to lanes carrying lysates infected with FP9 (WT or recombinant) (Figure 3.19). The ~56kD band

seen with 5D8 was not prominent. Once again, the pattern was not seen in the Marker lanes, though some faint, diffuse bands were present.

These results were unexpected and are not easy to explain. In none of these cases has a second antibody-only control yet been performed, to ascertain whether the cross reactivity is due to the primary or secondary antibody. A simple explanation for these results might be a failure to block adequately, or to wash adequately, so that low affinity cross-reactions are observed. Clearly ab25921 can distinguish between FP9-infected and uninfected cell lysates (via the ~60 kD band; Figure 3.19), even though the antiserum is claimed to be specific for an NP peptide. A different cross-reactive band was seen with DF1, at ~120 kD.

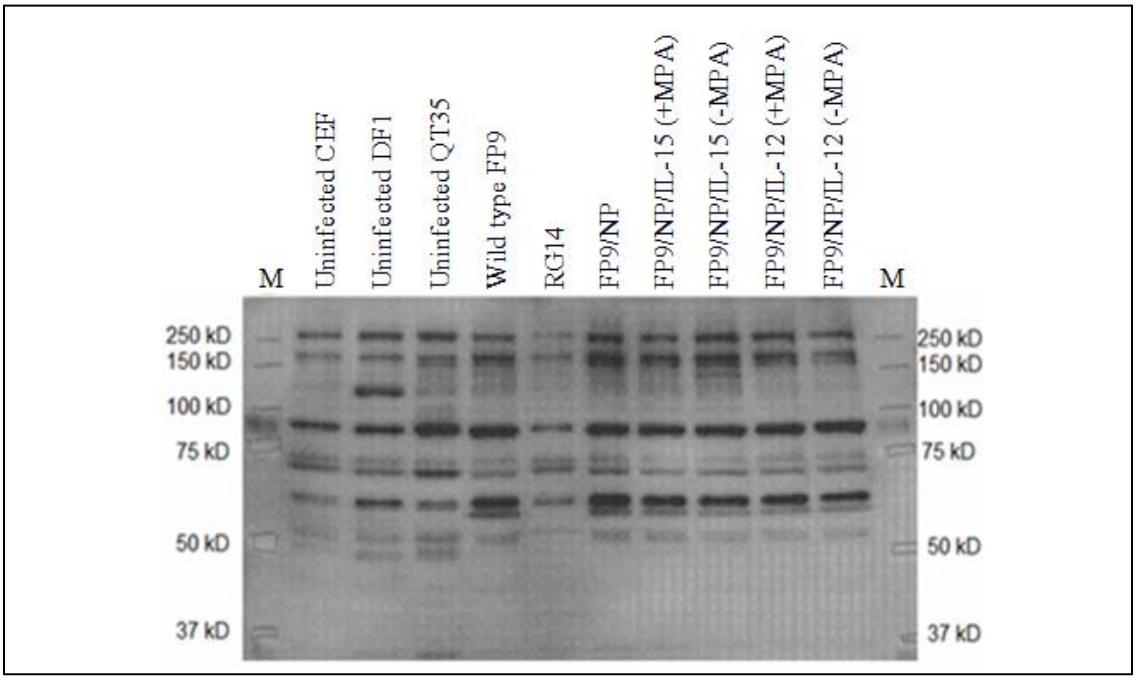


Figure 3.19. Western blot analysis of a mouse anti-nucleoprotein monoclonal antibody, ab25921 (Abcam). Lanes showed lysates of uninfected CEF, DF1 and QT35, or CEF infected with different viruses. M is a protein marker.

3.3.5.2 Analysis of H5 expression by rFWPV.

In order to detect H5 expression by the recombinants, ab62587 (Abcam) was used as a primary antibody at a final concentration of 1 μg/mL. The immunogen for this antibody was a synthetic peptide corresponding to 12 amino acids near the amino terminus (N-terminus) of the AIV HA protein. While the predicted size of unglycosylated H5 protein is 64 kD, the positive control (RG14-infected CEF cells) displayed a distinct protein size of ~80 kD, the difference probably being due to glycosylation (though this was not checked by the use of inhibitors or glycosidases). No such expression was observed for any of the rFWPV (Figure 3.20).

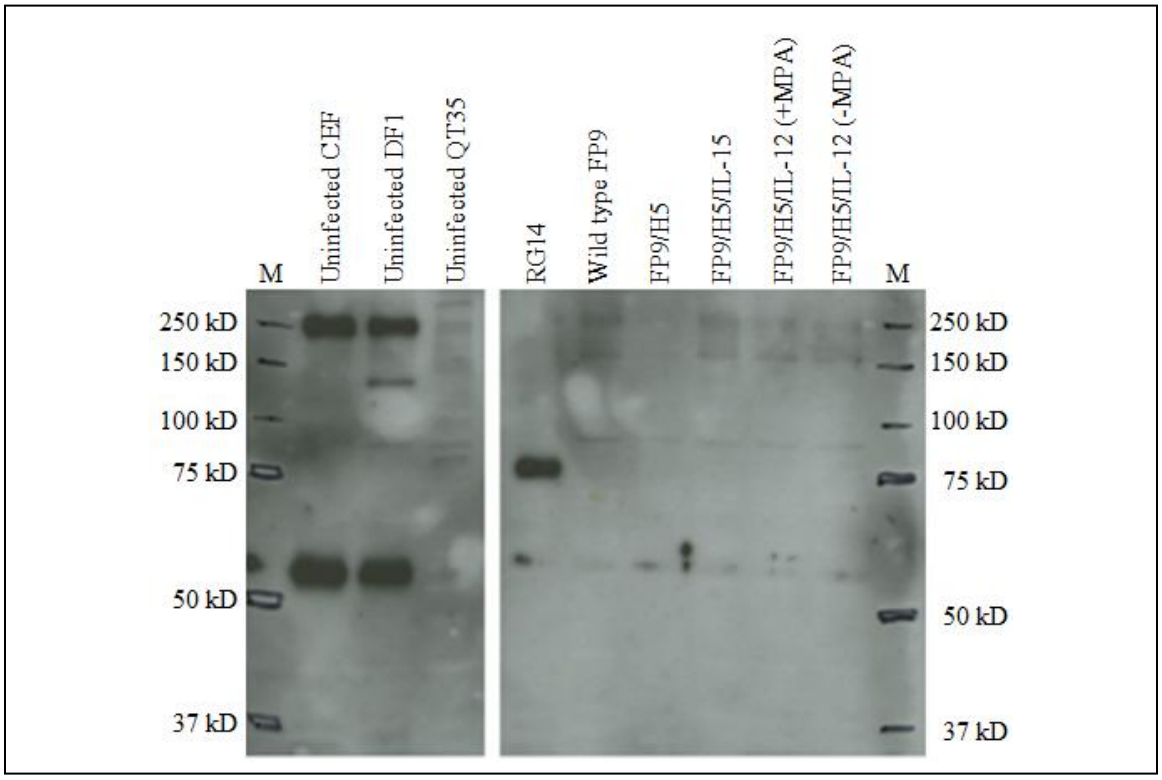


Figure 3.20. Western blot analysis of a goat anti-haemagglutinin polyclonal antibody, ab62587 (Abcam). Lanes showed lysates of uninfected CEF, DF1 and QT35, or DF1 infected with different viruses. M is a protein marker.

A subsequent analysis was performed using Abcam's ab70077, diluted to a final concentration of 1 µg/mL. The immunogen for this rabbit polyclonal primary antibody was a synthetic peptide corresponding to 14 amino acids near the middle of AIV H5 strain A/China/GD01/2006. A commercial kit using the chromogenic substance, WesternBreeze (Invitrogen) was used to replace the conventional western blot procedure. A faint band at ~50 kD was observed for H5 recombinant, none for uninfected cell lysate and negative control (Figure 3.21). Reproducibility was difficult due to limited virus stock. As an alternative approach, IFAT was performed using the same antibody. Fluorescent signals were detected only for CEF infected with H5 recombinant, no reactivity was observed for uninfected or negative control (WT FP9)-infected CEF (Figure 3.22).

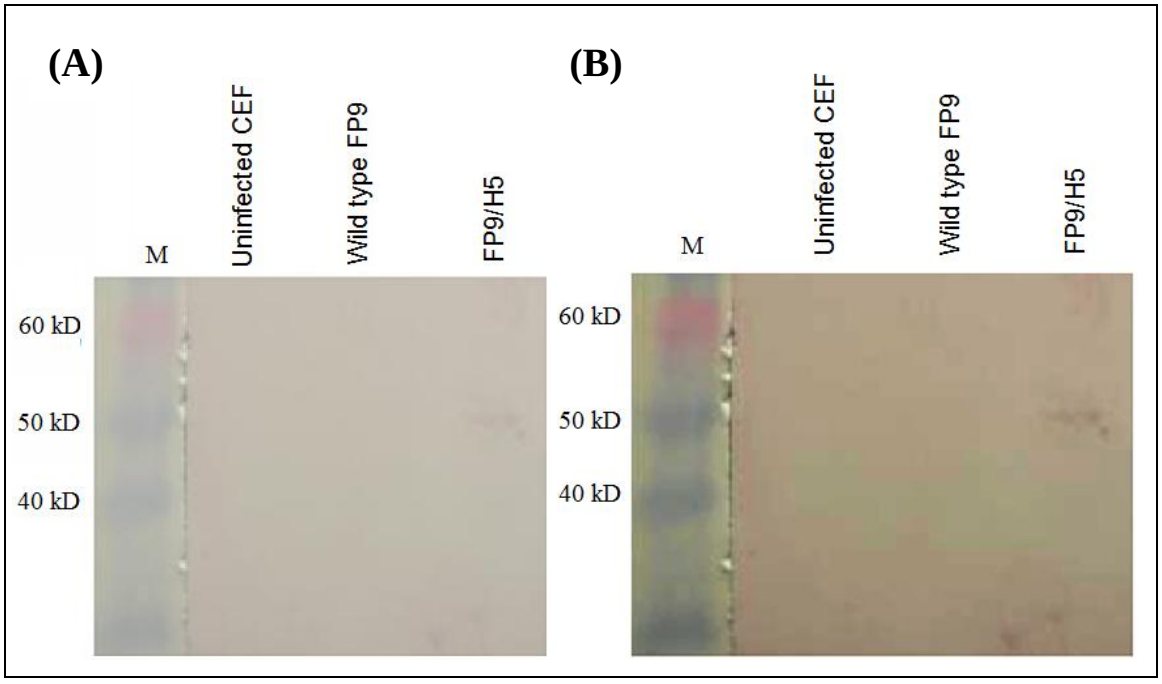


Figure 3.21. Western blot analysis using a rabbit anti-haemagglutinin polyclonal antibody, ab70077 (Abcam). Lanes showed lysates of uninfected CEF, CEF infected with wild type FP9 and CEF infected with recombinant FP9/H5. M is a protein marker. Blot was presented in an original version (A) or an edited version, to increase the contrast (B). Blot of cell lysates was darker than the Marker due to the final staining procedure using a chromogenic reagent (purple).

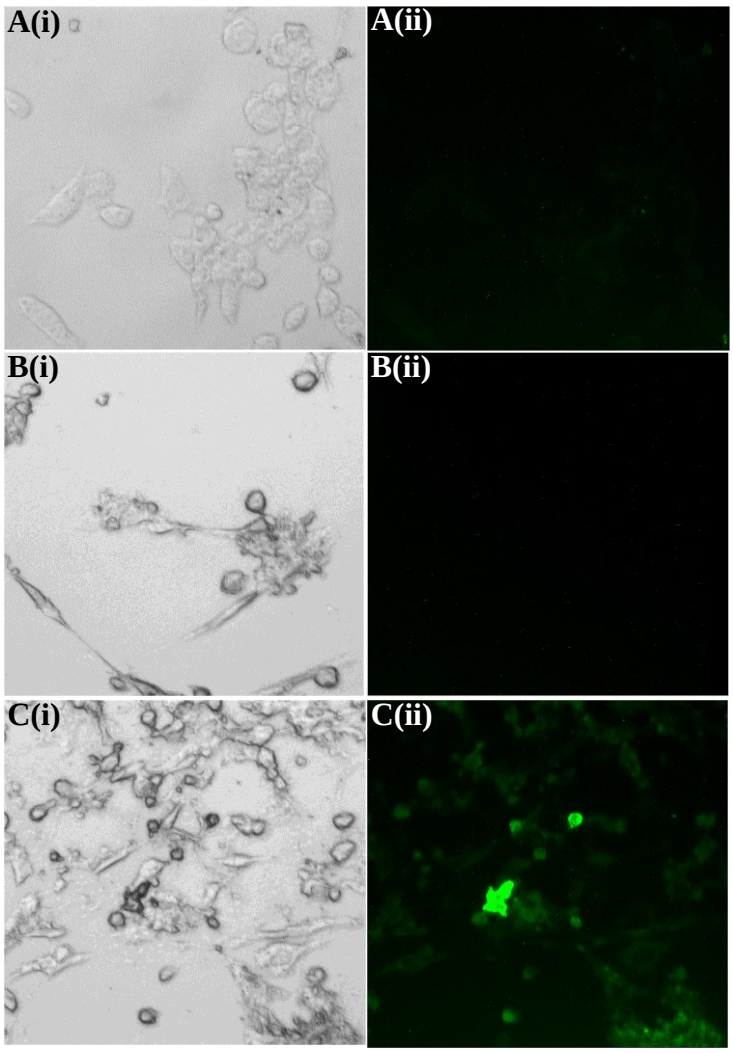


Figure 3.22. Indirect immunofluorescence assay, IFAT, of H5 recombinant. CEF cell cultures were grown until 80% confluent on coverslips, and either remained uninfected (A), infected with wildtype FP9 (B) or infected with recombinant FP9 carrying H5 gene (C). After 19 hours, the cells were incubated with Abcam's ab70077 primary antibody for 2 hours, before counterstained with a fluorescein-labeled anti-rabbit antibody, for an hour. (i) showed CEF under visible light, while (ii) showed cells under UV light. Positive fluorescence was observed only in C(ii). The images did not represent 80 % of cell confluency due to repeated washing of the cells (without fixing) in IFAT procedure.

3.3.5.3 Analysis of N1 expression by rFWPV.

To examine the expression of N1, we used the ab36566 primary antibody (Abcam). The immunogen for this antibody was a synthetic peptide, corresponding to 15 amino acids at the C terminus of AIV NA. The predicted size of the unglycosylated N1 protein is 49 kD, based on its deduced amino acid sequence. However, a faint, diffuse ~60 kD band, instead of 49 kD, was present in the positive control, suggesting a glycosylated product. Besides the band seen in the positive control, the analysis revealed no unique protein band among the recombinant virus lysates. Lysates from DF1s infected with FP9 (both WT and recombinant), but not from uninfected DF1s, generated two strong bands of ~65 kD and ~70 kD (presumably FWPV proteins), with no sign of the N1 band (Figure 3.23). A band at about 42 kD was present in all samples, infected or uninfected, CEF, DF1 or QT35 (in which it was stronger and accompanied by an equally strong band at 45 kD), but not in the Marker lanes.

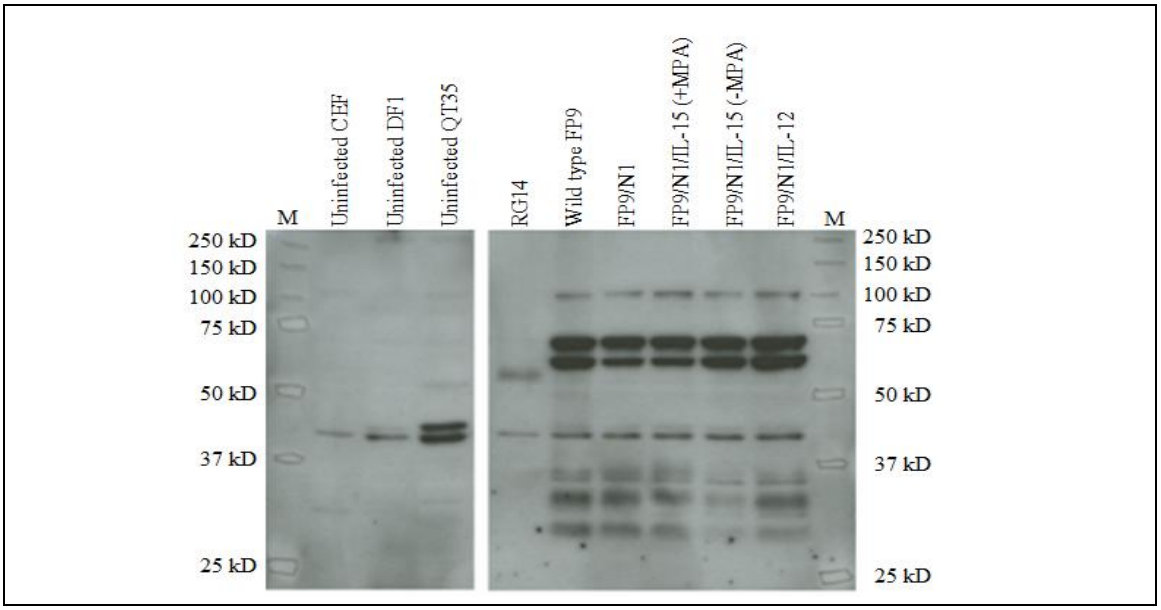


Figure 3.23. Western blot analysis of a rabbit anti-neuraminidase polyclonal antibody, ab36566 (Abcam). Lanes showed lysates of uninfected CEF, DF1 and QT35, or DF1 infected with different viruses. M is a protein marker.

Further western blot analysis was performed using WesternBreeze (Invitrogen). Abcam's rabbit polyclonal, ab70759, was diluted at a final concentration of 1 μg/mL. Immunogen for this primary antibody is a neuraminidase of influenza A/H5N1/Vietnam/1203/2004. A prominent band was observed at ~48 kD, which corresponding to the predicted unglycosylated size, 49 kD (Figure 3.24). Using the same primary antibody in IFAT, fluorescent signals were detected only for N1 recombinants, not for the negative controls (Figure 3.25).

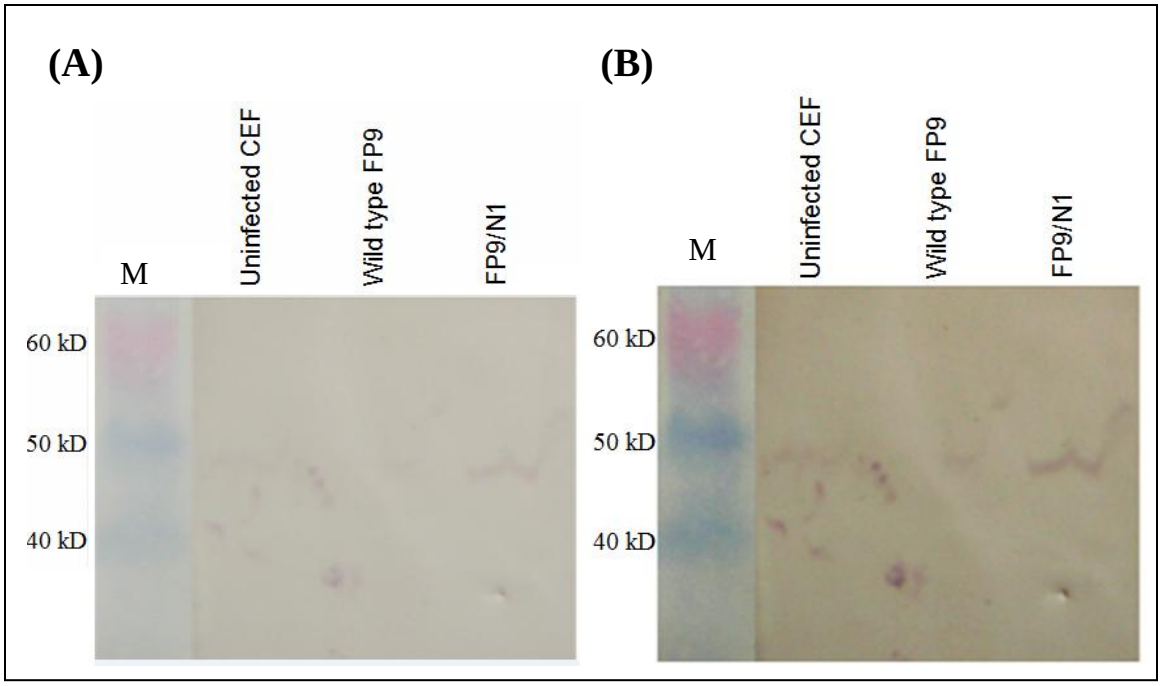


Figure 3.24. Western blot analysis of a rabbit anti-neuraminidase polyclonal antibody, ab70759 (Abcam). Lanes showed lysates of uninfected CEF, CEF infected with wild type FP9 and CEF infected with recombinant FP9 carrying N1 gene. M is a protein marker. Blot was presented in an original version (A) or an edited version, to increase the contrast (B). Blot of cell lysates was darker than the Marker due to the final staining procedure using a chromogenic reagent (purple).

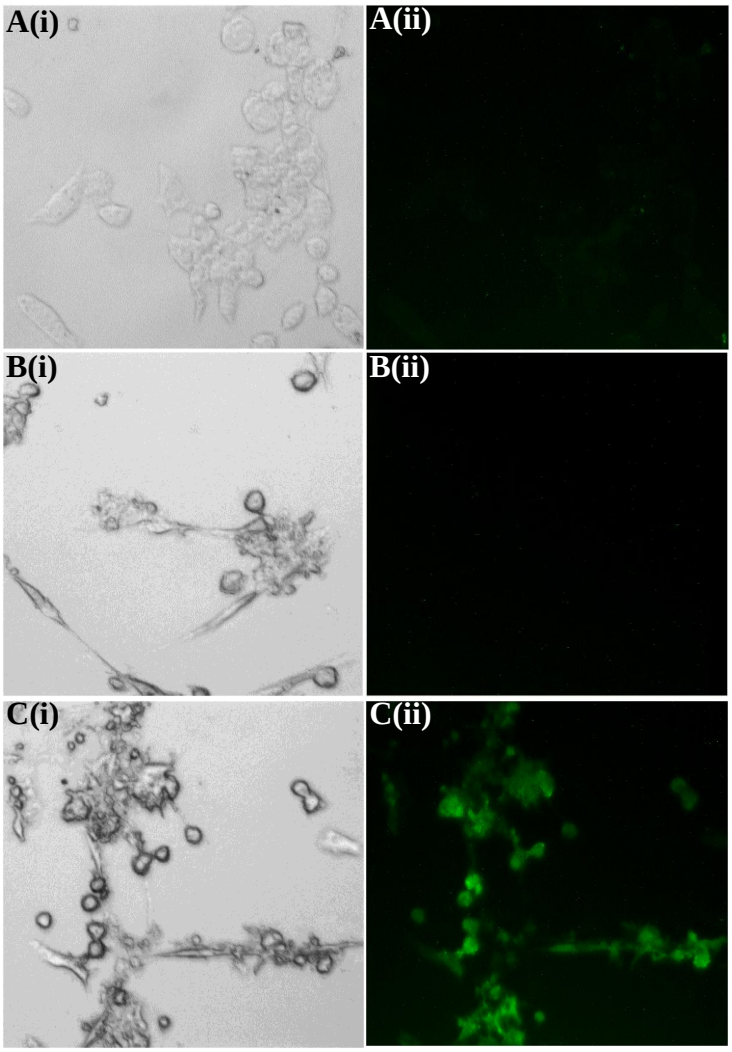


Figure 3.25. Indirect fluorescent antibody test of N1 recombinant. CEF cell cultures were grown until 80% confluent on coverslips, and either remained uninfected (A), infected with wildtype FP9 (B) or infected with recombinant FP9 carrying N1 gene (C). After 19 hours, the cells were incubated with Abcam's ab70759 primary antibody for 2 hours, before counterstained with a fluorescein-labeled anti-rabbit antibody, for an hour. (i) showed CEF under visible light, while (ii) showed cells under UV light. Positive fluorescence is observed only in C(ii). The images did not represent 80 % of cell confluency due to repeated washing of the cells (without fixing) in IFAT procedure.

In conclusion, expression of H5 and N1 by rFWPV has been detected using a commercial western blotting kit and IFAT. However, the RG14 positive control was not available for these analyses. It is not clear whether the difficulties experienced in AIV protein detection were a function of the antigens used to produce the primary antibody, affecting specificity, or a technological issue relating to the sensitivity of detection method.

There are several possible reasons for the inability to detect expression of H5 and N1 by the recombinants using conventional western blotting procedures. Firstly, the viruses might not actually be recombinant. This possibility was excluded by western blotting with an antibody against β-galactosidase protein (Promega), expressed by the *LacZ* selectable marker carried on pEFL29 (into which the AIV genes were inserted). A band of ~130 kD, similar to the predicted size of β-galactosidase expressed by pEFL29 (120 kD), was detected in FP9 recombinant for H5, N1 and NP. No band was observed for FP9 WT nor for uninfected CEF (Figure 3.26). Confirmation of the presence of the respective AIV genes by PCR therefore confirms that the viruses are actually recombinant and have integrated the respective expression cassettes from the corresponding pEFL29 derivatives. Naturally it does not per se demonstrate the function of the expression cassettes.

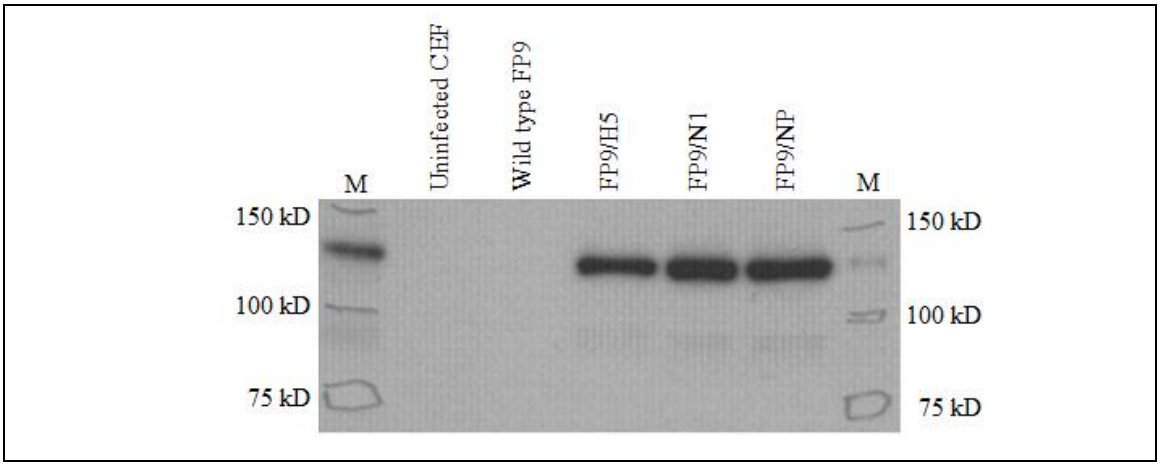


Figure 3.26. Western blot analysis of a mouse anti-β-galactosidase monoclonal antibody (Promega). Lanes showed lysates of uninfected CEF or CEF infected with different viruses. M is a protein marker.

The second possibility, and most likely, is that the actual level of expression (or, more precisely, the steady state level) was below the detectable limit. This would be somewhat surprising, given that the VACV p7.5 promoter used to express recombinant proteins in pEFL29 is, though not the strongest poxvirus promoter available, tried and tested with a good 'track record' of success for many diverse proteins. Increasing the MOI of the recombinant viruses might possibly should boost the expression to a level that could be detected.

Thirdly, mutations in the PCR-generated AIV gene inserts might lead to missense or frame-shift mutations, leading to truncation, loss of recognition, altered glycosylation or folding of the polypeptides. The resulting products might then have been masked by the cross-reacting bands seen in the western blot for N1, or might have become unstable leading to rapid turnover and undetectable steady-state levels. However, sequencing demonstrated that the genes' start codons were in-frame, that there were no insertion or

deletion mutations in the sequences that could disturb the reading frames and that there were no non-coding changes. Hence, these possible explanations could be rejected.

In the case of nucleoprotein detection, the specificity of the assay remains to be established, with cross-reactivity towards a protein of unknown nature and source obscuring the position expected for NP. Curiously the presence of NP in the positive control sample (RG14 in CEF) has not been unambiguously demonstrated.

3.3.5.4 Analysis of chicken IL-12 expression by rFWPV.

Monoclonal antibody HC8, specific for the chicken IL-12 p70 heterodimer (provided by Dr Pete Kaiser; Institute for Animal Health, Compton), was used to monitor for the presence of the soluble secreted chicken IL-12 protein 70 kD fusion protein in supernatants from infected and uninfected CEF. A doublet of bands (the upper band being fainter) was observed at about 70 kD for five recombinants (two H5/IL-12, one N1/IL-12 and two NP/IL-12) but not for uninfected CEF nor for FP9 WT (Figure 3.27). There were differences between the levels of IL-12 observed for the recombinants; it is not yet known how reproducible this is.

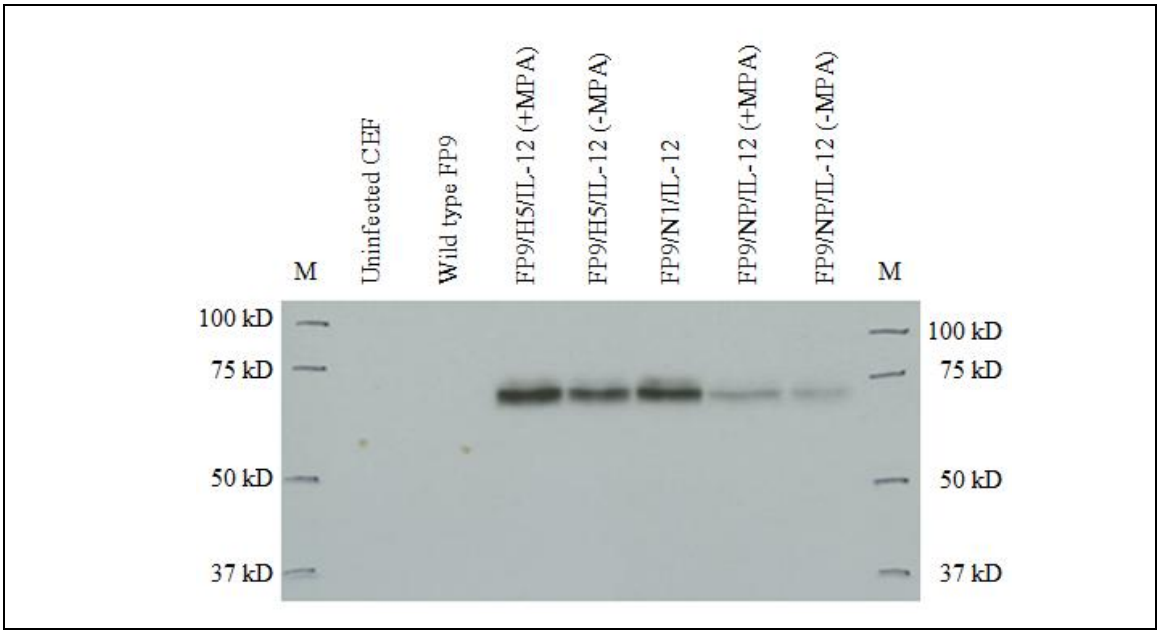


Figure 3.27. Western blot analysis of a mouse anti-chicken IL-12 monoclonal antibody, HC8 (Dr Kaiser, IAH). Lanes showed supernatants of uninfected CEF or CEF infected with different viruses. M is a protein marker.

3.3.5.5 Analysis of chicken IL-15 expression by rFWPV.

Although one resolved (FP9/H5/IL-15) and two unresolved recombinants containing IL-15 (FP9/N1/IL-15 and FP9/NP/IL-15) have been obtained, we have so far been unable to screen for expression of IL-15 protein as no antibodies are currently available. However, bioactive recombinant chicken IL-15 expression has been described (Lillehoj *et al.*, 2001), where the observed protein size corresponds to the deduced size of the studied chicken IL-15 (22 kD).

3.4 Summary

Recombinant FWPVs coexpressing AIV genes, H5 or N1, and chicken cytokine genes, IL-15 or IL-12, have been constructed using homologous recombination at two non-essential sites in the genome of FWPV strain FP9. Antibodies against chicken IL-15 are unavailable but expression of H5, N1 and IL-12 proteins was verified using western blotting and IFAT. The available recombinants after the verifications are FP9/H5 (rFWPV/H5), FP9/N1 (rFWPV/N1), FP9/H5/IL-15 (rFWPV/H5/IL-15) and FP9/N1/IL-12 (rFWPV/N1/IL-12). These recombinants were prepared ready to be inoculated into chickens to evaluate host immune responses.

Initial plans to include NP recombinants in animal trials were aborted due to the inability to specifically detect NP expression in avian cells, and failure to obtain resolved NP recombinants carrying either IL-15 or IL-12, during the time that recombinants were being constructed at Imperial College London.

CHAPTER 4

Co-immunostimulatory effect of IL-15 co-expressed in H5-recombinant fowlpox viruses on host immune responses

4.1 Introduction

Cell-mediated immune (CMI) responses of mice against viral infections have been widely described. However, the functional significance of CD4+ and CD8+ T cells as antiviral effectors in chickens needs more elucidation. In general, T cells displaying CD4 membrane glycoprotein at their surface, CD4+, are T helper (Th) cells, while those presenting CD8 marker, CD8+, function as T cytotoxic (Tc) cells. Th cells differentiate into memory cells or effector cells that enable or "help" the activation of B cells, Tc cells, macrophages and various other immune cells. Effector Th cells are divided into Th1 and Th2. While Th1 produce IFN-γ, which activates macrophages for CMI responses, the Th2 cell response results in the activation of B cells to produce antibodies that are able to neutralise (direct effect) or to opsonise (indirect effect) antigens, leading to humoral immune responses.

In *in vivo* studies conducted at Animal Experimental House, Faculty of Veterinary Medicine, UPM, Malaysia, two recombinant vaccines, rFWPV/H5 and rFWPV/H5/IL-15, the construction of which (at Imperial College London), has been described in Chapter 3, were inoculated into one-day-old SPF chicks, with parental, wild type (WT) FWPV strain FP9 and PBS-mock inocula implemented as controls. The experiments were carried out according to the international Guide for the Care and Use of Laboratory Animals (ILAR, 1996), under the approval of the local Institutional Animal Care and Use

Committee (IACUC) of UPM (Reference No.: UPM/FPV/PS/3.2.1.551/AUP-R72). Use of the rFWPV in laboratory of UPM was in accordance to guidelines from the local Biosafety Department, at Category 1 (elsewhere known as biosafety level, BSL, 1).

Upon inoculation of rFWPV carrying the H5 gene, infection of host cells will result and H5 will be expressed, along with FWPV proteins. Antigen presenting cells such as macrophages are responsible for identifying and engulfing virus-infected cells. These will then be lysed and then digested and peptides from the digested proteins will be transported to the cell surface by Class II MHC proteins for display to T or B lymphocyte cells. Presentation of H5 to T cells activates B cells to induce specific H5 antibodies which can be measured by haemagglutination inhibition test.

IL-15 is an immune-enhancing cytokine with profound effects on CD8 memory T cells, NK cells, B cells, mast cells and cytokine-chemokine networks. The ability of IL-15 to induce the expression of IFN-γ in both NK and T cells emphasizes its significant role in promoting Th1 response. Although chicken IL-15 was cloned and characterized in 1999 (Choi *et al.*, 1999), no subsequent study has been reported since. It therefore seemed valid to investigate the effect of IL-15 co-expressed in rFWPV/H5 on host CMI response, in comparison to rFWPV expressing H5 gene alone, as a major aim of this study. Although the level of IL-15 was not assessed, populations of CD4+ and CD8+ cells was measured as indicator of CMI response elicited by the cytokine.

Wing web puncture and brushing of chicken's exposed-follicles with the vaccine have been universally accepted in commercial FWPV vaccination (Mockett *et al.*, 1990; Boyle and Heine, 1994). However, we inoculated our vaccines (10^5 PFU in 100 μL) subcutaneously at the nape of the chicks, without any booster. This approach standardizes the vaccine dose as well alleviates inoculation of one-day-old chicks. Qiao *et al.* (2009) have demonstrated that rFWPV carrying HA and NA genes given by this subcutaneous route can induce HI antibodies and protect chickens against challenge with a pathogenic H5N1 strain A/Goose/Guangdong/1/96 (GS/GD/96), as efficiently as if they were given by wing web puncture or intramuscular injection.

4.2 Results

4.2.1 Humoral immune responses following rFWPV/H5 and rFWPV/H5/IL-15 vaccination

To measure H5 antibody levels in host serum samples post vaccination, the haemagglutination inhibition (HI) test was performed using influenza virus H5N2 strain A/Duck/Malaysia/8443/2004 as a heterologous antigen. The nucleotide (89.0 %) and amino acid (91.1 %) identities between HA genes of the agglutinating antigen virus and the vector insert strain (A/Chicken/Malaysia/5858/2004) were determined by pair-wise sequence alignment using BioEdit Version 7.0.5.3 (Figure 4.1).

As presented in Table 4.1, none of the nine control chickens inoculated with PBS or WT FP9 showed any evidence of HI antibody responses. Four chickens vaccinated with rFWPV/H5 started to develop HI antibody titre at 3 weeks post inoculation (p.i.), ranging

from a low $\log_2$ 1 to $\log_2$ 32. A week later, the titres had decreased in two birds ($\log_2$ 32 to 0, and $\log_2$ 32 to $\log_2$ 16), and increased in one bird ($\log_2$ 1 to $\log_2$ 8). Another bird elicited a constant HI titre of $\log_2$ 8 at both timepoints. No antibodies were detected at Week 5.

In the rFWPV/H5/IL-15-vaccinated group, two chickens developed antibodies as early as 2 weeks post-vaccination ($\log_2$ 1 and $\log_2$ 4). At Week 3, six other birds elicited HI antibody titre ranging from $\log_2$ 1 to $\log_2$ 16. A week later, the titre in one bird remained constant at $\log_2$ 16. Three birds demonstrated a decrease in titre to $\log_2$ 4, while titres in another four birds dropped to undetectable levels. At Week 5, no HA-specific antibodies were detected for any birds.

Mean HI titres, in $\log_2$, of all groups were calculated for general comparison (Table 4.2). The data showed that H5 antibodies are induced at the highest level at Week 3 in both recombinant vaccine-treated chickens. However, the antibodies were undetectable at Week 5 onwards. In summary, fewer chickens in rFWPV/H5 elicited H5 antibodies compared to rFWPV/H5/IL-15. Moreover, contrary to expectations, chickens vaccinated with rFWPV/H5 developed antibodies one week later than those vaccinated with rFWPV/H5/IL-15.

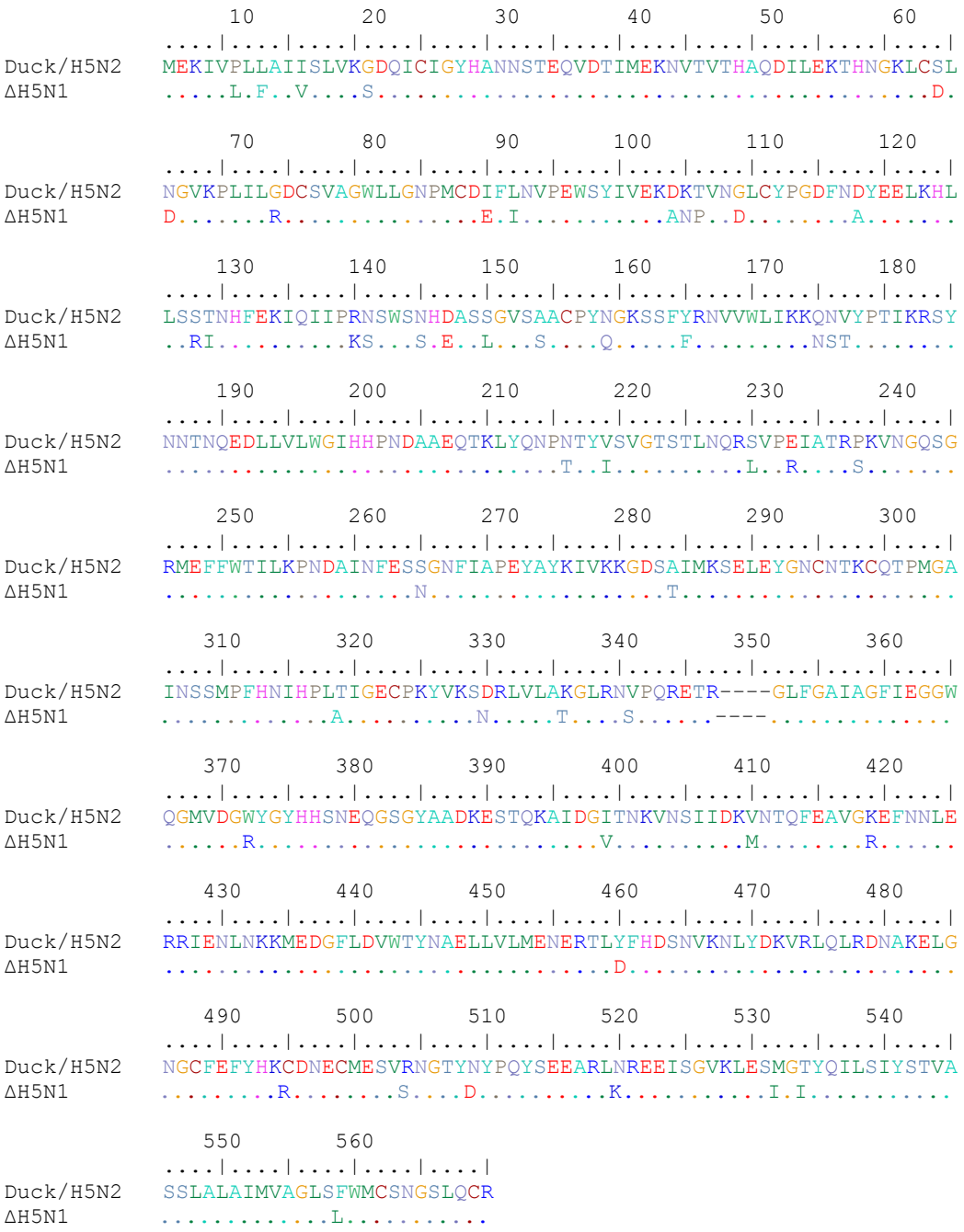


Figure 4.1. Haemagglutinin (HA) amino acid identities between the studied, mutated H5 of A/Chicken/Malaysia/5858/2004, denote ΔH5N1, and H5N2 strain A/Duck/Malaysia/8443/2004, represented by Duck/H5N2. Duck/H5N2 is used as heterologous antigen for haemagglutination inhibition (HI) test. The sequences were determined by pair-wise sequence alignment using BioEdit Version 7.0.5.3. Nucleotide identities between both sequences are not shown.

Table 4.1. HI titre, log_2, of serum samples obtained from chickens immunized by different vaccines, in one week interval, for seven weeks.

Vaccines	Number of chickens	Day, post inoculation						
		7	14	21	28	35	42	49
PBS-treated control group	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0
WT FP9	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0
rFWPV/H5	1	0	0	32	0	0	0	0
	2	0	0	32	16	0	0	0
	3	0	0	1	8	0	0	0
	4	0	0	8	8	0	0	0
	5	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0
	9	0	0	0	0	0	0	0
rFWPV/H5/ IL-15	1	0	1	16	4	0	0	0
	2	0	0	16	16	0	0	0
	3	0	0	16	4	0	0	0
	4	0	4	8	4	0	0	0
	5	0	0	8	0	0	0	0
	6	0	0	1	0	0	0	0
	7	0	0	8	0	0	0	0
	8	0	0	0	0	0	0	0
	9	0	0	16	0	0	0	0

Table 4.2. Mean of haemagglutination inhibition (HI) titre, log_2, of sera from immunized chickens upon weekly bleeding.

Group	Day, post immunization						
	7	14	21	28	35	42	49
Control	ND (0/9)*	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)
WT FP9	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)	ND (0/9)
rFWPV/H5	ND (0/9)	ND (0/9)	8.11±4.60 (4/9)	3.56±1.94 (3/9)	ND (0/9)	ND (0/9)	ND (0/9)
rFWPV/H5/IL-15	ND (0/9)	0.56±0.44 (2/9)	9.89±2.16 (8/9)	3.11±1.74 (4/9)	ND (0/9)	ND (0/9)	ND (0/9)

ND indicates undetected titre. Each value represents the means ± SEM.
*The proportion of individual chickens which induce detectable HI antibody titre, to total chickens in each group.

4.2.2 Cell-mediated immune response following rFWPV/H5 and rFWPV/H5/IL-15 vaccination

Immune competence of a host can be evaluated from several parameters, including circulating T lymphocyte populations. Typical CD4+ and CD8+ T lymphocyte cell enumeration is via flow cytometric analysis, using fluorescent-conjugated monoclonal antibodies that recognise expressed cell surface markers. In our immunophenotypic flow cytometry analysis, isolated lymphocytes were triple-stained with anti-chicken CD3 (R-Phycoerythrin(PE)-conjugated), anti-chicken CD4 (Fluorescein(FITC)-conjugated) and anti-chicken CD8 (Peridinin Chlorophyll Protein Complex (PerCP)-Cy5-conjugated) monoclonal antibodies, allowing eight cell subsets to be characterized concurrently; CD3-CD4-, CD3-CD4+, CD3+CD4-, CD3+CD4+, CD3-CD8-, CD3-CD8+, CD3+CD8-, CD3+CD8+. The estimation of CD4+ and CD8+ T cells captured by the cytometer, in percentages, was determined by the measurement of T cells having CD3+CD4+ or CD3+CD8+ receptors. We assume that mature and functional CD4+ and CD8+ T cells are constantly bound to T-cell receptor-CD3 complex. A typical flow cytometric pattern of CD4+ and CD8+ T cell that present positive signals is shown in Figure 4.2.

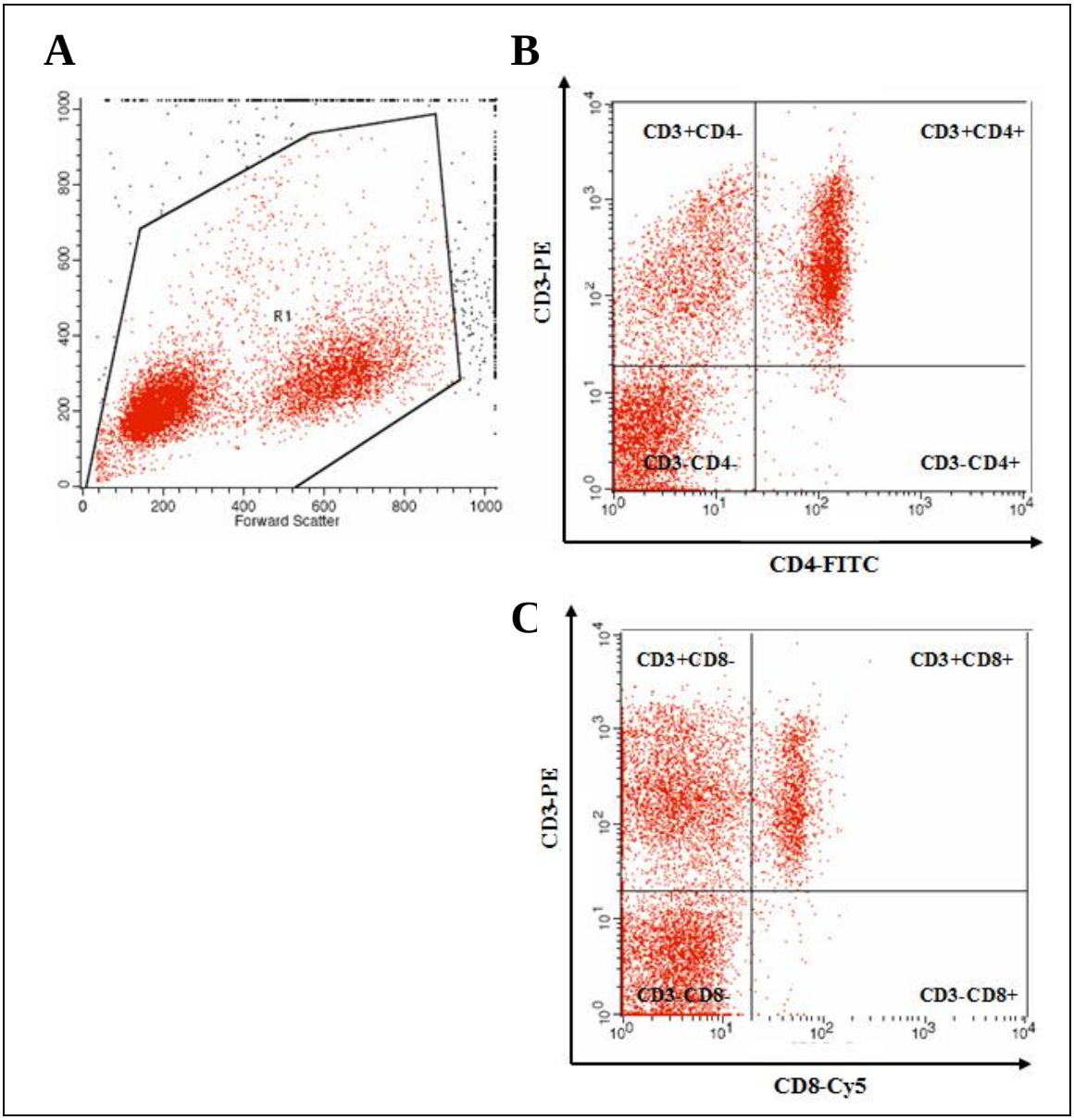


Figure 4.2. Typical flow cytometric pattern of chicken CD4 and CD8 cell expression in a single tube of 10000 captured cell events. Cells were gated using forward and side scatter to distinguish physical properties of cells and cellular contaminants (A). Percentages of CD3+CD4+ (B) and CD3+CD8+ (C) T cells amongst chicken PBMC were calculated from the upper right quadrant. The upper left quadrant represents CD3+ T cells which were CD4- (B) or CD8- (C). Anti-chicken CD3 monoclonal antibodies were conjugated with R-Phycoerythrin (PE), CD4 with Fluorescein (FITC) and CD8 with Peridinin Chlorophyll Protein Complex-Cy5 (Cy5).

In the first animal experiment, three pools of whole blood were established for each group of nine birds (n=3), each pool representing three individual chickens. This practise was selected to facilitate the ficoll gradient-isolation of lymphocytes from fresh peripheral blood mononuclear cells (PBMC). A minimal blood volume (will be discussed in Section 7.2) was drawn from birds to reduce overall stress and prevent anaemia. CD4 and CD8 T cell phenotypes were determined at Weeks 2 and Week 5. In the second animal experiment, whole blood was sampled from five individual chickens (n=5) at Weeks 2 and 4.

To present the difference, which is either an increment or reduction of a particular cell population, a fraction of 100 (percentage) is calculated in equation as below:

$$\left(\frac{\text{Final population percentage} - \text{initial population percentage}}{\text{Initial population percentage}} \right) \text{X } 100$$

In the equation, 'initial population percentage' refers to flow cytometry values of a particular T cell population at two weeks post-vaccination, while 'final population percentage' refers to values at Weeks 5 or 4, post-vaccination, for Experiments 1 or 2, respectively.

4.2.2.1 PBMC CD4+ T cell population

In the first animal experiment, in which *n*=3, the levels of CD4+ in the control group remained relatively constant at Weeks 2 (15.64 ± 1.75 %) and 5 (16.54 ± 3.33 %), showing an increase of only 0.9 % points, or 5.75 %. Samples from groups vaccinated with WT FP9 or rFWPV/H5/IL-15 demonstrated increases in CD4+ levels of 10.99 % (18.75 ± 2.53 % to 20.81 ± 0.84 %; 2.06 % points) and 21.97 % (14.38 ± 0.70 % to 17.54 ± 3.48 %; 3.16 % points), respectively. However, the rFWPV/H5 vaccinated group showed a higher CD4+ population at Week 2 (23.67 ± 1.11 %), than at Week 5 (16.09 ± 2.72 %), a fall of 7.58 % points, or 32.02 %. The level of the CD4+ population in the rFWPV/H5 group at Week 2 was significantly higher ($P \leq 0.05$) compared to the control. No statistically significant difference was observed for other groups at either sampling point (Table 4.3, Figure 4.3(A)).

In the second animal experiment (in which *n*=5), the CD4+ cell population increased only in the WT FP9-vaccinated group (from 9.94 ± 1.17 % to 10.93 ± 0.89 %, an increase of 0.99 % points or 9.96 %). The CD4+ population in the control group decreased from 10.49 ± 1.29 % to 7.37 ± 0.91 % (by 3.12 % points, or 29.74 %), while those in the rFWPV/H5 and rFWPV/H5/IL-15 groups decreased from 10.37 ± 0.82 % to 9.65 ± 0.71 % (0.72 % points, or 6.94 %) and from 13.05 ± 1.05 % to 8.44 ± 0.62 % (4.61 % points, or 35.32 %), respectively. The 48.3 % difference of CD4+ cell population between WT FP9 and control at Week 4 was the only statistically significant comparison (Table 4.3, Figure 4.3(B)).

Overall, the pattern of results is similar for pooled and individual samples, except for control and rFWPV/H5/IL-15. The relative levels, and direction of change, of CD4+ in the rFWPV/H5/IL-15 group compared to the rFWPV/H5 were different in the two experiments. However, the data obtained from the individual samples in experiment 2 may be more reliable since they represented a larger sample size (*n*) and lower standard deviations (SD). The only significant change in CD4+ T cells observed during the time course in either experiment was the decrease observed for the rFWPV/H5/IL-15 group in experiment 2. Therefore, it could be assumed that IL-15 co-expression does not induce a persistent Th1 response in chickens.

Table 4.3. CD4+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15.

Vaccine	Animal experiment			
	1 (*n*=3)		2 (*n*=5)	
	Week 2	Week 5	Week 2	Week 4
Control	15.64 ± 1.75	16.54 ± 3.33	10.49 ± 1.29	7.37 ± 0.91
Wild Type FP9	18.75 ± 2.53	20.81 ± 0.84	9.94 ± 1.17	10.93 ± 0.89*
rFWPV/H5	23.67 ± 1.11*	16.09 ± 2.72	10.37 ± 0.82	9.65 ± 0.71
rFWPV/H5/IL-15	14.38 ± 0.70	17.54 ± 3.48	13.05 ± 1.05^	8.44 ± 0.62^

Animal experiment 1 represents PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. Animal experiment 2 represents individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated and control groups, indicated by asterisks (*), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a caret (^), were determined by paired-samples T-test ($P \leq 0.05$).

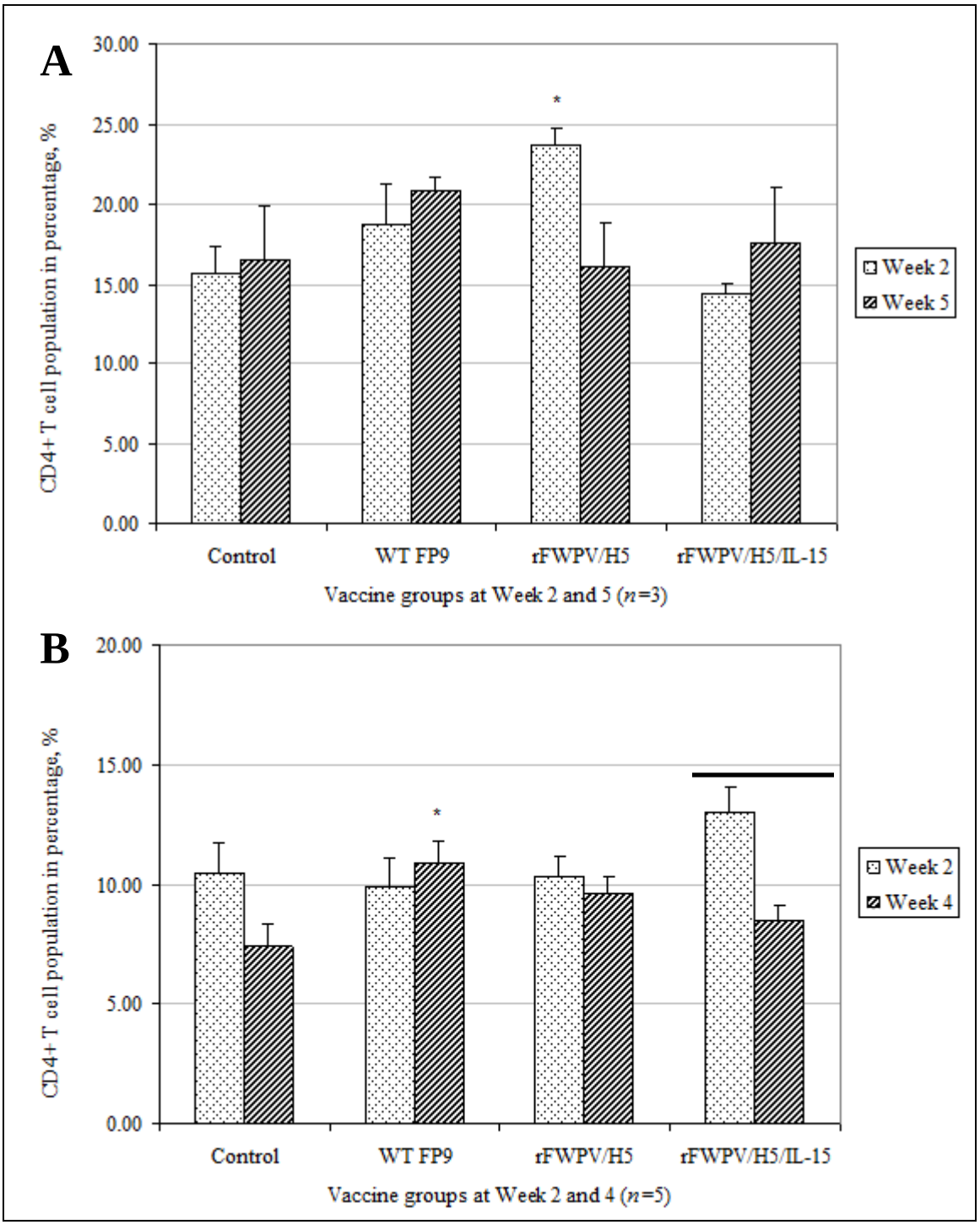


Figure 4.3. CD4+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15. A) PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. B) Individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated and control groups, indicated by asterisks (*), were determined by one-way ANOVA (P≤0.05). Significant differences within the same group at different points, indicated by a horizontal line, were determined by paired-samples T-test (P≤0.05).

4.2.2.2 PBMC CD8+ T cell population

In experiment 1, pooled blood samples at Weeks 2 and 5 revealed relatively constant CD8+ populations for unimmunized control (8.54 ± 0.55 % and 8.17 ± 1.15 %, respectively) and WT FP9-vaccinated (12.71 ± 0.82 % and 13.28 ± 1.06 %, respectively) chickens, rising 4.3 % and falling 4.5 %, respectively. The population in rFWPV/H5/IL-15 group demonstrated decreases in CD8+ levels of 7.82 % (10.49 ± 1.17 % to 9.67 ± 2.51 %; 0.82 % points). A remarkable fall of 42.44 % (or 6.91 % points) of CD8+ cell in rFWPV/H5-vaccinated group from 16.28 ± 1.58 % to 9.37 ± 1.32 % is surprisingly not statistically significant. This is due to the small sample size (n=3), which needed a greater difference in the value and a smaller standard deviation (SD), to be considered as statistically significant (Table 4.4, Figure 4.4(A)).

In the second animal experiment (n=5), the levels of CD8+ T cell in control group, rFWPV/H5 and rFWPV/H5/IL-15 decreased 34.61 % (or 2.89 % points; 8.35 ± 0.99 % to 5.46 ± 0.69 %), 22.91 % (or 2.68 % points; 11.70 ± 0.61 % to 9.02 ± 0.51 %) and 45.78 % (or 4.94 % points; 10.79 ± 0.67 % to 5.85 ± 0.87 %), respectively. The CD8+ T cell population increased only in WT FP9-vaccinated group (from 8.36 ± 0.73 % to 11.01 ± 1.21 %; an increase of 31.70 % or 2.65 % points), although not statistically significant (Table 4.4, Figure 4.4(B)).

Overall, chicken IL-15 co-expression in rFWPV/H5 did not promote a higher CD8+ cell percentage significantly, in comparison to rFWPV carrying H5 alone. In fact, it appears that the CD8+ population in rFWPV/H5/IL-15-vaccinated birds diminished relative to those in birds vaccinated with WT FP9 (significant) or FWPV/H5 (insignificant) at Week 4, though only in Experiment 2.

Table 4.4. CD8+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15.

Vaccine	Animal experiment			
	1 (*n*=3)		2 (*n*=5)	
	Week 2	Week 5	Week 2	Week 4
Control	8.54 ± 0.55	8.17 ± 1.15	8.35 ± 0.99	5.46 ± 0.69
Wild Type FP9	12.71 ± 0.82*	13.28 ± 1.06*	8.36 ± 0.73	11.01 ± 1.21*
rFWPV/H5	16.28 ± 1.58*	9.37 ± 1.32	11.70 ± 0.61*,**^	9.02 ± 0.51^
rFWPV/H5/IL-15	10.49 ± 1.17	9.67 ± 2.51	10.79 ± 0.67^	5.85 ± 0.87**^

Animal experiment 1 represents PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. Animal experiment 2 represents individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated groups and control (*), or between vaccinated groups and WT FP9 (**) were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a caret (^), were determined by paired-samples T-test ($P \leq 0.05$)

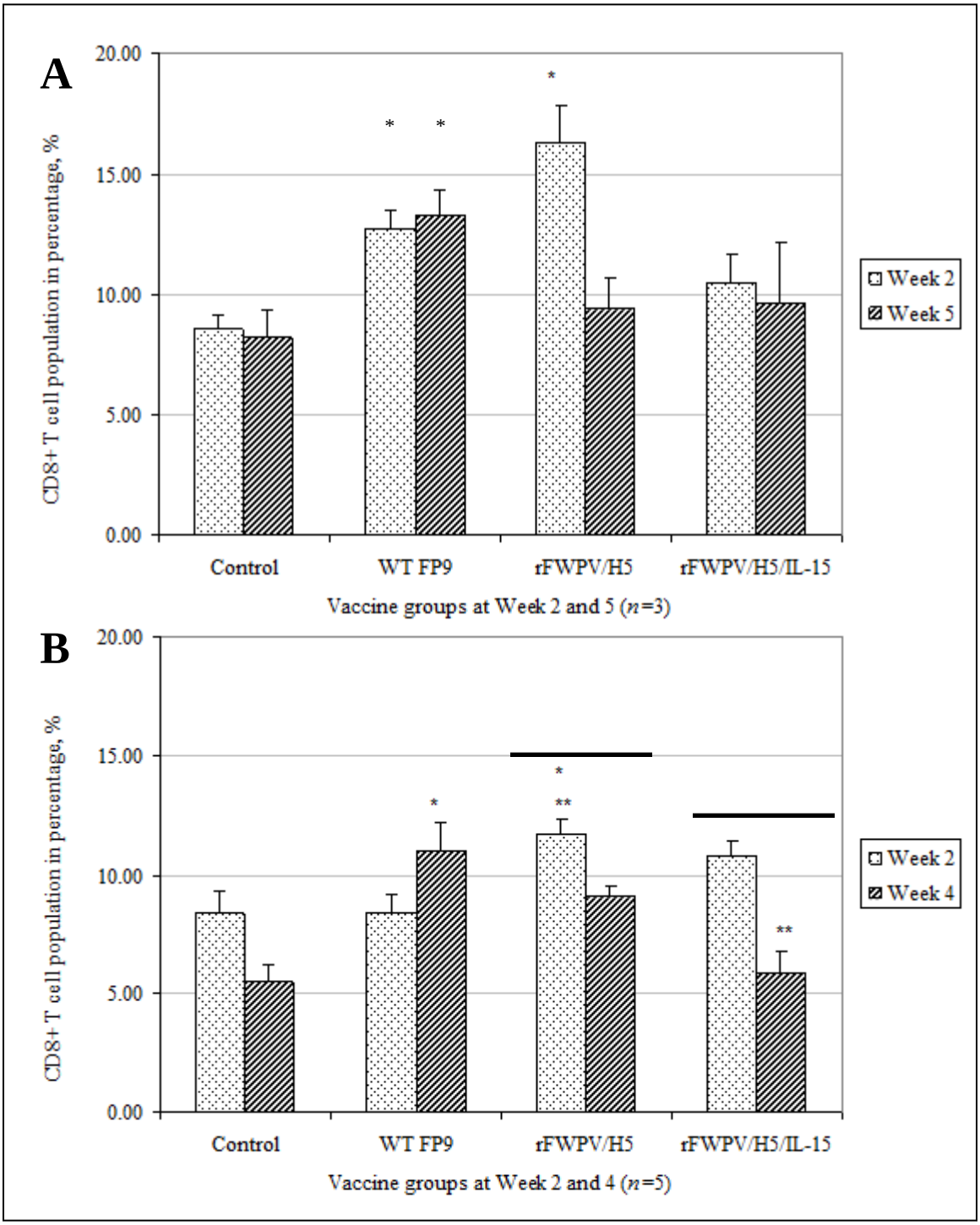


Figure 4.4. CD8+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15. A) PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. B) Individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated groups and control (*), or between vaccinated groups and WT FP9 (**) were determined by one-way ANOVA (P≤0.05). Significant differences within the same group at different points, indicated by a horizontal line, were determined by paired-samples T-test (P≤0.05)

4.2.2.3 CD4+/CD8+ T cell population ratio at different weeks

Ratios of CD4+ to CD8+ T cells (CD4+/CD8+), obtained from the flow cytometric analyses described above, were calculated to determine the relative fluctuation of CD8 cells in comparison to CD4 cells. A higher ratio indicates a smaller CD8 cell fraction present in circulating T lymphocytes. As presented in Table 4.5, the control group had the highest ratio of CD4+ to CD8+ at Week 2 of both experiments (1.83 ± 0.24 and 1.26 ± 0.21), and Week 5 (2.02 ± 0.50) of the first animal experiments. This is to be expected as unvaccinated host does not trigger CD8 cells which respond to an antigen presented by Class I MHC molecule. Chickens vaccinated with rFWPV/H5 and rFWPV/H5/IL-15 had lower ratio at Week 2, 0.89 ± 0.08 and 1.37 ± 0.17, respectively, in comparison to WT FP9. However, the ratios augmented over time, suggesting less CD8 cell populations or more CD4 cell populations were present in both vaccine groups than WT FP9. Importantly, rFWPV/H5 carrying IL-15 had the highest ratio between vaccinated groups at later weeks. This suggests that IL-15 co-expression in rFWPV either suppresses CD8 cell population or promotes CD4 cell population, in a delayed manner.

Table 4.5. Ratios of CD4+ to CD8+ T lymphocyte cells, CD4/CD8, post vaccination with different vaccines, at different weeks in two animal experiments.

Vaccine	Animal experiment			
	1		**2**	
	Week 2	Week 5	Week 2	Week 4
Control	1.83 ± 0.24	2.02 ± 0.50	1.26 ± 0.21	1.35 ± 0.24
WT FP9	1.48 ± 0.22	1.58 ± 0.14	1.19 ± 0.18	0.99 ± 0.14
rFWPV/H5	1.45 ± 0.15	1.72 ± 0.38	0.89 ± 0.08	1.07 ± 0.10
rFWPV/H5/IL-15	1.37 ± 0.17	1.81 ± 0.59	1.21 ± 0.12	1.44 ± 0.24

Pooled PBMC samples of nine chickens into three, n=3, were used in animal experiment 1, while individual PBMC samples of five chickens (*n*=5) in animal experiment 2. CD4+ and CD8+ cell population percentage was obtained from flow cytometry immunophenotyping analysis. Each value represents the ratios of the means ± SEM.

4.3 Discussion

Studies have shown that the most important component host immune reponse that confers protection against AIV is the humoral response against the haemagglutinin (HA) protein (Swayne *et al.*, 2008). To achieve this, several different types of vaccines have been developed with some implemented in the field. Although vaccines which are based on inactivated low pathogenic AIV of H5 subtypes have traditionally been used, a replicative virus-based vector expressing H5 offers several advantages to becoming a favourable vaccination strategy. The advantages include (i) the ability to differentiate between infected and vaccinated birds (DIVA) using a commercial assay, (ii) the lesser risk of accidental influenza virus release, (iii) the potency of providing bivalent protection against different pathogens (depending on the vector), and (iv) the avoidance of adjuvant, such as mineral oil, which is a component of an inactivated vaccine.

In this study, a safe, laboratory-adapted fowlpox virus (FWPV)-based vector expressing the HA of AIV strain H5N1 was modified to co-express a chicken IL-15 cytokine gene to test if it would in any way enhance the host CMI response, which is likely to be critical in clearance of AIV during primary infection. Since we did not perform any protective or challenge study, the evaluation of immunogenicity elicited by the constructed vaccines should also consider the host response against typical FWPV infection.

4.3.1 Humoral immune response

As shown in Table 4.2, inoculation of chickens with PBS or WT FP9 did not induce the production of antibody against the HA protein, a result which was expected and is comparable to results demonstrated by Taylor *et al.* and Webster *et al.* using different strains of FWPV (Taylor *et al.*, 1988; Webster *et al.*, 1991). Low but detectable levels of antibodies specific for H5 were observed as early as Week 2 in chickens vaccinated with rFWPV/H5 carrying IL-15. The vaccine also produced a higher antibody titre at Week 3, compared to rFWPV expressing H5 alone, which suggests that IL-15 could play a role in enhancing the host humoral immune response. This finding corresponds with several studies conducted in mice, including that by Perera *et al.* (2007) who observed a two-fold higher neutralizing antibody titre in human IL-15-integrated recombinant vaccines against Vaccinia virus (VV). The group also showed that recombinant VV strain Wyeth adjuvanted with human IL-15 and five influenza genes induced stronger neutralizing antibodies against AIV H5 (Poon *et al.*, 2009). Further studies should be done before we can assume that chicken IL-15 induces a co-stimulatory effect similar to that of human (Armitage *et al.*, 1995) or mouse (Gill *et al.*, 2009) IL-15 on B cell proliferation and differentiation, as well as IgA antibody synthesis (Hiroi *et al.*, 2000).

The mean HI titres induced by vaccination with either rFWPV/H5 or rFWPV/H5/IL-15 decreased after reaching the highest reading at Week 3, and no antibodies were detected from Week 5 onwards. The result might have been influenced by our use of a heterologous antigen (A/Duck/Malaysia/8443/2004 (H5N2)), sharing 91.1 % amino acid identity with the H5 in our recombinant vaccine in the HI assays. Taylor and co-workers

(Taylor *et al.*, 1988) reported that mean HI titres of less than 10 were observed in 6-week-old chickens vaccinated with rFWPV expressing the H5 from an H5N8 strain, when heterologous antigen (H5N2) was used in the HI test. When homologous antigen was used, a higher mean HI titre of 15 was observed. In another report, heterologous antigen with 83.5 % to 93.2 % amino acid identity to the studied HA gene, failed to produce consistent HI titres for 3-week-old chickens, post-vaccination with rFWPV expressing H5 of the H5N1 strain (Swayne *et al.*, 2007). However, in recent report, Bublot *et al.* showed the highest HI titres induced by a rFWPV expressing H5 of the H5N1 strain were detected with heterologous H5 from the H5N8 strain or from an H5N1 virus of a different clade (Bublot *et al.*, 2010). Phylogenetic relationships of the HA genes of nine representative influenza A viruses isolated in Asia in 2004, including that of the strain (Chicken/Malaysia/5858/2004) used as a source of HA for this study and the H5N2 strain (A/Duck/Malaysia/8443/2004) used as a heterologous antigen for the HI test, are shown in Figure 4.5.

Since the template HA used for the recombinant vaccines was derived from HPAI virus Chicken/Malaysia/5858/2004, high-level biosafety containment is needed to produce the antigen, which is a limitation in our study. The same is of course true of virus neutralisation assays used to measure antibodies capable of virus neutralization. Reverse-genetic technology could be used to incorporate the HA gene into an apathogenic virus background (for instance PR8, as used in the case of the RG16 pre-pandemic vaccine candidate produced at NIBSC, UK) but this has not yet been done. Although the most suitable antigen might not have been used for HI testing, the results provide useful comparisons of HI antibody levels elicited by rFWPV/H5 and rFWPV/H5/IL-15.

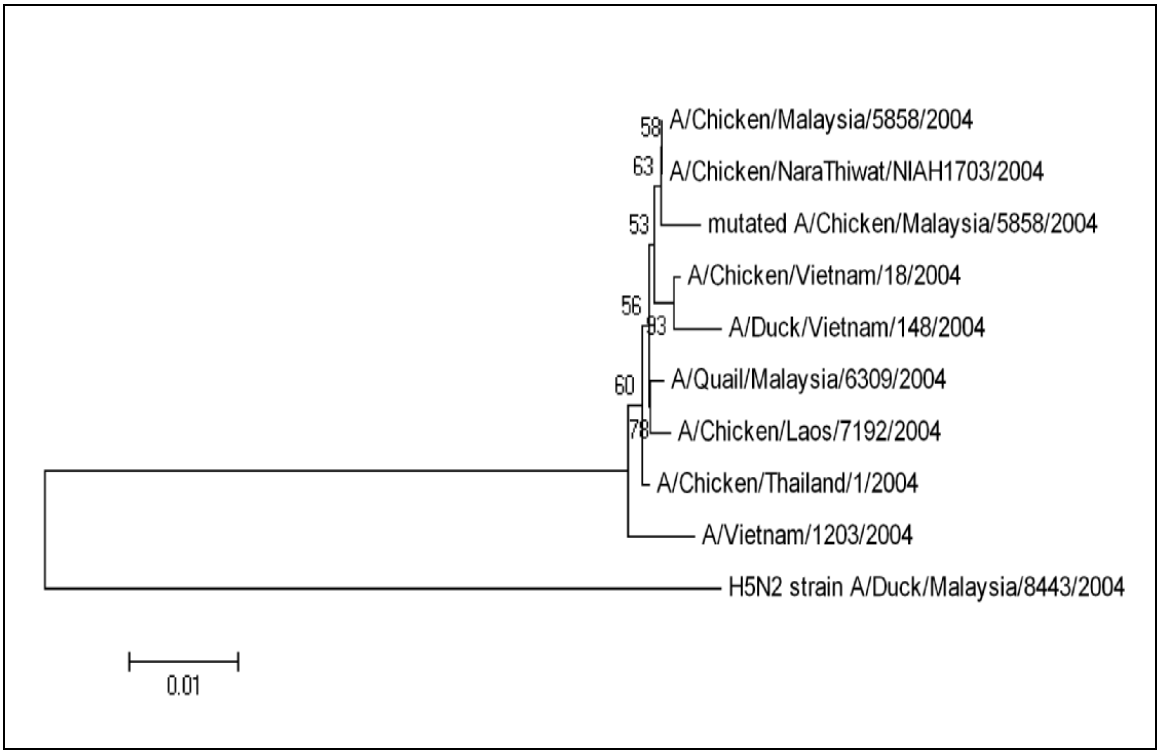


Figure 4.5. Phylogenetic relationships of the haemagglutinin (HA) genes of nine representative influenza A viruses isolated in Asia in 2004, including that of the strain (Chicken/Malaysia/5858/2004) used as a source of HA for this study. The mutated HA sequence of the study strain is also included. The tree was generated by the neighbour-joining method in MEGA 4.0 (Kumar and Nei, 1994) with bootstrap of 1000 replications. Numbers on the branches indicate neighbour-joining bootstrap values. Analysis was based on nucleotide sequences of the HA genes. Viruses are isolates from poultry during the H5N1 outbreak in 2004, with Vietnam/1203/2004, Malaysia/5858/2004 and Malaysia/6309/2004 belonging to sublineage VTM (Vietnam/Thailand/Malaysia), which is closely related to the Guangdong sublineage of viruses isolated from domestic and migratory birds in Hong Kong in early 2002 and late 2003 (Chen *et al.*, 2006). The H5N2/Malaysia/8443/2004 sequence has not been published elsewhere. The scale bar represents 0.01 nucleotide per site.

Several studies have shown rFWPV expressing HA can provide complete or nearly complete protection against lethal challenge, even when achieving pre-challenge HI titres of as low as 3 $\log_2$ (Beard *et al.*, 1991; Webster *et al.*, 1991; Qiao *et al.*, 2006; Bublot *et al.*, 2010). Though not directly comparable, rFWPV FP9 expressing the F gene of NDV were able to induce 86 % protection with HI titres of less than 2 (Boursnell *et al.*, 1990). These findings indicate that post-vaccination protection of chickens against AIV is not dependent entirely on HI antibodies but also on non-HI antibodies and possibly also on cellular mediated immunity. Although cellular immunity appears unable to protect chickens from infection with lethal virus, it may help in viral clearance before virus-induced pathology reaches a critical stage, thus reducing the rate of morbidity and mortality of immunized chickens. It may also play a more critical role in eradication of LPAI viruses from the individual bird and from the flock.

4.3.2 Cell-mediated immune response

4.3.2.1 CD4+ T cells

In comparison to its documented effects on CD8+ T cells (Oh *et al.*, 2003; Mueller *et al.*, 2005; Tang *et al.*, 2009), the effect of IL-15 expression on CD4+ T cells is not yet well established. Studies of phenotypic CD4+ T cells in mice suggest that IL-15 has little or no effect on naïve (Kanegane *et al.*, 1996; Zhang *et al.*, 1998; Marks-Konczalik *et al.*, 2000; Nishimura *et al.*, 2000; Picker *et al.*, 2006), memory (Tan *et al.*, 2002; Mueller *et al.*, 2005) and central memory (Picker *et al.*, 2006) CD4+ T cells. However, there are contradictory reports that IL-15 enhances *in vitro* priming of naïve CD4+ T cells (Niedbala *et al.*, 2002).

The flow cytometry results for Experiment 1 (Table 4.3, Figure 4.3(A)) demonstrated that rFWPV/H5/IL-15 did not increase CD4+ T cell population but results for Experiment 2 (Table 4.3, Figure 4.3(B)) showed a slight increase of the CD4+ T cell population at Week 2 (n=5) upon rFWPV/H5/IL-15 vaccination, although not statistically significant. These findings are consistent with previous reports that IL-15 only has profound effects on the proliferation and survival of memory CD8+ T cells, not on CD4+ T cells (Zhang *et al.*, 1998; Marks-Konczalik *et al.*, 2000; Nishimura *et al.*, 2000). It is not known whether antigenic or immunomodulatory proteins from FP9 can influence the IL-15 cytokine milieu in vaccinated chickens. Several reports showed that IL-15 can only activate CD4+ T cell proliferation when at high concentration (Kanegane *et al.*, 1996; Seder, 1996). Niedbala *et al.* (2002) showed that 2 to 4 fold higher concentrations of IL-15 are required to achieve optimal CD4+ T cell proliferation, compared to the concentrations required to promote CD8+ T cell response. However, overexpression of IL-15 has also been observed to lower the number of Herpes Simplex Virus-2 specific CD4+ T cells (Gill and Ashkar, 2009).

We observed no increase in CD4+ T cells in individual chickens vaccinated with WT FP9, a result similar to those described in other reports using chickens (Chen *et al.*, 2010a) and mice (Jin *et al.*, 2004). More importantly, although pooled blood samples from chickens vaccinated with rFWPV/H5 in Experiment 1 showed a significant increase of CD4+ T cell compared to the control, CD4+ T cells from individual samples showed no augmentation. This contradicts the basis of vaccination, where CD4+ T cells population should increase in response to foreign antigens, in this case haemagglutinin

H5 gene of the vaccine, since they are the basis of Th cells. Furthermore, CD4+ T cells of mice have been shown to be induced following influenza virus infection (Swain *et al.*, 2006), although they are not essential to "help" cytotoxic T lymphocyte activation (Ridge *et al.*, 1998). We speculate that individual chickens elicit different level of lymphoproliferative response towards rFWPV/H5. It should be noted that despite the extensive study on immune response against influenza in mammals, understanding of chicken T cell responses against influenza virus is very limited (Haghighi *et al.*, 2009).

4.3.2.2 CD8+ T cells

The co-stimulatory effects of IL-15 on CD8 cells have been studied widely, especially with regard to proliferation and survival of memory CD8+ T cells. IL-15 has been found to directly stimulate purified CD8+ memory cells *in vitro* (Zhang *et al.*, 1998; Ku *et al.*, 2000; Becker *et al.*, 2002; Oh *et al.*, 2004). Transgenic mice which constitutively expressed a significant level of IL-15 in the serum had higher numbers of memory CD8+ T cells (Nishimura *et al.*, 2000; Marks-Konczalik *et al.*, 2000), while a correspondingly inverse effect was observed in IL-15-deficient mice (Kennedy *et al.*, 2000). As presented in Table 4.4 and Figure 4.4, pooled and individual samples from chickens vaccinated with rFWPV/H5 co-expressing IL-15 showed an increase, although not significant, in CD8+ cell population in comparison to unvaccinated chickens. This result suggests chicken and mouse IL-15 share a similar capability of enhancing CD8+ T cells. The higher, significant increase of CD8+ T cells in rFWPV/H5 compared to rFWPV/H5/IL-15 raises two possible explanations:

(i) Over-expression of IL-15 in rFWPV/H5/IL-15 decreases the population percentage of CD8+ T cells specific for H5 antigen expressed by the recombinant vaccine. This possibility is compatible with the results of Yin *et al.*, who showed that low doses of plasmid encoding macaque IL-15, co-inoculated with an influenza DNA-based vaccine, enhanced CD4+ and CD8+ T cell population, but that high doses lead to a decrease in the production of both classes of T cells (Yin *et al.*, 2009). Unfortunately, in this study, we did not measure the levels of IL-15, secreted by cells infected with an initial dose of 10^5 PFU rFWPV/H5/IL-15, in peripheral blood prior to flow analysis. Since a strong synthetic/hybrid promoter was used for IL-15 co-expression, over-expression is possible, with consequential influence on the level of immune responses generated (Boyle and Heine, 1993).

(ii) FWPV infection, possibly by expression of as yet undefined immunomodulatory proteins encoded by this avipoxvirus, might down-regulate expression of chicken IL-15. Therefore, the heterologous co-expression of chicken IL-15 in cells infected with rFWPV/H5/IL-15 might tend to compensate for the down-regulation of levels of IL-15 seen after vaccination with rFWPV/H5, achieving a more optimal CD4+/CD8+ ratio. Although human IL-15 has been shown to stimulate CD8+ T cells population and promote the maintenance of CD8+CD44hi memory T cells, the responsiveness of CD8+ T cells to IL-15 might depends on the cytokine background (Niedbala *et al.*, 2002; Oh *et al.*, 2003).

A pattern of decreasing CD4+ and CD8+ T cell populations over time suggests that incorporation of the IL-15 gene in the rFWPV does not induce a sustained T cell response

in chickens. This is in agreement with an *in vivo* study examining T cell populations in the peripheral blood of rhesus macaques treated with rhesus IL-15, where the level of CD4+ and CD8+ memory, but not naïve, T cells peaked at Week 1 to 2 and returned to baseline by Weeks 3 to 4. In addition, extended treatment with IL-15 after day 10 often failed to maintain the peak lymphoproliferative response (Picker *et al.*, 2006).

4.3.2.3 CD4+/CD8+ T cell population ratio

In healthy humans, the ratio of CD4+ to CD8 + cells is between 0.9 and 1.9, which is equivalent to 1 to 2 CD4 cells for every CD8 cell. In HIV-infected individuals, the CD4+/CD8+ ratio is used as an indication of immune suppression, where a lower ratio represents higher immune suppression. In the context of very limited knowledge on chicken CD4+/CD8+ ratios upon FWPV infection, Chen *et al.* (2010) demonstrated an elevated CD4+/CD8+ ratio at Weeks 2 and 5 in chickens vaccinated with a parental Chinese FWPV (strain S-FPV-017), a recombinant S-FPV-017 expressing the S1 gene of IBDV, and recombinant S-FV-017/S1 carrying recombinant chicken IL-18, compared to an unvaccinated group, with fluctuations at Weeks 3 and 4. We also noted that the CD4+/CD8+ ratios of PBS-treated, White Leghorn SPF chickens from Experiment 2 were 1.26 ± 0.21 at Week 2 and 1.35 ± 0.24 at Week 5, which is consistent with observation by Chen *et al.* (1.26 ± 0.05 at Week 2; 1.33 ± 0.09 at Week 4) (Chen *et al.*, 2010a). Thus, we considered the results of Experiment 2 to be more reliable for data interpretation.

From our study (Table 4.5), we observed a lower CD4+/CD8+ ratio in birds vaccinated with FWPV carrying recombinant IL-15, compared to mock-vaccinated control. However, the ratio for both rFWPV/H5 and rFWPV/H5/IL-15 groups increases over time. Bridle *et al.* reported that commercially raised chickens with genetic selection, intense immunization and other environmental factors involved in commercial poultry operations have significantly altered T lymphocyte subpopulations, manifest as lower CD4+/CD8+ ratios, compared to unimmunized, laboratory-raised, outbred chickens (Bridle *et al.*, 2006).

CHAPTER 5

Co-immunostimulatory effect of IL-12 co-expressed in N1-recombinant fowlpox viruses on host immune responses

5.1 Introduction

Neuraminidase (NA) is enzymatically active to cleave α-2,3 or α-2,6-linked sialic acid residues from carbohydrate moieties on the surfaces of infected cells, promoting the release of budded virus particles from the cell membrane. In comparison to the extensive characterization of haemagglutinin (HA) antibodies to protect against influenza virus infection, the role of NA-specific antibodies as protective agents is less well understood. Several factors might influence the bias between the study of HA and NA as protective agents. Firstly, the levels of HA glycoprotein on the surface of infectious influenza virions are four (Webster and Pereira, 1968), six or even seven (Harris *et al.*, 2006) times higher than those of NA, with a consequently skewed humoral response towards HA after influenza virus infection. The higher level response against HA has been demonstrated in mice by Johansson and colleagues (1987), who showed that responses to HA and NA are competitive towards each other, with that against HA dominating in the priming of B and T cells (Johansson *et al.*, 1987a). Although the effect is less clear in birds and other mammals, susceptible species tend to respond with higher levels of serum antibodies against HA than NA. Secondly, the quantity of NA in licensed vaccines is not standardized (Gerentes *et al.*, 1999). This might be due to the lability of NA during storage, and exposure to various chemical treatments during its production, or the difficulty in measuring the level of NA in each vaccine (Tanimoto *et al.*, 2005).

Consequently, the response against HA has been viewed as a more important subject for study than that against NA, and has therefore been the subject of more intense scrutiny. However, it has been shown that humans exposed to H7N2 virus produced higher levels of antibodies against N2 than did those exposed to H3N2. This suggests that host immune responses against the NA protein of a vaccine can be influenced by existing humoral immunity against HA subtypes (Johansson *et al.*, 1987b).

Despite the overall paucity of studies on NA, vaccination with purified recombinant NA protein (Deroo *et al.*, 1996; Martinet *et al.*, 1997) or NA-encoding DNA (Chen *et al.*, 2000) has been demonstrated to protect mice against homologous, but not heterologous, lethal influenza virus challenge. Similar results were obtained using NA-expressing recombinant virus vaccines, including VV (Webster *et al.*, 1988), adenovirus (Guo *et al.*, 2006) and alphavirus replicon-based virus-like particles (Sylte *et al.*, 2007). A rFWPV co-expressing HA and NA of AIV has been shown to offer complete protection upon lethal homologous challenge in poultry (Qiao *et al.*, 2003). No NA-only rFWPV was included in the study, making it difficult to evaluate the specific role of NA-specific antibodies. However, it has been shown that induced antibodies against HA and NA confer different functions in defending against influenza. Unlike HA antibodies, which neutralize and block the interaction between viral HA and host receptors directly, NA antibodies still permit infection but limit viral replication and prevent the release of progeny virus from infected cells, thus reducing viral spread within the host and viral shedding into the environment (Kilbourne *et al.*, 1968; Deroo *et al.*, 1996).

The main aim of this chapter was to characterize the immunogenicity of two recombinant vaccines, rFWPV/N1 and rFWPV/N1/IL-12, which were constructed at Imperial College London, as described in Chapter 3. The *in vivo* studies were conducted at the Experimental House, Faculty of Veterinary Medicine, UPM, Malaysia, with parental, 'wild type' (WT) FWPV strain FP9 and PBS-mock inocula implemented as controls.

IL-12 was chosen as a co-stimulatory molecule due to its significant role in promoting cellular mediated immunity (CMI) responses. In humans and mice, IL-12 induces tyrosine phosphorylation of Janus kinase 2 and STAT4 selectively in Th1, but not Th2 cells (Szabo *et al.*, 1995; Rogge *et al.*, 1999). This is probably due to the inability of Th2 cells to express the β-subunit of the high-affinity IL-12 receptor, IL-12R, which is important for IL-12 signaling to activate STAT4 and induce the expression of IFN-γ in T lymphocytes or NK cells (Rogge *et al.*, 1999; Smits *et al.*, 2001). However, in a few contradictory reports, IL-12 was shown to bind directly to B cells and induce the expression of transcripts for β1 and β2-chains of the IL-12 receptor (Vogel *et al.*, 1996; Airoldi *et al.*, 2000) and induce production of Th2 IL-4 cytokine (Skok *et al.*, 1999). These stimulatory effects of IL-12 suggest its potential for use as an immunomodulator in vaccine development.

5.2 Results

5.2.1 Humoral immune responses following rFWPV/N1 or rFWPV/N1/IL-12 vaccination

A commercial competitive enzyme-linked immunosorbent assay (c-ELISA) kit, ID Screen Influenza N1 Antibody Competition, obtained from ID.Vet (France), was used to detect NA-specific antibodies in the sera of vaccinated chickens. Negative and positive controls were provided in the kit to validate the test.

To determine the competition percentage, the OD (optical density) value of each sample, which was read using a spectrophotometer at 450 nm, was used in the equation below:

$$\left(\frac{\text{OD of sample}}{\text{OD of negative control}} \right) \text{X } 100$$

N1 antibodies were considered to be present in the sample if the competition percentage of the sample was less than 60 %, and absent if the percentage was equal to or more than 60 %.

As expected, all sera from chickens inoculated with PBS (control) or WT FP9 had competitive percentages of more than 60 % for five weeks post-vaccination, which indicated the absence of N1-specific antibodies (Table 5.1). Chickens vaccinated with rFWPV/N1 and rFWPV/N1/IL-12 developed N1 antibodies after 28 days post vaccination, with competition percentages of 53.9 ± 1.9 % and 51.9 ± 1.2 %, respectively. The antibodies persisted until the following week, 54.3 ± 1.6 % for rFWPV/N1 and 55.6 ± 1.6 % for rFWPV/N1/IL-12.

Table 5.1. Mean competitive ELISA percentage, %, for N1 antibodies detected from chicken sera at interval weeks, post vaccination with different treatments.

Vaccine	Days, post inoculation				
	7	14	21	28	35
Control	63.4 ± 1.0	71.6 ± 1.8	70.5 ± 1.3	73.4 ± 2.5	74.6 ± 1.8
WT FP9	68.2 ± 2.2	74.1 ± 1.8	72.2 ± 1.2	94.4 ± 17.9	84.8 ± 4.1
rFWPV/N1	62.8 ± 1.1	70.2 ± 1.4	70.4 ± 2.1	53.9 ± 1.9	54.3 ± 1.6
rFWPV/N1/IL-12	64.4 ± 1.8	66.3 ± 1.1	77.2 ± 13.3	51.9 ± 1.2	55.6 ± 1.6

Each value represents the means ± SEM. Competitive percentages were calculated by dividing the sample OD with the negative control OD, and multiply by 100, where each OD value was read at 450 nm. N1 antibodies are considered present in the sera if the percentage is ≤60%, while absent if >60%.

5.2.2 Cell-mediated immune responses following rFWPV/N1 or rFWPV/N1/IL-12 vaccination

In order to understand the immunomodulatory effect of chicken IL-12 on T cell populations, T cell immunophenotyping was carried out and the results are presented in Figure 5.1. Circulating CD4+ and CD8+ T lymphocyte cells in rFPWPV/N1 and rFWPV/N1/IL-12-vaccinated chickens were enumerated using a triple-staining, flow cytometric counting analysis. To represent changes of the T cell populations, a fraction of 100 (percentage) was calculated using the equation below:

$$\left(\frac{\text{Final population percentage} - \text{initial population percentage}}{\text{Initial population percentage}} \right) \text{X } 100$$

In the equation, 'initial population percentage' refers to flow cytometry values of a particular T cell population at two weeks post-vaccination, while 'final population percentage' refers to values at Weeks 5 or 4, post-vaccination, for Experiments 1 or 2, respectively.

However, to represent differences of population percentage between similar T cell subset of a particular vaccine groups, at a particular week, 'initial population percentage' was referred to values from the control or WT FP9, while 'final population percentage' was referred to a particular vaccine group.

5.2.2.1 PBMC CD4+ T cell population

In the first animal experiment, pooled peripheral blood of chickens vaccinated with rFWPV/N1 or rFWPV/N1/IL-12, in which *n*=3, showed no significant increase in the level of CD4+ compared to the control group (Table 5.2, Figure 5.1 (A)). However, WT FP9 demonstrated a significant increase at Week 2 and 5 (described in Section 4.2.2.1). In term of changes in population level over time, CD4+ T cells of rFWPV/N1/IL-12 decreased from 16.36 ± 1.97 % to 12.41 ± 1.29 % (24.14 % or 3.95 % points), but this was not statistically significant. The CD4+ T cell population in the control (15.64 ± 1.75 % and 16.54 ± 3.33 %) and rFWPV/N1 (14.92 ± 1.06 % and 14.09 ± 0.82 %) groups was consistent at Week 2 and 5, with differences of only 5.75 % (0.9 % points) and 5.56 % (0.83 % points), respectively.

At Week 2, individual peripheral blood samples obtained from the second experiment, in which *n*=5, rFWPV/N1 and rFWPV/N1/IL-12 groups showed an increase of 20.4 % and 7.82 % in CD4+ T cell population, respectively, compared to the control. The levels of CD4+ T cells in rFWPV/N1 and rFWPV/N1/IL-12 groups showed a drastic decrease from 12.64 ± 0.55 % to 7.79 ± 1.19 % (38.37 % or 4.85 % points) and from 11.31 ± 0.90 % to 6.94 ± 2.57 % (38.64% or 4.37 % points) over time, respectively. However, the WT FP9 group showed a 9.96 % of increase, from 9.94 ± 1.17 % to 10.93 ± 0.89 % (Table 5.2, Figure 5.1 (B)).

Overall, co-expression of IL-12 in rFWPV/N1 does not influence persistence of total CD4+ T cells in PBMC. Increase of CD4+ T cell populations was observed in

rFWPV/N1 and rFWPV/N1/IL-12, although not at a significant level. The patterns of results for pooled and individual samples were divergent. However, the second experiment in which the WT FP9-vaccinated chickens showed levels of CD4+ T cells relatively comparable to the control, as previously reported by others (Jin *et al.*, 2004; Chen *et al.*, 2010a), may be more reliable.

Table 5.2. CD4+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/N1 or rFWPV/N1/IL-12.

Vaccine	Animal experiment			
	1 (*n*=3)		2 (*n*=5)	
	Week 2	Week 5	Week 2	Week 4
Control	15.64 ± 1.75	16.54 ± 3.33	10.49 ± 1.29	7.37 ± 0.91
Wild Type FP9	18.75 ± 2.53	20.81 ± 0.84	9.94 ± 1.17	10.93 ± 0.89
rFWPV/N1	14.92 ± 1.06	14.09 ± 0.82**	12.64 ± 0.55^	7.79 ± 1.19^
rFWPV/N1/IL-12	16.36 ± 1.97	12.41 ± 1.29**	11.31 ± 0.90	6.94 ± 2.57

Animal experiment 1 represents PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. Animal experiment 2 represents individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.

Each value represents the means ± SEM (error bars). Significant differences between vaccinated and WT FP9 groups, indicated by double asterisks (**), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a caret (^), were determined by paired-samples T-test ($P \leq 0.05$). No statistically significant differences observed between vaccinated and control groups.

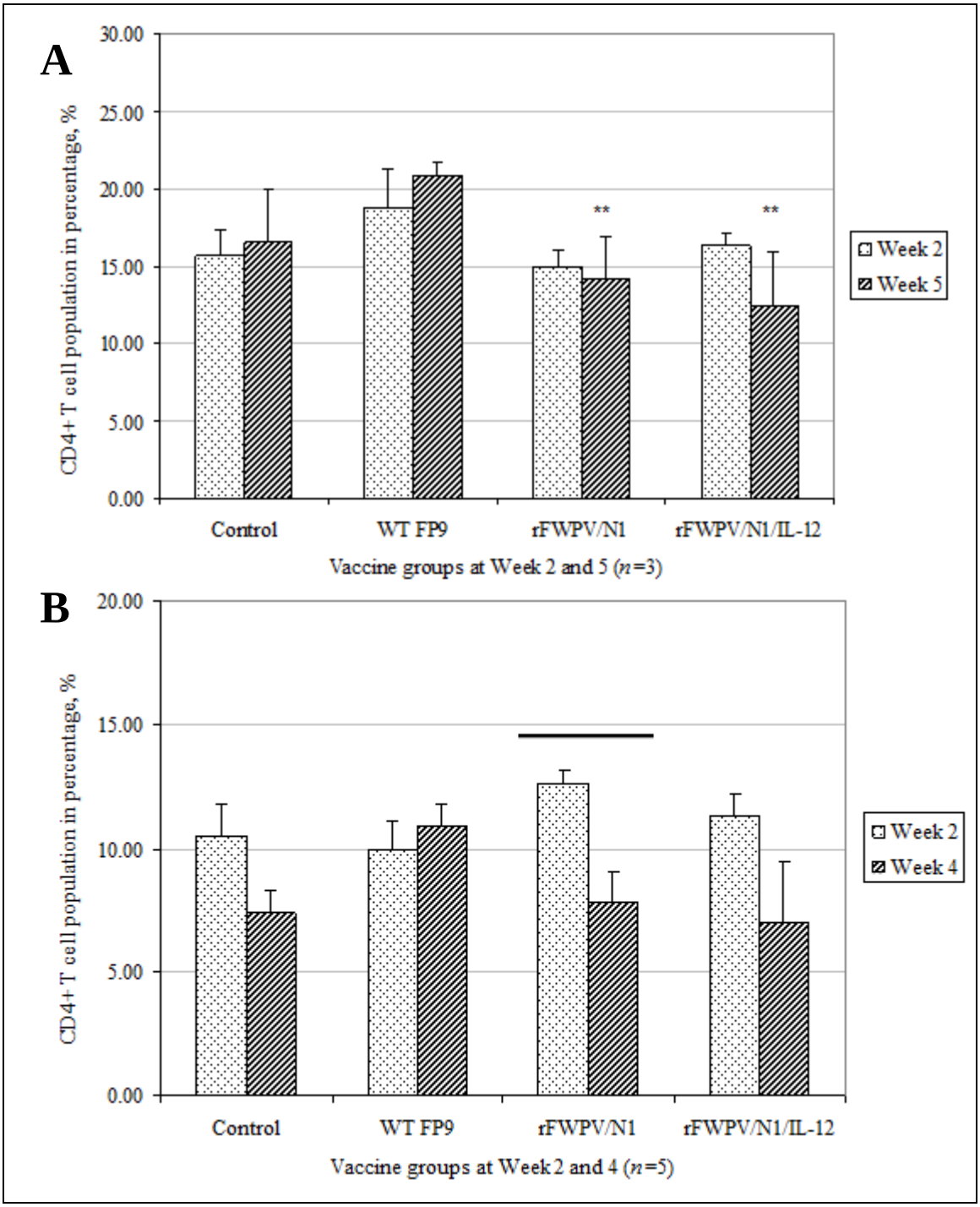


Figure 5.1. CD4+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/N1 or rFWPV/N1/IL-12. A) PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. B) Individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated and WT FP9 groups, indicated by double asterisks (**), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a horizontal line, were determined by paired-samples T-test ($P \leq 0.05$).

5.2.2.2 PBMC CD8+ T cell population

In the first animal experiment, in which *n*=3, the CD8+ T cell population increased only in the WT FP9-vaccinated group (as described in Section 4.2.2.2). These levels were higher than those of the control (8.54 ± 0.55 % and 8.17 ± 1.15 %), rFWPV/N1 (9.49 ± 1.93 % and 8.56 ± 1.43 %) and rFWPV/N1/IL-12 (9.91 ± 1.89 % and 8.73 ± 0.71 %) groups. No statistically significant increase was observed for rFWPV/N1 and rFWPV/N1/IL-12 groups relative to the control at either sampling point (Table 5.3, Figure 5.2 (A)).

A different pattern was observed for T-cell populations in individual samples (in which *n*=5) from animal experiment 2 (Table 5.3, Figure 5.2 (B)). At Week 2, the level of CD8+ T cells in the control (8.35 ± 0.99 %) and WT FP9 (8.36 ± 0.73 %)-vaccinated chickens was similar, making the results of this experiment more consistent than the first experiment with the results of others (Jin *et al.*, 2004; Chen *et al.*, 2010a). At the same time point, rFWPV/N1 and rFWPV/N1/IL-12 showed an increase of 37.96 % (or 3.17 % points) and 50.18 % (or 4.19 % points), respectively, compared to the control. However, those CD8+ T cell levels decreased by 41.84 % or 4.82 % points for rFWPV/N1 (from 11.52 ± 1.37 % to 6.7 ± 1.33 %), and by 38.76 % or 4.86 % points for rFWPV/N1/IL-12 (from 12.54 ± 1.79 to 7.68 ± 2.36 %), at the following sampling point. No significant difference in the CD8+ T cell population was observed between groups vaccinated with rFWPV/N1 or rFWPV/N1/IL-12 (Table 5.3, Figure 5.2 (B)).

In conclusion, population percentage of CD8+ T cells was increased in response to rFWPV/N1 and rFWPV/N1/IL-12. However, the raised levels did not persist until Week 4. Co-expression of IL-12 did induce a higher level of CD8+ T cells, but not statistically significant compared to rFWPV expressing N1 antigen alone, and only in experiment 2.

Table 5.3. CD8+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/N1 or rFWPV/N1/IL-12.

Vaccine	Animal experiment			
	1 (*n*=3)		2 (*n*=5)	
	Week 2	Week 5	Week 2	Week 4
Control	8.54 ± 0.55	8.17 ± 1.15	8.35 ± 0.99	5.46 ± 0.69
Wild Type FP9	12.71 ± 0.82*	13.28 ± 1.06*	8.36 ± 0.73	11.01 ± 1.21*
rFWPV/N1	1.49 ± 1.93	8.56 ± 1.43	11.52 ± 1.37^	6.70 ± 1.33^
rFWPV/N1/IL-12	9.91 ± 1.89	8.73 ± 0.71**	12.54 ± 1.79	7.68 ± 2.36

Animal experiment 1 represents PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. Animal experiment 2 represents individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.

Each value represents the means ± SEM (error bars). Significant differences between vaccinated and control groups, indicated by asterisks (*), or between vaccinated and WT FP9 groups, indicated by double asterisks (**), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a caret (^), were determined by paired-samples T-test ($P \leq 0.05$).

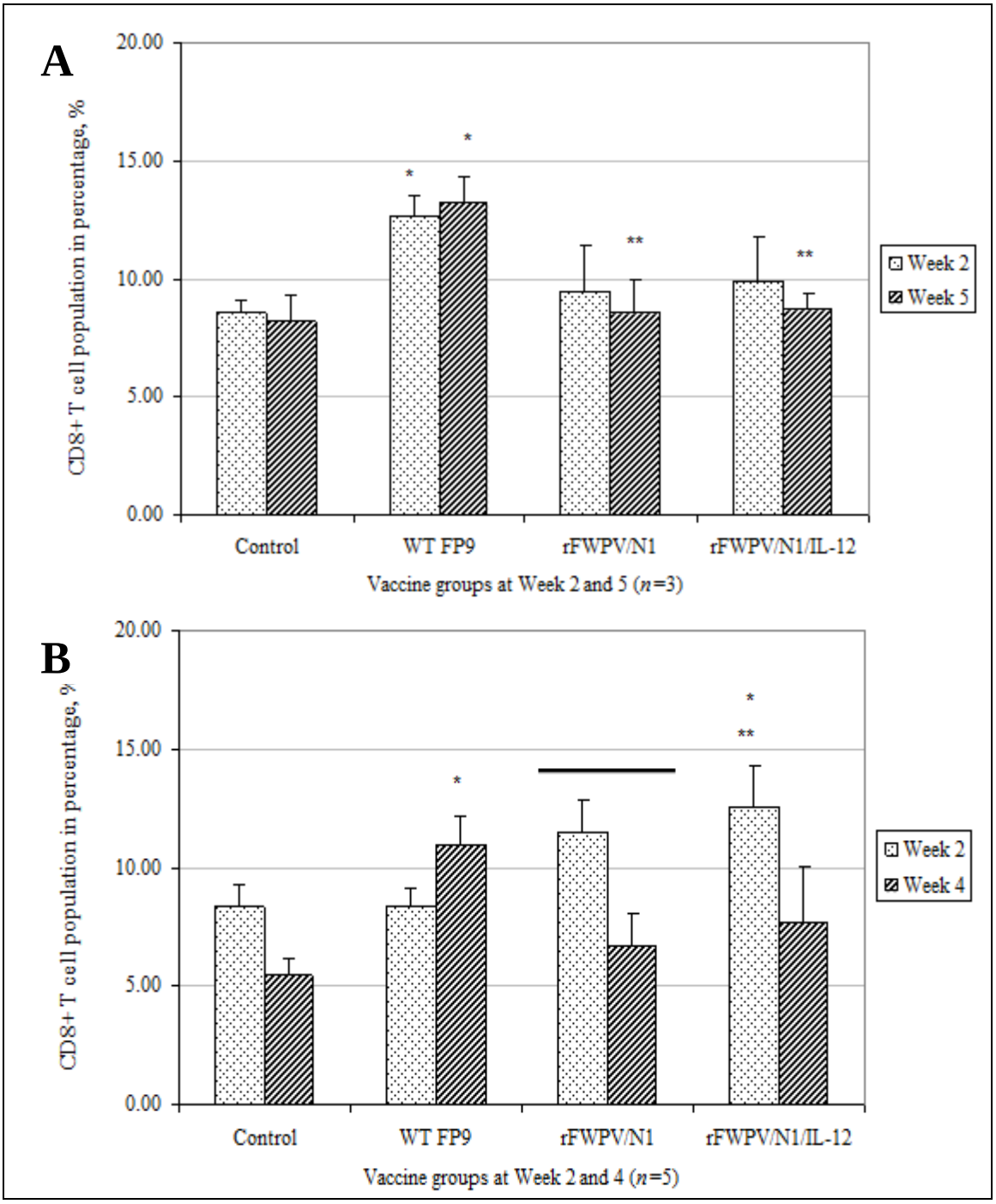


Figure 5.2. CD8+ T cell immunophenotyping of lymphocytes from chickens after mock-treatment with PBS (Control), or vaccination with WT FP9, rFWPV/N1 or rFWPV/N1/IL-12. A) PBMC samples of nine chickens pooled in threes (*n*=3), sampled at Weeks 2 and 5. B) Individual PBMC samples of five chickens (*n*=5), sampled at Weeks 2 and 4.
Each value represents the means ± SEM (error bars). Significant differences between vaccinated and control groups, indicated by asterisks (*), or between vaccinated and WT FP9 groups, indicated by double asterisks (**), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points, indicated by a horizontal line, were determined by paired-samples T-test ($P \leq 0.05$).

5.2.2.3 CD4+/CD8+ T cell population ratios

To study the relative flux of CD4+ and CD8+ T cell in a sample, ratios of CD4+ to CD8+ T cell percentages (CD4+/CD8+) were calculated (Table 5.4). A higher ratio represents a decrease in CD8+ or an increase in CD4+ T lymphocyte cells in peripheral blood samples.

According to the results obtained, the control group had the highest ratio of CD4+ to CD8+ in both experiments at all sampling points; Week 2 (1.83 ± 0.24 and 1.26 ± 0.21), Week 4 (1.35 ± 0.24) and Week 5 (2.02 ± 0.50). In the first experiment, in which *n*=3, the CD4+/CD8+ ratio increased in the WT FP9 (from 1.48 ± 0.22 at Week 2 to 1.58 ± 0.14 at Week 5) and rFWPV/N1 (from 1.57 ± 0.34 to 1.71 ± 0.29) groups, while that in the rFWPV/N1/IL-12 group decreased from 1.65 ± 0.37 to 1.42 ± 0.19. However, in the second experiment (in which *n*=5), both groups showed a decrease; from 1.19 ± 0.18 to 0.99 ± 0.14 for WT FP9, and from 1.20 ± 0.14 to 1.16 ± 0.29 for rFWPV/N1. The CD4+/CD8+ ratio of the rFWV/N1/IL-12 group showed a constant CD4+/CD8+ ratio in the following experiment (0.9 ± 0.15 and 0.9 ± 0.43, respectively).

Table 5.4. Ratios of CD4+ to CD8+ T lymphocyte cells, CD4/CD8, post vaccination with different vaccines, at different sample points in two animal experiments.

Vaccine	Animal experiment			
	1 (*n*=3)		2 (*n*=5)	
	Week 2	**Week 5**	**Week 2**	**Week 4**
Control	1.83 ± 0.24	2.02 ± 0.50	1.26 ± 0.21	1.35 ± 0.24
WT FP9	1.48 ± 0.22	1.58 ± 0.14	1.19 ± 0.18	0.99 ± 0.14
rFWPV/N1	1.57 ± 0.34	1.71 ± 0.29	1.20 ± 0.14	1.16 ± 0.29
rFWPV/N1/IL-12	1.65 ± 0.37	1.42 ± 0.19	0.90 ± 0.15	0.90 ± 0.43

PBMC samples from nine chickens, pooled in threes (giving *n*=3), were used in animal experiment 1, while individual PBMC samples from five chickens (*n*=5) were used in animal experiment 2. CD4+ and CD8+ cell population percentages were obtained by flow cytometry immunophenotyping analysis. Each value represents the ratio of the means ± SEM.

5.3 Discussion

NA inhibitors have been developed as antiviral drugs and have been used widely in humans, to combat circulating influenza virus. They are not used in poultry because of their cost, the speed with which resistant viruses would then develop and because antibiotic residues in meat and eggs are undesirable.

In poultry, NA normally performs as an additional component alongside HA in whole, killed or inactivated AIV vaccines, since HA-mediated immunity is more effective than NA-mediated immunity for AIV protection. Consequently, potential infection-permissive immunity (i.e. NA antibodies permit infection of influenza viruses, but block the release of infectious virions from the apical surface of the infected cells) upon administration of NA as a primary component is less exploited. In this study, the immunogenicity of rFWPV co-expressing NA N1 and chicken IL-12 vaccines was evaluated by assessing the antibody and cellular response of the host. Since no protective or challenge study has yet been performed, it was only possible to evaluate host humoral and cellular responses after FWPV inoculation.

5.3.1 The humoral immune response

As expected, control or WT FP9-vaccinated chickens did not induce antibodies to NA antigen throughout the experiment (Table 5.1). Chickens inoculated with rFWPV/N1 or rFWPV/N1/IL-12 developed N1 antibodies 4 weeks post-vaccination. This response is somewhat late for a typical host antibody response induced by rFWPV against a foreign antigen. As a comparison, HA antibodies can be detected as early as one week, after

vaccination with rFWPV co-expressing H5 and N1 genes (Qiao *et al.*, 2003). There are several possible explanations for this result. Firstly, the rFWPV might not have expressed N1 at levels sufficient to stimulate antibody response in the first few weeks post inoculation. Secondly, circulating antibodies against N1 during the initial weeks might have been present, but below detection limits for the c-ELISA. Liu *et al.* has demonstrated that Neuraminidase Inhibition (NI) is a more sensitive assay for NA detection, probably due to the different substrate systems (Liu *et al.*, 2010). However, unlike c-ELISA, which is rapid and effective for high numbers of serum samples, the NI assay is laborious and requires propagation and handling of infectious virus, with consequential biosafety implications. Thirdly, the result might be specific to NA, as a unique feature of this particular antigen, though possibly in combination with the particular (FWPV) expression system. It may be that there are unusual aspects to the presentation of NA by APC to induce MHC Class II restricted CD4+ T cells that account for this observation, particularly in the context of the largely uncharacterised immunomodulation that is undoubtedly induced by the FWPV vector.

The same expression vector (pEFL29) and promoter (VV 7.5 early/late) combination has been used successfully for a range of diverse proteins, including the H5 protein considered in the previous chapter. In this study, the scale and kinetics of N1 expression in vaccinated birds was not addressed but it has been shown that in skin sections from the inoculation site of birds vaccinated with rFWPV FP9 expressing an IBDV VP2/lacZ fusion protein, B cells started to aggregate in germinal centre (GC)-like structures at 8 days post vaccination onwards (Eldaghayes, 2005). GC are sites for the growth and

differentiation of B cells into immunoglobulin-producing plasma cells, and are thus important for the humoral immune response. That particular system was also poor at inducing VP2-specific antibodies compared to antibodies against parental FP9, with none detectable even 31 days post vaccination (Shaw and Davison, 2000) but this is viewed as a consequence of the artificial nature of the expression of the capsid protein of this icosahedral virus as a fusion with the large β-galactosidase protein (M. A. Skinner, personal communication). Other expression systems, particularly those expressing VP2 in the context of virus-like particles, have successfully induced high levels of VP2-specific antibodies (Fernandez-Arias, 1998).

It is possible that the host B cell receptors were biased against N1 towards immunodominant FWPV antigens (such as fpv140, fpv168 and fpv191; Boulanger *et al.*, 1998) in the first few weeks post-vaccination. As such effects have not been observed with other antigens, it is possible that it is a unique feature of the expression of NA in rFWPV that led to the observed delay in host seroconversion. The same observation or other NA expression has not been reported elsewhere. Although anti-β-galactosidase responses could have been evaluated, clear demonstration of this effect would ideally have required the inclusion of another, similar antigen in the rFWPV, to serve as a control for the timing of seroconversion.

5.3.2 Cellular immune response

IL-12 has been demonstrated to induce priming for high production of IFN-γ in both CD4+ and CD8+ T cell clones (Manetti *et al.*, 1995; Paganin *et al.*, 1995). However, the cytokine has a short life-span (5 to 6 hours) in blood circulation, thus leading to a dramatic decrease of IFN-γ levels after peak release (Lui *et al.*, 2002). However, it was conceivable that co-expression of chicken IL-12 from rFWPV/N1 might initiate and maintain circulating IFN-γ concentration for a longer duration. This was assessed by measuring the preponderance of predominantly IFN-γ producers, namely CD4+ and CD8+ T cells.

5.3.2.1 CD4+ T cells

For Experiment 1, flow cytometry results showed that neither rFWPV/N1 nor rFWPV/N1/IL-12 increased CD4+ T cell population percentage (Table 5.2, Figure 5.1A). For Experiment 2, a slight, although not statistically significant, increase in the CD4+ T cell population was observed at Week 2 (n=5) upon rFWPV/N1 or rFWPV/N1/IL-12 vaccination (Figure 5.1B). This result suggests that the presence of NA antigen alone might be able to increase CD4+ T cell population. In fact, *de novo* synthesis of NA is not required to enhance T cell proliferation (Oh and Eichelberger, 1999). Several studies have demonstrated that sialic acid cleavage by NA contributes to distortion of charge at dendritic, T lymphocyte or B cell surface, thus enhance the avidity of APC for T cells, T cell responses and proliferation (Oh and Eichelberger, 1999). It was later demonstrated that sialidase from *Clostrodium perfringens* (Garcia *et al.*, 2005) and *Salmonella typhimurium* (Berger *et al.*, 2006) improves CD4+ T cell function in both young and old

mice by cleaving α2,3- and/or α2,6-linked terminal sialic acid residues of specific proteins that regulate interaction between T cell receptor and APC. However, the effect of the cleavage on CD4+ T cell proliferation was not addressed in either report. Their findings suggested that desialylation might be a useful strategy to enhance immunogenicity of a vaccine.

We observed similar levels of percentage of CD4+ T cells in rFWPV/N1 and rFWPV/N1/IL-12, indicating that IL-12 co-expression did not influence CD4+ T cell populations significantly, at least not in PBMC samples at 2 or 4 weeks post-vaccination. A pattern of decreasing CD4+ T cell populations over time suggests that incorporation of the IL-12 gene in rFWPV does not lead to sustained T cell response in chickens. This is consistent with recent *in vivo* findings, where IFN-γ appears rapidly in circulation on day 3 post-vaccination with rFWPV expressing IBDV VP2 plus rFWPV expressing recombinant chicken IL-12, but decreased to the basal level 14 and 28 days later (Su *et al.*, 2011). Upon lethal IBDV challenge at day 28 post-vaccination, 83 to 100 % protection was observed in these dually-vaccinated chickens, in comparison to 17 % and 50 % protection in chickens vaccinated only with rFWPV expressing VP2 (Su *et al.*, 2011). Their finding suggests that the level of protection is increased upon IL-12 administration, even though only a low IFN-γ level was detected prior to challenge.

5.3.2.2 CD8+ T cells

Although no significant, augmentation of CD8+ T cell level was observed for rFWPV/N1 in Experiment 1, the vaccine led to an increase, although not statistically significant, in the CD8+ T cell population in Experiment 2. NA-specific CD8+ T cell-mediated responses upon influenza virus infection have been described (Oh *et al.*, 2001) but that is in a very different context. The proliferations of the self-regulating CD8+ T cells, which are not dependent on CD4+ T cell activation, indicate that influenza virus alters the capacity of antigen-presenting cells (APC) to stimulate T cell proliferation (Oh and Eichelberger, 1999) though it is not clear how large a specific role NA might play in this mechanism.

Increasing proliferation levels or nonspecific expansion of CD8+ T cells after IL-12 treatment have been reported extensively, including in *in vitro* studies using Myeloid-derived suppressor cells (Steding *et al.*, 2011) and Lewis lung carcinoma cells (Yin *et al.*, 2011) from mice, describing the pivotal role of IL-12 in CMI responses. *In vivo* studies using recombinant VV expressing IL-12-infected mice showed that reduction of VV titres correlate with the increased number of specific CD8+ T cells seen after expression of high levels of IL-12 (Gherardi *et al.*, 1999; Gherardi *et al.*, 2003). Our flow cytometry results for Experiment 1 (Figure 5.2A) demonstrated that rFWPV/N1/IL-12 did not increase CD8+ T cell percentage but results for Experiment 2 (Figure 5.2B) showed a significant increase of the CD8+ T cell population at Week 2 (n=5) after rFWPV/N1/IL-12 vaccination. The significant differences in population levels between rFWPV/N1/IL-12 and control or WT FP9 indicates that co-expression of chicken IL-12 induced high

levels of circulating CD8+ T cells present even at 2 weeks post vaccination. Two weeks later, we observed that the level had dropped to levels comparable to the control. It is possible that this was due to over-expression of IL-12 from the strong synthetic/hybrid promoter. High levels of recombinant chicken IL-12 have been shown to elicit lower levels of IBDV-specific IFN-γ production (Su *et al.*, 2011).

A slight increase over time in the CD8+ T cell population percentage in birds vaccinated with WT FP9 was noted, contradicting the observations with rFWPV/N1 and rFWPV/N1/IL-12. There are two possible explanations for this apparent discrepancy:

(i) WT FP9 encodes undefined immunomodulatory proteins, which can induce persistent CD8+ T cell proliferation, although not markedly high. This is not surprising since FWPV strain FP9 has been shown to be more immunogenic in eliciting CD8+ T cell responses in mice against the circumsporozoite protein of a liver-stage, *Plasmodium berghei* malaria, than the commercially available Webster's FWPV vaccine strain (Anderson *et al.*, 2004; Cottingham *et al.*, 2006).

(ii) Expression of NA N1 from rFWPV triggers, by an unknown mechanism, interaction between immune cells, leading to the reduction of the CD8+ T cell population to the basal state. It has been reported that pre-existing CD8+ T cell responses against viral epitopes in a boosting agent can inhibit the boosting of the CD8+ T cell response against the recombinant antigen (Anderson *et al.*, 2004). It is not known whether the immediate CD8+ T cell responses against N1 after vaccination suppress further increase of of CD8+ T cells.

5.3.2.3 CD4+/CD8+ T cell population ratio

As reported in the previous chapter, the ratio of CD4+ to CD8+ cells was shown to provide a general comparison on immune fluctuation after vaccination (Table 5.3). Since there is no standard range of CD4+/CD8+ ratio for SPF White Leghorn chickens in any literature, ratios of control group served as benchmarks. We considered the results of Experiment 2 to be more reliable for data interpretation (Section 4.3.2.3). In comparison to the control, we observed a lower level of CD4+/CD8+ ratio in WT FP9, rFWPV/N1 and rFWPV/N1/IL-12-vaccinated birds at both sampling points. Interestingly, the CD4+/CD8+ ratio of rFWPV/N1/IL-12-vaccinated birds was indistinguishable at Week 2 and Week 4 of the second animal experiment, indicating that IL-12 might be a potent CMI-regulator for the flux of both CD4+ and CD8+ T cells in circulating blood.

CHAPTER 6

Effect on host body weight of chicken IL-15 or IL-12 coexpressed from recombinant fowlpox viruses

6.1 Introduction

In the intensive and massive global poultry industry, vaccination is the most commonly applied strategy to control infectious diseases. Recombinant fowlpox virus (rFWPV) has been identified as a safe, important vaccine vector for numerous infectious pathogens of poultry, including avian influenza virus (e.g. Bublot *et al.*, 2006), Newcastle disease virus, NDV (e.g. Sun *et al.*, 2006), Marek's disease virus (e.g. Lee *et al.*, 2003), Infectious bursal disease virus, IBDV (e.g. Su *et al.*, 2011) and Infectious bronchitis virus (Chen *et al.*, 2010a). Despite the efficacy, vaccination with rFWPV *in ovo* or in young chickens can slow the rate of growth and induce weight loss (Springer and Truman, 1981; Karaca *et al.*, 1998; Mingxiao *et al.*, 2006). This condition might not give a significant impact on small flocks. However, in the intensive commercial industry, a minor weight loss or retarded growth of large numbers of broiler chickens can influence the meat production greatly.

Co-expression of chicken Type I IFN (Karaca *et al.*, 1998), IFN-γ (Wang *et al.*, 2009) or IL-18 (Mingxiao *et al.*, 2006; Chen *et al.*, 2010b) from rFWPV expressing various antigens has been shown to normalize the negative effect of FWPV vaccination on body weight. In our study, an animal experiment was carried out, described in Chapter 2.4.2.1, to demonstrate the effect of IL-15 or IL-12 co-expression from rFWPV vaccines, on

chicken body weight. Briefly, fifty-four 1-day-old chicks were randomly assigned into six groups. Each group of nine chicks was inoculated with rFWPV constructed previously, namely; rFWPV/H5, rFWPV/H5/IL-15, rFWPV/N1 or rFWPV/N1/IL-12. Another two groups were inoculated with WT FP9 or PBS, as controls. Weights of the chickens were measured in one-week intervals, for four weeks.

In order to represent the differences in body weight, the percentage change was calculated using the equation below:

$$\left(\frac{\text{Weight of a particular vaccine group} - \text{Weight of PBS-treated control}}{\text{Weight of PBS-treated control}} \right) \text{ X } 100$$

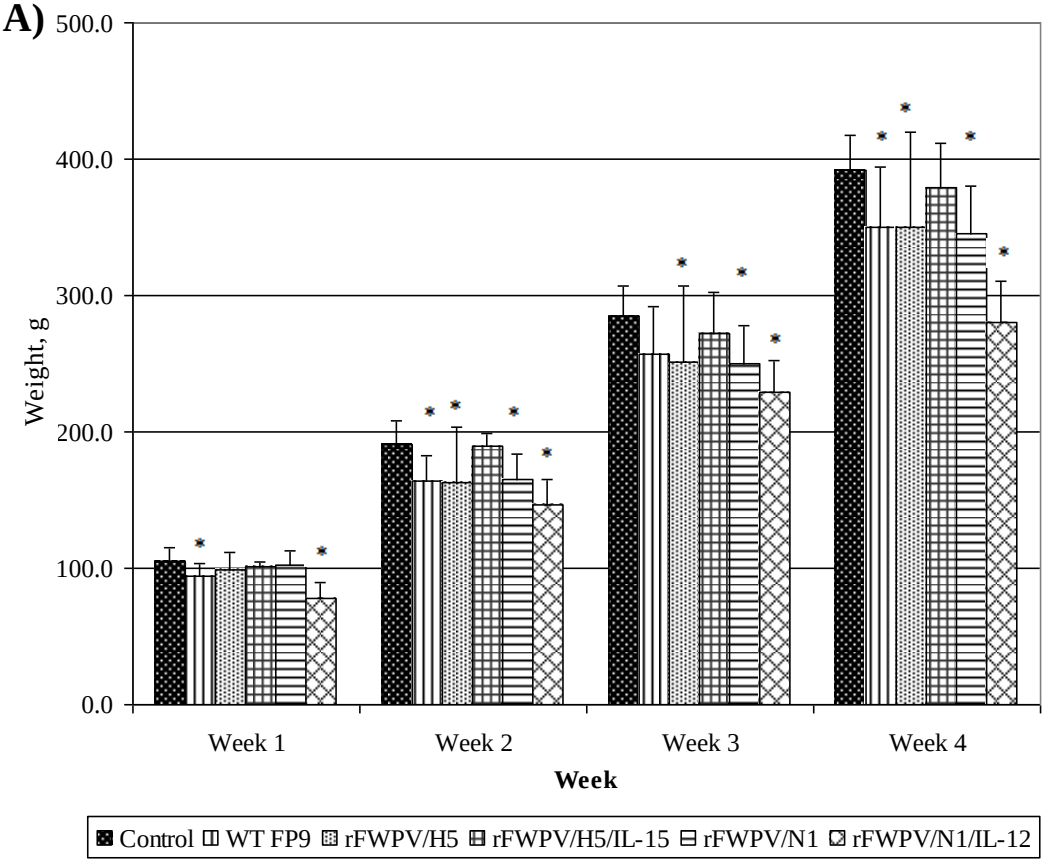
6.2 Results

To measure body weights, all chickens were weighed at Week 1 until Week 4, post-vaccination. The chickens were left until Week 7 to distinguish their sexes (Table 6.1). As shown in Figure 6.2, body weights of chickens in WT FP9 (93.9 ± 9.9 g), rFWPV/H5 (98.9 ± 12.7 g), rFWPV/H5/IL-15 (101.1 ± 3.5 g) and rFWPV/N1 (102.2 ± 10.9 g) decreased 10.6 %, 5.8 %, 3.7 % and 2.7 %, respectively, compared to control chickens (105.0 ± 10.0 g), with rFWPV/N1/IL-12 having the highest weight loss of 25.9 % or 27.2 g (77.8 ± 11.5 g), after one week post-vaccination. At Week 2, the relative weight reduction was irreversible for WT FP9 (164.2 ± 18.4 g), rFWPV/H5 (162.2 ± 41.6 g), rFWPV/N1 (165.1 ± 19.0 g) and rFWPV/N1/IL-12 (146.3 ± 19.0 g). However, rFWPV/H5/IL-15 (189.7 ± 9.5 g) showed a weight similar to the control (190.9 ± 16.9 g), with difference of only 0.6 % or 1.2 g. This weight pattern was consistent for all groups until Week 4, with WT FP9, rFWPV/H5, rFWPV/N1, rFWPV/N1/IL-12 having a statistically significant weight loss compared to the control ($P \leq 0.05$). The body weight of chickens vaccinated with rFWPV/H5/IL-15 was higher compared to other vaccine groups, indicating a potential effect of IL-15 in reversing the rFWPV side effect of weight loss. The weight gain was gender independent, as the control group had two more male chickens than the rFWPV/H5/IL-15 group.

Table 6.1. Sex of nine experimental chickens, determined at Week 7 post immunization.

Vaccine	Males	Females	Total
Mock-treated (Control)	6	3	9
WT FP9	5	4	9
rFWPV/H5	5	4	9
rFWPV/H5/IL-15	4	5	9
rFWPV/N1	5	4	9
rFWPV/N1/IL-12	3	6	9

A)



B)

Vaccine	Mean body weight in grams at different weeks, post-vaccination			
	Week 1	Week 2	Week 3	Week 4
Control	105.0 ± 10.0	190.9 ± 16.9	285.0 ± 21.9	392.1 ± 25.0
WT FP9	93.9 ± 9.9*	164.2 ± 18.4*	256.8 ± 35.3	350.0 ± 44.5*
rFWPV/H5	98.9 ± 12.7	162.2 ± 41.6*	251.1 ± 56.3*	350.1 ± 70.2*
rFWPV/H5/IL-15	101.1 ± 3.5	189.7 ± 9.5	272.1 ± 30.0	378.6 ± 32.7
rFWPV/N1	102.2 ± 10.9	165.1 ± 19.0*	249.6 ± 28.4*	345.3 ± 35.0*
rFWPV/N1/IL-12	77.8 ± 11.5*	146.3 ± 19.1*	228.7 ± 24.0*	280.4 ± 30.3*

Figure 6.1. Effect of WT FP9 or rFWPV inoculation of 1-day-old chicks on mean body weight (grams) at Weeks 1, 2, 3 and 4. Data were presented in a bar graph (A) or table (B). Each value represents the means ± SD (error bars) of nine samples (n=9). Significant differences between control and vaccinated groups were determined by one-way ANOVA ($P \leq 0.05$) and indicated by an asterisk (*).

6.3 Discussion

6.3.1 Effect of IL-15 co-expression from rFWPV on body weight of chickens

IL-15 is among the most abundant cytokines in skeletal muscle. In humans and other mammals, IL-15 has been shown to regulate the fat-to-lean pathway by several means, including; (i) stimulating protein synthesis and inhibiting proteolysis of skeletal muscle (Quinn *et al.*, 2002; Busquets *et al.*, 2005), (ii) increasing glucose uptake into skeletal muscle (Busquets *et al.*, 2006), (iii) stimulating lipid oxidation of skeletal muscle (Almendro *et al.*, 2006) and (iv) reducing fat composition in adipocytes (Alvarez *et al.*, 2002). In one study, lean human subjects showed higher levels of muscle IL-15 mRNA expression and circulating/plasma IL-15, compared to obese subjects. However, muscle IL-15 protein level of obese subjects was increased, suggesting that the role of IL-15 in regulating obesity involves processes downstream of transcription (Nielsen *et al.*, 2008). The involvement of avian IL-15 in fat decomposition and muscle anabolism has not been described.

From our experiment, the growth of chickens immunized with rFWPV/H5/IL-15 was similar to that of the control group, indirectly suggesting IL-15 can ameliorate the weight loss effect of rFWPV vaccines. Similar observations were reported after *in ovo* (Ding *et al.*, 2004; Lillehoj *et al.*, 2005) and intramuscular leg injection (Ma *et al.*, 2011) of DNA vaccines coexpressing chicken IL-15 with the 3-1E gene of *Eimeria acervulina*, a parasite which can also cause weight loss. It is clear that the host responses to rFWPV expressing AIV H5 and to the DNA vaccine expressing the parasite *E. acervulina* 3-1E

gene are likely to be very different. However, both experiments indicate that IL-15 can reverse the weight-suppressive effect of pathogens of chickens.

Similar results have also been shown in mice. Despite the effect of recombinant IL-15 protein treatment in significantly reducing the body weight of IL-15-deficient mice (Barra *et al.*, 2010), the same treatment reversed the suppressive effect of Coxsackievirus B3 on body weight (Bigalke *et al.*, 2009). However, in macaques infected with Simian Immunodeficiency Virus, which does not induce weight loss, no weight gain was observed upon treatment of IL-15 protein, despite an increase in effector memory CD8+ T cells and NK cells (Mueller *et al.*, 2005).

It is interesting to note that co-administration of chicken IL-18 (expressed from a different plasmid DNA vector) with a DNA vaccine expressing the 3-1E gene of *E. acervulina* introduces weight loss upon *E. acervulina* infection, relative to PBS-vaccinated group (Lillehoj *et al.*, 2005), though this contradicts its observed effect of inducing weight gain in chickens vaccinated with rFWPV expressing AIV H5 (Ding *et al.*, 2004). It appears that, compared to IL-18, IL-15 plays a more consistent role in sparing disease-related weight loss in chickens. It is of course possible that IL-15 reduced weight loss by inhibiting FWPV replication. The replication of FWPV, which would have required sampling at the site of inoculation as FP9 does not spread, was not monitored but the induction of HI titres observed by rFWPV/H5/IL-15 (Section 4.2.1) argues against this explanation.

6.3.2 Effect of IL-12 co-expression from rFWPV on body weight of chickens

It has been reported that IL-12-deficient mice have normal size and weight, despite having defective IFN-γ production and type 1 cytokine responses (Magram *et al*., 1996). However, weight loss of more than 10 % in uninfected, with more than 20 % in lymphocytic choriomeningitis virus-infected, IL-12-deficient mice, was observed upon high-dose administration of recombinant IL-12, following 6 days of daily treatment (Orange *et al.*, 1995). This finding suggests that high concentrations of IL-12 might increase host stress, and severity of disease, as indicated by weight loss.

In this study, exacerbated weight loss was observed as early as one week post-inoculation with rFWPV/N1/IL-12. The weight loss was not accompanied by loss of appetite, suggesting the suppressive effect of IL-12 co-expression from rFWPV/N1/IL-12 on body weight of chickens did not involve restricted food consumption. *In vivo* studies in mice have suggested that administration of high dose IL-12 can increase the level of TNF-α (Orange *et al.*, 1994; Orange *et al.*, 1995; Ciftci *et al.*, 2010), a cytokine that can regulate physiological and pathological changes, and can promote weight loss or anorexia (Langhans and Hrupka, 1999). Unfortunately, the levels of TNF-α were not not measured during the course of the experiment, so it is not possible to conclude whether or not the weight loss was TNF-α-dependent. There is also a possibility that the effect of IL-12 in our study involved either FWPV or AIV antigen-specific interaction. In the latter case, we did not managed to construct rFWPV co-expressing H5 gene of AIV and IL-12, which can serve as a comparison to rFWPV/N1/IL-12. Hence, more studies involving

measurement of viral load, viral antigens, host antibodies and specific cytokine productions are needed to support such theory.

CHAPTER 7

General Discussion and Conclusions

7.1 Poultry vaccination against avian influenza virus

The poultry sector has been a steady-growing industry in most parts of the world developed and developing, due to an increasing global population and purchasing power. In addition, poultry products are highly demanded due to their relatively low pricing compared to other meat, absence of religious barriers and nutritional (protein) qualities (Magdelaine *et al.*, 2008). Moreover poultry production is viewed as more environmentally sustainable than alternative sources of animal protein. The trend is reciprocal to a constant production of healthy chickens, thus indicating the need to control the occurrence and circulation of infectious diseases of poultry. Although several disease pathogens only cause mild distress, and consequent reduction in production levels, several other important pathogens including avian influenza virus (AIV), Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV), can lead to severe illness with high mortality rate. In commercial production of poultry, the risk of rapid spread of infectious diseases is increased due to: (i) the centralisation of chick production, (ii) the close-rearing environment of large numbers of the birds and (iii) the frequent requirement for migratory labour to handle large numbers of poultry. The most common preventive tool in controlling the occurence of infectious disease, especially viral, is vaccination, normally implemented in the first few weeks of the animal's life to provide lifelong protection (though for the modern broiler this need only be as little as 6 to 7 weeks). However, depending on the nature of the particular vaccine, they can possess

several disadvantages, such as: slow stimulation of host immunity, weak cross protection against diverse field viruses or subtypes, difficulty in large scale production in a short period of time, variable antigen quantity, requirement for adjuvant, and difficult to differentiate between infected or vaccinated birds in routine serological tests. New vaccination strategies, including recombinant viral vectors, are designed to minimise these weaknesses thus improving overall vaccine efficacy.

In this study, we constructed recombinant fowlpox virus (rFWPV) vaccines co-expressing the H5 or N1 antigens of AIV with chicken cytokines, IL-15 or IL-12. AIV is an important pathogen for poultry and still enzootic in certain parts of the world, particularly in Asia. Of the greatest concern is the highly pathogenic avian influenza A (H5N1), which is species-adapted and may be evolving inconspicuously to start a new panzootic. The vaccines were constructed to allow laboratory-based studies to provide more knowledge on chicken immune responses upon infection of recombinant vaccines against AIV. The cytokines were chosen due to their profound effect on Th1 responses, which are critical for host cellular-mediated immune (CMI) response for viral clearance and defence against intracellular pathogens (AIV). Expression of AIV antigens proteins, HA or NA, in the recombinant vaccines normally triggers host Th2 responses. Hence, we hypothesized that our recombinant vaccines might also be able to induce strong cellular responses for efficient virus clearance associated with reduced virus shedding in vaccinated, challenged chickens.

7.2 Restrictions of chicken experiment

In comparison to mammals, less is known about modulation of the avian immune system in response to physiological processes or infections. In this study, we tried to provide more perspectives on chicken immune system upon recombinant viral infection. Despite some advantages, notably the direct relevance of the avian pathogen antigens, conducting animal experiments in chickens rather than mammals actually poses several major challenges. Firstly, more effort is needed to rear chickens compared to smaller mammalians, such as mice or ferrets. Mammalian model studies are performed in a smaller environment, allowing are easier care and management. Unlike chickens, those small animals require less effort for sanitizing, feeding and blood sampling purposes. Secondly, the availability of avian research reagents, including antibodies to avian cell surface markers and cytokine proteins, is limited. Despite an increasing number of reagents and a growing profile of avian cytokines, the accessibility still lags far behind.

To negate some of these issues and to study whether the rFWPV vaccines could elicit good immune responses at an early stage of life, comparable to the gold standard rFWPV vaccines developed by Merial (Bublot *et al.*, 2007), the vaccines were inoculated into one-day-old chicks. This practice allowed the study of larger numbers of birds, while limiting the total volume of whole blood and sera that could be sampled from them.

To determine the blood volume to be sampled from each chicken, we considered the chicken body weight, frequency (once a week) and period (seven weeks) over which the blood will be collected. In this study, we sampled 0.2 mL blood at Week 1 and 2, 0.3 mL

at Week 3 and 4, 0.5 mL at Week 5 and 1 mL at Week 6 and 7. Extra blood volume was sampled for flow cytometry analysis (0.2 mL at Week 2 and Week 5 for Experiment 1; 0.5 mL at Week 2 and 4 for Experiment 2). This etiquette was advised by veterinarians and approved by the local IACUC. The general guide allows a maximum of 10 % of the total blood volume for weekly-repeat bleeds, in which ten percent of chicken body weight (g) is estimated to represent the total blood volume (mL) of an individual chicken (Dr Mustapha Abu Bakar and Dr Kartini Ahmad, personal communication). Frequent samplings of a large volume of blood may allow a short-term hypovolaemic shock or in a longer term, anaemia (British Veterinary Association (BVA), 1993).

7.2.1 Limitations and suggestions for improved evaluation of cellular mediated immune responses

For the assessment of CMI responses, T lymphocyte cell subsets were measured in peripheral blood mononuclear cells (PBMC). Other than lymphocytes (T cells, B cells and NK cells), PBMC of whole blood extracted using ficoll contains monocytes. Therefore, in our flow cytometry results, we do not know the absolute number of PBMC per volume of blood, and thus it is impossible to deduce accurate estimates of cell proliferation in response to vaccination For example, variation in the number of monocytes would change the apparent % of T cells, even if the absolute number of T cells had not changed. To improve the assay, cell counting should be done in addition to antibody staining, either by using an automated analyzer or manually. To increase the assay specificity, antigen-specific responses could be measured by staining for intracellular cytokine such as IFN-γ after in vitro stimulation of PBMC with appropriate

antigen. However, availability of commercialised anti-chicken cytokine markers is the major limitation compared to those of mammals.

An alternative approach of not using PBMC would have been to measure the magnitude of T lymphocyte cell populations from organs such as the spleen, the largest secondary lymphoid organ. However, this approach would have restricted the experiment to single time-point data, requiring higher numbers of birds.

7.3 Experimental design

In this study, four recombinant vaccines, rFWPV/H5, rFWPV/H5/IL-15, rFWPV/N1 and rFWPV/N1/IL-12 were characterized. It had originally been the intention to isolate more recombinants for comparative study, namely rFWPV/H5/IL-12, rFWPV/N1/IL-15, rFWPV/NP/IL-15 and rFWPV/NP/IL-12 but it proved impossible to isolate resolved and stable recombinant clones within the limited time available for rFWPV construction in the UK (Chapter 3). It had also been the intention to challenge chickens vaccinated with homologous HPAIV. Unfortunately delays to the construction and licensure of appropriate facilities in Malaysia, together with the scarcity of and high demand upon such facilities elsewhere, means that this objective has had to be deferred. Therefore, the study did not evaluate the protective efficacy of the vaccines against lethal viral challenge, but only their immunogenicity and general effects upon vaccinated hosts. Those vaccines which induced stronger humoral and cellular-mediated responses will be subjected to protection/challenge studies when the facilities eventually become available.

7.4 Characterization of rFWPV/H5 and rFWPV/H5/IL-15

The results showed that inoculation of rFWPV/H5/IL-15 did not induce any adverse effect in the host. In fact, co-expression of IL-15 induced gain in body weight relative to birds vaccinated with rFWPV not expressing IL-15, indicating its ameliorating effect on the suppressive effect of rFWPV on body weight. rFWPV/H5/IL-15 showed a stronger humoral response, compared to rFWPV/H5, indicated by a higher HI titre at peak week (Week 3). The vaccine induced higher, although not statistically significant, CD4+ T cells and similar CD8+ T cells level compared to rFWPV/H5. This suggests that the level of IL-15 co-expressed from rFWPV/H5/IL-15 was sufficient to enhance CD4+ T cell population (Kanegane *et al.*, 1996; Seder, 1996; Niedbala *et al.*, 2002). CD4+ T cell proliferation is a multifaceted process, which relies on activation of recognition and verification signals. These signals are critical in recognizing extracellular, soluble materials that are released from infected cells and ensuring that a T cell is responding to that foreign antigen. Once the signals are activated, proliferation of CD4+ T cells is allowed through a further complex process involving IL-2 (also called T cell growth factor) and expression of IL-2 receptor. Since Lillehoj *et al.* showed that chicken IL-15 and chicken IL-2 share similar functional characteristic in increasing the level of T cells (Lillehoj *et al.*, 2001), we showed indirectly that the chicken IL-15 co-expressed from rFWPV/H5/IL-15 was biologically active, indicated by its preferential increase of CD4+ T cells compared to rFWPV/H5. Interestingly, no significant difference in the level of CD8+ T cells of rFWPV/H5 and rFWPV/H5/IL-15 was observed. It is possible that the host cytotoxic response is H5 antigen-dependent and IL-15-independent. Alternatively

over-expression of IL-15 might have been at such levels that CD8+ responses were not stimulated (Niedbala *et al.*, 2002; Oh *et al.*, 2003).

Collectively, rFWPV/H5/IL-15 showed enhanced immunogenicity and might prove to be a valuable adjunct to recombinant vaccines against AIV. However, further study should be done to evaluate its effect on the protective efficacy of the vaccines against lethal challenge infection.

7.5 Characterization of rFWPV/N1 and rFWPV/N1/IL-12

Neuraminidase (NA) is less-well recognized as a potentially protective antigen in AIV vaccine candidates. Considering the ability of NA antibodies to limit viral replication and reduce the release of progeny virus from infected cells, we constructed rFWPV/N1 and rFWPV/N1/IL-12 and evaluated their levels of immunogenicity. Humoral responses induced by each vaccine appeared four weeks after vaccination, suggesting a late induction of immunity, which is a disadvantage in vaccine development. It is likely that the delayed induction of immunity was attributable to the expression of the neuraminidase itself. CD4+ T cell population percentage in rFWPV/N1 and rFWPV/N1/IL-12-vaccinated groups was indistinguishable, whereas the CD8+ T cell population was higher in the rFWPV/N1/IL-12 group, suggesting a predominant effect of IL-12 in modulating cytotoxic T cell activity against foreign antigens.

Despite the enhanced CD8+ T cell response, rFWPV/N1/IL-12 would not be desirable as a vaccine candidate due to the unforeseen, suppressive effect on chicken body weight.

Until further tests are done on the levels of TNF-α, thymic athrophy or corticosterone (Orange *et al.*, 1995; Matsushita *et al.*, 1999) concurrently with weighing, the loss of weight and possible toxicities, which could be triggered by IL-12, could not be explained mechanistically. In conclusion, the study showed that IL-12 co-expression from a recombinant poultry vaccine would require careful consideration due to the detrimental effect on body weight, despite possible enhancements of host CMI responses.

7.6 Future experiments

The animal experiments conducted have allowed evaluation of immunogenicity of the vaccines pre-challenge. Further assays would be necessary to provide a broader perspective, particularly to the cellular responses in chickens post-vaccination. Harvested organs from the 7-week-old experimental chickens have been reserved for real-time quantitative reverse transcription-PCR assay. The assay will allow quantification of cytokine targets, including IFN-α, IFN-γ, IL-1β, IL-2, IL-10, IL-12, IL-15, IL-18 (Th1 or Th1-related cytokines) and TGF-β4, IL-4 (Th2 cytokines) (Hong *et al.*, 2006; Park *et al.*, 2008). A transcription factor, named lipopolysaccharide-induced TNF-α factor (LITAF) can be used for a direct indication of TNF-α levels in the host (Hong *et al.*, 2006), which may be related to body weight (Orange *et al.*, 1995; Tanaka *et al.*, 2001). An alternative experimental design for this assay would be to quantitate the mRNA levels in specific organs every week, post vaccination, although it will not reflect responses of the same, individual chicken.

Due to constraints of time and containment facilities, it was not possible to conduct a protective/challenge study using live, homologous H5N1 virus strain A/Chicken/Malaysia/5858/2004. This final stage will allow measurements of viral replication, viral shedding and mortality/survival rate, thus indicating whether the enhanced humoral and CMI responses, which were elicited by the vaccines, can confer protection against lethal infection.

7.7 Concluding remarks

In summary, this study showed diverse immunogenicity of H5N1-rFWPV co-expressing IL-12 or IL-15, with rFWPV/H5/IL-15 being a better vaccine candidate compared to rFWPV/N1/IL-12. It also demonstrates a weight sparing effect of co-expressing IL-15 in rFWPV vaccines. The results provide the basis for future homologous challenge studies, using live H5N1 virus to evaluate the protective efficacy of the rFWPV vaccines.

Appendix 1

Primers used in this study. Restriction enzyme sites are shown in bold.

Primer	Description	Sequence (5 ’- 3’)
H5-F	+*EcoRV* site	ATCG**GATATC**ATGGAGAAAATAGTGC
H5-R	+*EcoRV* site	GACT**GATATC**TTAAATGCAAATTCTGC
N1-F	+*Ssp* site	ACCG**AATATT**ATGAATCCAAATAAGAAG
N1-R	+*Ssp* site	AGGC**AATATT**CTACTTGTCAATGGTG
NP-F	+*EcoRV* site	ACT**GATATC**ATGGCGTCTCAAGG
NP-R	+*EcoRV* site	ACGT**GATATC**TCAATTGTCATATTCCTC
S(2-F)	Mutagenic primer	CAAAGAGAGACAAGAGGATTATTTGGAGCTATAG
S(1-R)	Mutagenic primer	CAAATAATCCTCTTGTCTCTCTTTGAGGGCTATTTC
H5-F1	For screening	ATGGAGAAAATAGTGCTTCTTTTTG
H5-R1	For screening	TTAAATGCAAATTCTGCATTGTAACG
N1-F1	For screening	ATGAATCCAAATAAGAAGATAATAACCATCG
N1-R1	For screening	CTACTTGTCAATGGTGAATGGCAACT
NP-F1	For screening	ATGGCGTCTCAAGGCACCA
NP-R1	For screening	TCAATTGTCATATTCCTCTGCATTG
pEFL29-F	For screening	CGGAGACCATATCCATACGC
pEFL29-R	For screening	CGTAAAAGTAGAAAATATATTC
pEFGPT12S-F	For screening	AGTAAGAGAACCGGGAGCG
pEFGPT12S-R	For screening	ACCCACATGATAAGAGATTGTATC
pPC1.X-F	For screening	ATGAAAAATAGTACCACTATGG
pPC1.X-R	For screening	ATCCGATACTAGTATTAGGTTAGC
IL15-F	For screening	ATGCTGGGGATGGCACAGCC
IL15-R	For screening	ACAGAGTTTTGTAAAGGTTATACAGAGG
IL12-F	For screening	ATGTCTCACCTGCTATTTGCC
IL12-R	For screening	ACCACCCTTGGCTCCTTCCAGG

Appendix 2

Media Constituents

Media I – 2 % Newborn bovine serum (NBBS) in DMEM

Component	Volume
1 bottle DMEM (Sigma)	500 mL
Newborn bovine serum (NBBS) (Autogen Bioclear)	10 mL
Nystatin (Sigma)	5 mL
Pen-Strep	500 uL
Tryptose Phosphate Broth (TPB)	5 mL

Media II – 10 % Newborn bovine serum (NBBS) in DMEM

Component	Volume
1 bottle DMEM (Sigma)	500 mL
Newborn bovine serum (NBBS) (Autogen Bioclear)	10 mL
Nystatin (Sigma)	5 mL
Pen-Strep	500 uL
Tryptose Phosphate Broth (TPB)	5 mL

Media III – For agarose overlay

2 % Newborn bovine serum (NBBS) in 2X MEM

Component	Volume
1 bottle 2X MEM (Sigma)	250 mL
Newborn bovine serum (NBBS) (Autogen Bioclear)	10 mL
Nystatin (Sigma)	5 mL
Pen-Strep	500 uL

Media III was mixed with 2 % Low Gelling Agarose, LGA (diluted in distilled water), to make the agarose medium.

Appendix III

Data of CD4+ and CD8+ T cell population (*n*=3 for Experiment 1, *n*=5 for Experiment 2)

Experiment 1			
Vaccine	Number of Chickens	CD4+ T cell population, %	
		Week 2	Week 5
Control	1	12.26	10.14
	2	16.51	21.36
	3	18.14	18.11
Wild Type FP9	1	22.80	19.17
	2	14.10	21.31
	3	19.35	21.96
rFWPV/H5	1	21.74	17.80
	2	25.60	10.77
	3	23.66	19.71
rFWPV/H5/IL-15	1	15.74	24.14
	2	13.97	12.33
	3	13.43	16.16

Experiment 1			
Vaccine	Number of Chickens	CD8+ T cell population, %	
		Week 2	Week 5
Control	1	7.56	5.93
	2	9.47	9.75
	3	8.58	8.84
Wild Type FP9	1	14.12	13.74
	2	11.29	14.84
	3	12.71	11.27
rFWPV/H5	1	14.12	11.21
	2	19.35	6.82
	3	15.38	10.08
rFWPV/H5/IL-15	1	12.53	13.08
	2	8.46	11.16
	3	10.47	4.77

Experiment 1			
Vaccine	Number of Chickens	CD4+ T cell population, %	
		Week 2	Week 5
Control	1	12.26	10.14
	2	16.51	21.36
	3	18.14	18.11
Wild Type FP9	1	22.80	19.17
	2	14.10	21.31
	3	19.35	21.96
rFWPV/N1	1	16.50	15.64
	2	15.37	12.86
	3	12.90	13.76
rFWPV/N1/IL-12	1	12.57	13.58
	2	19.20	9.84
	3	17.30	13.82

Experiment 1			
Vaccine	Number of Chickens	CD8+ T cell population, %	
		Week 2	Week 5
Control	1	7.56	5.93
	2	9.47	9.75
	3	8.58	8.84
Wild Type FP9	1	14.12	13.74
	2	11.29	14.84
	3	12.71	11.27
rFWPV/N1	1	13.16	11.19
	2	8.66	8.23
	3	6.64	6.26
rFWPV/N1/IL-12	1	8.52	8.63
	2	7.56	7.54
	3	13.64	10.01

Experiment 2			
Vaccine	Number of Chickens	CD4+ T cell population, %	
		Week 2	Week 4
Control	1	13.73	5.54
	2	7.96	10.16
	3	7.03	8.57
	4	11.33	5.43
	5	12.40	7.15
Wild Type FP9	1	7.90	11.34
	2	8.06	7.71
	3	14.25	12.73
	4	8.98	12.29
	5	10.49	10.58
rFWPV/H5	1	13.65	9.49
	2	9.83	10.90
	3	9.38	10.12
	4	9.50	10.75
	5	9.48	6.99
rFWPV/H5/IL-15	1	13.33	7.42
	2	11.78	7.51
	3	15.57	8.74
	4	14.80	7.80
	5	9.77	10.73

Experiment 2			
Vaccine	Number of Chickens	CD8+ T cell population, %	
		Week 2	Week 4
Control	1	10.40	4.43
	2	6.30	5.20
	3	11.05	4.60
	4	6.78	5.23
	5	7.20	7.83
Wild Type FP9	1	7.10	14.62
	2	7.14	9.46
	3	9.05	10.51
	4	10.94	7.75
	5	7.59	12.72
rFWPV/H5	1	11.81	9.87
	2	13.84	9.08
	3	11.88	7.17
	4	10.45	10.02
	5	10.54	8.96
rFWPV/H5/IL-15	1	11.47	4.59
	2	10.88	8.88
	3	12.84	4.75
	4	9.01	4.30
	5	9.75	6.74

Experiment 2			
Vaccine	Number of Chickens	CD4+ T cell population, %	
		Week 2	Week 4
Control	1	13.73	5.54
	2	7.96	10.16
	3	7.03	8.57
	4	11.33	5.43
	5	12.40	7.15
Wild Type FP9	1	7.90	11.34
	2	8.06	7.71
	3	14.25	12.73
	4	8.98	12.29
	5	10.49	10.58
rFWPV/N1	1	12.40	12.45
	2	14.20	6.81
	3	11.06	6.68
	4	12.09	5.79
	5	13.46	7.23
rFWPV/N1/IL-12	1	13.79	6.39
	2	12.81	3.72
	3	11.22	3.65
	4	9.18	3.92
	5	9.55	17.01

Experiment 2			
Vaccine	Number of Chickens	CD8+ T cell population, %	
		Week 2	Week 4
Control	1	10.40	4.43
	2	6.30	5.20
	3	11.05	4.60
	4	6.78	5.23
	5	7.20	7.83
Wild Type FP9	1	7.10	14.62
	2	7.14	9.46
	3	9.05	10.51
	4	10.94	7.75
	5	7.59	12.72
rFWPV/N1	1	14.08	11.46
	2	10.13	7.76
	3	9.92	5.08
	4	15.36	4.66
	5	8.12	4.55
rFWPV/N1/IL-12	1	12.08	3.82
	2	15.87	3.23
	3	17.37	4.49
	4	8.46	13.90
	5	8.92	12.98

BIBLIOGRAPHY

Ada, G. L., and P. D. Jones. 1986. The immune response to influenza infection. Curr Top Microbiol Immunol **128:**1-54.

Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, G. F. Kutish, and D. L. Rock. 2000. The genome of fowlpox virus. J Virol **74:**3815-31.

Airoldi, I., G. Gri, J. D. Marshall, A. Corcione, P. Facchetti, R. Guglielmino, G. Trinchieri, and V. Pistoia. 2000. Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. J Immunol **165:**6880-8.

Alcami, A., and G. L. Smith. 1995. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. J Virol **69:**4633-9.

Almendro, V., N. Carbo, S. Busquets, J. Lopez-Soriano, M. Figueras, E. Ametller, J. M. Argiles, and F. J. Lopez-Soriano. 2005. Interleukin-15 decreases lipid intestinal absorption. Int J Mol Med **15:**963-7.

Alvarez, B., N. Carbo, J. Lopez-Soriano, R. H. Drivdahl, S. Busquets, F. J. Lopez-Soriano, J. M. Argiles, and L. S. Quinn. 2002. Effects of interleukin-15 (IL-15) on adipose tissue mass in rodent obesity models: evidence for direct IL-15 action on adipose tissue. Biochim Biophys Acta **1570:**33-7.

Anderson, R. J., C. M. Hannan, S. C. Gilbert, S. M. Laidlaw, E. G. Sheu, S. Korten, R. Sinden, G. A. Butcher, M. A. Skinner, and A. V. Hill. 2004. Enhanced CD8+ T cell immune responses and protection elicited against Plasmodium berghei malaria by prime boost immunization regimens using a novel attenuated fowlpox virus. J Immunol **172:**3094-100.

Armitage, R. J., B. M. Macduff, J. Eisenman, R. Paxton, and K. H. Grabstein. 1995. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. J Immunol **154:**483-90.

Arulanandam, B. P., V. H. Van Cleave, and D. W. Metzger. 1999. IL-12 is a potent neonatal vaccine adjuvant. Eur J Immunol **29:**256-64.

Balu, S., and P. Kaiser. 2003. Avian interleukin-12beta (p40): cloning and characterization of the cDNA and gene. J Interferon Cytokine Res **23:**699-707.

Banadyga, L., J. Gerig, T. Stewart, and M. Barry. 2007. Fowlpox virus encodes a Bcl-2 homologue that protects cells from apoptotic death through interaction with the proapoptotic protein Bak. J Virol **81:**11032-45.

Banks, J., E. S. Speidel, E. Moore, L. Plowright, A. Piccirillo, I. Capua, P. Cordioli, A. Fioretti, and D. J. Alexander. 2000. Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol **146:**963-73.

Barber, M. R., J. R. Aldridge, Jr., R. G. Webster, and K. E. Magor. 2010. Association of RIG-I with innate immunity of ducks to influenza. Proc Natl Acad Sci U S A **107:**5913-8.

Baron, S. D., R. Singh, and D. W. Metzger. 2007. Inactivated Francisella tularensis live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. Infect Immun **75:**2152-62.

Barra, N. G., S. Reid, R. MacKenzie, G. Werstuck, B. L. Trigatti, C. Richards, A. C. Holloway, and A. A. Ashkar. 2010. Interleukin-15 contributes to the regulation of murine adipose tissue and human adipocytes. Obesity (Silver Spring) **18:**1601-7.

Beard, C. W., W. M. Schnitzlein, and D. N. Tripathy. 1991. Protection of chickens against highly pathogenic avian influenza virus (H5N2) by recombinant fowlpox viruses. Avian Dis **35:**356-9.

Becker, T. C., E. J. Wherry, D. Boone, K. Murali-Krishna, R. Antia, A. Ma, and R. Ahmed. 2002. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. J Exp Med **195:**1541-8.

Bender, B. S., T. Croghan, L. Zhang, and P. A. Small, Jr. 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J Exp Med **175:**1143-5.

Berger, S. B., A. A. Sadighi Akha, R. A. Miller, and G. G. Garcia. 2006. CD43-independent augmentation of mouse T-cell function by glycoprotein cleaving enzymes. Immunology **119:**178-86.

Betakova, T. 2007. M2 protein-a proton channel of influenza A virus. Curr Pharm Des **13:**3231-5.

Bigalke, B., P. L. Schwimmbeck, C. S. Haas, and S. Lindemann. 2009. Effect of interleukin-15 on the course of myocarditis in Coxsackievirus B3-infected BALB/c mice. Can J Cardiol **25:**e248-54.

Bodewes, R., Osterhaus, A. D., and Rimmelzwaan, G. F. 2010. Targets for the induction of protective immunity against influenza A viruses. Viruses **2(1):**166-188.

Bolte, A. L., J. Meurer and E. F. Kaleta. 1999. Avian host spectrum of avipoxviruses. Avian Pathology **28:**415-432.

Boltz, D. A., B. Douangngeun, S. Sinthasak, P. Phommachanh, S. Rolston, H. Chen, Y. Guan, J. S. Peiris, J. G. Smith, and R. G. Webster. 2006. H5N1 influenza viruses in Lao People's Democratic Republic. Emerg Infect Dis **12:**1593-5.

Boulanger, D., P. Green, T. Smith, C. P. Czerny, and M. A. Skinner. 1998. The 131-amino-acid repeat region of the essential 39-kilodalton core protein of fowlpox virus FP9, equivalent to vaccinia virus A4L protein, is nonessential and highly immunogenic. J Virol **72:**170-9.

Bouloy, M., C. Janzen, P. Vialat, H. Khun, J. Pavlovic, M. Huerre, and O. Haller. 2001. Genetic evidence for an interferon-antagonistic function of rift valley fever virus nonstructural protein NSs. J Virol **75:**1371-7.

Boursnell, M. E., P. F. Green, A. C. Samson, J. I. Campbell, A. Deuter, R. W. Peters, N. S. Millar, P. T. Emmerson, and M. M. Binns. 1990. A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge by NDV. Virology **178:**297-300.

Boyle, D. B., and H. G. Heine. 1994. Recombinant fowlpox virus vaccines for poultry. Immunol Cell Biol **71 (Pt 5):**391-7.

Bridle, B. W., R. Julian, P. E. Shewen, J. P. Vaillancourt, and A. K. Kaushik. 2006. T lymphocyte subpopulations diverge in commercially raised chickens. Can J Vet Res **70:**183-90.

Brown, D. W., Y. Kawaoka, R. G. Webster, and H. L. Robinson. 1992. Assessment of retrovirus-expressed nucleoprotein as a vaccine against lethal influenza virus infections of chickens. Avian Dis **36:**515-20.

Brown, M., Y. Zhang, S. Dermine, E. A. de Wynter, C. Hart, H. Kitchener, P. L. Stern, M. A. Skinner, and S. N. Stacey. 2000. Dendritic cells infected with recombinant fowlpox virus vectors are potent and long-acting stimulators of transgene-specific class I restricted T lymphocyte activity. Gene Ther **7:**1680-9.

Bublot, M., D. E. Swayne, P. Selleck, E. Montiel, N. Pritchard, and M. Lee 2005. TROVAC AI H5, an avian influenza fowlpox vector vaccine, as an alternative vaccine for hatcheries. *In* K. Dev Biol, Basel (ed.), OIE/FAO International Scientific Conference on Avian Influenza, Paris, France.

Bublot, M., F. X. Le Gros, D. Nieddu, N. Pritchard, T. R. Mickle, and D. E. Swayne. 2007. Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. Avian Dis **51:**332-7.

Bublot, M., R. J. Manvell, W. Shell, and I. H. Brown. 2010. High level of protection induced by two fowlpox vector vaccines against a highly pathogenic avian influenza H5N1 challenge in specific-pathogen-free chickens. Avian Dis **54:**257-61.

Bublot, M., N. Pritchard, D. E. Swayne, P. Selleck, K. Karaca, D. L. Suarez, J. C. Audonnet, and T. R. Mickle. 2006. Development and use of fowlpox vectored vaccines for avian influenza. Ann N Y Acad Sci **1081:**193-201.

Buchanan, R. M., D. E. Briles, B. P. Arulanandam, M. A. Westerink, R. H. Raeder, and D. W. Metzger. 2001. IL-12-mediated increases in protection elicited by pneumococcal and meningococcal conjugate vaccines. Vaccine **19:**2020-8.

Bui, M., E. G. Wills, A. Helenius, and G. R. Whittaker. 2000. Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins. J Virol **74:**1781-6.

Busquets, S., M. Figueras, V. Almendro, F. J. Lopez-Soriano, and J. M. Argiles. 2006. Interleukin-15 increases glucose uptake in skeletal muscle. An antidiabetogenic effect of the cytokine. Biochim Biophys Acta **1760:**1613-7.

Buttigieg, K. R. 2009. Identifying a molecular determinant of Fowlpox virus resistance to avian type I interferon. Imperial College London, PhD thesis.

Capua, I., and F. Mutinelli. 2001. A Colour Atlas and Text on Avian Influenza, p. 13-20, Papi Editore, Bologna.

Capua, I., and D. J. Alexander. 2007. Animal and human health implications of avian influenza infections. Biosci Rep **27:**359-72.

Capua, I., and D. J. Alexander. 2004. Avian influenza: recent developments. Avian Pathol **33:**393-404.

Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med **180:**1395-403.

Chan, M. C., C. Y. Cheung, W. H. Chui, S. W. Tsao, J. M. Nicholls, Y. O. Chan, R. W. Chan, H. T. Long, L. L. Poon, Y. Guan, and J. S. Peiris. 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res **6:**135.

Chen, H., Y. Li, Z. Li, J. Shi, K. Shinya, G. Deng, Q. Qi, G. Tian, S. Fan, H. Zhao, Y. Sun, and Y. Kawaoka. 2006. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J Virol **80:**5976-83.

Chen, H. Y., M. F. Yang, B. A. Cui, P. Cui, M. Sheng, G. Chen, S. J. Wang, and J. W. Geng. 2010a. Construction and immunogenicity of a recombinant fowlpox vaccine coexpressing S1 glycoprotein of infectious bronchitis virus and chicken IL-18. Vaccine **28:**8112-9.

Chen, H. Y., H. Y. Zhang, X. S. Li, B. A. Cui, S. J. Wang, J. W. Geng, and K. Li. 2010b. Interleukin-18-mediated enhancement of the protective effect of an infectious laryngotracheitis virus glycoprotein B plasmid DNA vaccine in chickens. J Med Microbiol **60:**110-6.

Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell. 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med **7:**1306-12.

Chen, Z., S. Kadowaki, Y. Hagiwara, T. Yoshikawa, K. Matsuo, T. Kurata, and S. Tamura. 2000. Cross-protection against a lethal influenza virus infection by DNA vaccine to neuraminidase. Vaccine **18:**3214-22.

Cheung, C. Y., L. L. Poon, A. S. Lau, W. Luk, Y. L. Lau, K. F. Shortridge, S. Gordon, Y. Guan, and J. S. Peiris. 2002. Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet **360:**1831-7.

Choi, K. D., H. S. Lillehoj, K. D. Song, and J. Y. Han. 1999. Molecular and functional characterization of chicken IL-15. Dev Comp Immunol **23:**165-77.

Ciftci, O., S. Tanyildizi, and A. Godekmerdan. 2010. Protective effect of curcumin on immune system and body weight gain on rats intoxicated with 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). Immunopharmacol Immunotoxicol **32:**99-104.

Conenello, G. M., J. R. Tisoncik, E. Rosenzweig, Z. T. Varga, P. Palese, and M. G. Katze. 2010. A Single N66S Mutation in the PB1-F2 Protein of Influenza A Virus Increases Virulence by Inhibiting the Early Interferon Response In Vivo. J Virol **85:**652-62.

Conenello, G. M., D. Zamarin, L. A. Perrone, T. Tumpey, and P. Palese. 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog **3:**1414-21.

Cottingham, M. G., A. van Maurik, M. Zago, A. T. Newton, R. J. Anderson, M. K. Howard, J. Schneider, and M. A. Skinner. 2006. Different levels of immunogenicity of two strains of Fowlpox virus as recombinant vaccine vectors eliciting T-cell responses in heterologous prime-boost vaccination strategies. Clin Vaccine Immunol **13:**747-57.

Crawford, J., B. Wilkinson, A. Vosnesensky, G. Smith, M. Garcia, H. Stone, and M. L. Perdue. 1999. Baculovirus-derived hemagglutinin vaccines protect against lethal influenza infections by avian H5 and H7 subtypes. Vaccine **17:**2265-74.

Cullen, S. P., and S. J. Martin. 2008. Mechanisms of granule-dependent killing. Cell Death Differ **15:**251-62.

Davidson, I., I. Shkoda, and S. Perk. 2008. Integration of the reticuloendotheliosis virus envelope gene into the poultry fowlpox virus genome is not universal. J Gen Virol **89:**2456-60.

Davies, M. V., O. Elroy-Stein, R. Jagus, B. Moss, and R. J. Kaufman. 1992. The vaccinia virus K3L gene product potentiates translation by inhibiting double-stranded-RNA-activated protein kinase and phosphorylation of the alpha subunit of eukaryotic initiation factor 2. J Virol **66:**1943-50.

Daviet, S., S. Van Borm, A. Habyarimana, M. L. Ahanda, V. Morin, A. Oudin, T. Van Den Berg, and R. Zoorob. 2009. Induction of Mx and PKR failed to protect chickens from H5N1 infection. Viral Immunol **22:**467-72.

Davis, A. R., T. Bos, M. Ueda, D. P. Nayak, D. Dowbenko, and R. W. Compans. 1983. Immune response to human influenza virus hemagglutinin expressed in Escherichia coli. Gene **21:**273-84.

de Jong, M. D., and T. T. Hien. 2006. Avian influenza A (H5N1). J Clin Virol **35:**2-13.

Degen, W. G., N. van Daal, H. I. van Zuilekom, J. Burnside, and V. E. Schijns. 2004. Identification and molecular cloning of functional chicken IL-12. J Immunol **172:**4371-80.

Denton, A. E., P. C. Doherty, S. J. Turner, and N. L. La Gruta. 2007. IL-18, but not IL-12, is required for optimal cytokine production by influenza virus-specific CD8+ T cells. Eur J Immunol **37:**368-75.

Deroo, T., W. M. Jou, and W. Fiers. 1996. Recombinant neuraminidase vaccine protects against lethal influenza. Vaccine **14:**561-9.

Ding, X., H. S. Lillehoj, M. A. Quiroz, E. Bevensee, and E. P. Lillehoj. 2004. Protective immunity against Eimeria acervulina following in ovo immunization with a recombinant subunit vaccine and cytokine genes. Infect Immun **72:**6939-44.

Doherty, P. C., D. J. Topham, R. A. Tripp, R. D. Cardin, J. W. Brooks, and P. G. Stevenson. 1997. Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. Immunol Rev **159:**105-17.

Donelan, N. R., C. F. Basler, and A. Garcia-Sastre. 2003. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J Virol **77:**13257-66.

Dredge, K., J. B. Marriott, S. M. Todryk, and A. G. Dalgleish. 2002. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. Cancer Immunol Immunother **51:**521-31.

Drillien, R., D. Spehner, D. Villeval, and J. P. Lecocq. 1987. Similar genetic organization between a region of fowlpox virus DNA and the vaccinia virus HindIII J fragment despite divergent location of the thymidine kinase gene. Virology **160:**203-9.

Dupuis, S., E. Jouanguy, S. Al-Hajjar, C. Fieschi, I. Z. Al-Mohsen, S. Al-Jumaah, K. Yang, A. Chapgier, C. Eidenschenk, P. Eid, A. Al Ghonaium, H. Tufenkeji, H. Frayha, S. Al-Gazlan, H. Al-Rayes, R. D. Schreiber, I. Gresser, and J. L. Casanova. 2003. Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. Nat Genet **33:**388-91.

Edelman, R. 2002. The development and use of vaccine adjuvants. Mol Biotechnol **21:**129-48.

Edelman, R. 1980. Vaccine adjuvants. Rev Infect Dis **2:**370-83.

Ehrhardt, C., T. Wolff, S. Pleschka, O. Planz, W. Beermann, J. G. Bode, M. Schmolke, and S. Ludwig. 2007. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J Virol **81:**3058-67.

Eldaghayes, I. 2005. Use of chicken interleuin-18 as a vaccine adjuvant with a recombinant fowlpox virus fpIBD1, a subunit vaccine giving partial protection against IBDV. PhD Thesis, University of Bristol.

Fair, J. M., K. J. Taylor-McCabe, Y. Shou, and B. L. Marrone. 2008. Immunophenotyping of chicken peripheral blood lymphocyte subpopulations: individual variability and repeatability. Vet Immunol Immunopathol **125:**268-73.

Falcon, A. M., R. M. Marion, T. Zurcher, P. Gomez, A. Portela, A. Nieto, and J. Ortin. 2004. Defective RNA replication and late gene expression in temperature-sensitive influenza viruses expressing deleted forms of the NS1 protein. J Virol **78:**3880-8.

Falkner, F. G., and B. Moss. 1990. Transient dominant selection of recombinant vaccinia viruses. J Virol **64:**3108-11.

Fan, J., X. Liang, M. S. Horton, H. C. Perry, M. P. Citron, G. J. Heidecker, T. M. Fu, J. Joyce, C. T. Przysiecki, P. M. Keller, V. M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M. A. Chastain, J. H. Condra, M. E. Davies, J. Liao, E. A. Emini, and J. W. Shiver. 2004. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine **22:**2993-3003.

Fawaz, L. M., E. Sharif-Askari, and J. Menezes. 1999. Up-regulation of NK cytotoxic activity via IL-15 induction by different viruses: a comparative study. J Immunol **163:**4473-80.

Fernandez-Arias, A., C. Risco, S. Martinez, J. P. Albar, and J. F. Rodriguez. 1998. Expression of ORF A1 of infectious bursal disease virus results in the formation of virus-like particles. J Gen Virol **79 (Pt 5):**1047-54.

Ferrara, J. L., S. Abhyankar, and D. G. Gilliland. 1993. Cytokine storm of graft-versus-host disease: a critical effector role for interleukin-1. Transplant Proc **25:**1216-7.

Fiers, W., M. De Filette, A. Birkett, S. Neirynck, and W. Min Jou. 2004. A "universal" human influenza A vaccine. Virus Res **103:**173-6.

Flint, S. J., Enquist, L. W., Racaniello, V. R., and Skalka, A. M. 2004. Principles of virology. Molecular biology, pathogenesis, and control of animal viruses., 2 ed. ASM Press, Washington, DC, USA.

Fouchier, R. A., V. Munster, A. Wallensten, T. M. Bestebroer, S. Herfst, D. Smith, G. F. Rimmelzwaan, B. Olsen, and A. D. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol **79:**2814-22.

Fouchier, R. A., P. M. Schneeberger, F. W. Rozendaal, J. M. Broekman, S. A. Kemink, V. Munster, T. Kuiken, G. F. Rimmelzwaan, M. Schutten, G. J. Van Doornum, G. Koch, A. Bosman, M. Koopmans, and A. D. Osterhaus. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci U S A **101:**1356-61.

Gabriel, G., B. Dauber, T. Wolff, O. Planz, H. D. Klenk, and J. Stech. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci U S A **102:**18590-5.

Gall-Recule, G. L., F. X. Briand, A. Schmitz, O. Guionie, P. Massin, and V. Jestin. 2008. Double introduction of highly pathogenic H5N1 avian influenza virus into France in early 2006. Avian Pathol **37:**15-23.

Garcia, G. G., S. B. Berger, A. A. Sadighi Akha, and R. A. Miller. 2005. Age-associated changes in glycosylation of CD43 and CD45 on mouse CD4 T cells. Eur J Immunol **35:**622-31.

Garcia, M., J. M. Crawford, J. W. Latimer, E. Rivera-Cruz, and M. L. Perdue. 1996. Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J Gen Virol **77 (Pt 7):**1493-504.

Garcia, M. A., J. Gil, I. Ventoso, S. Guerra, E. Domingo, C. Rivas, and M. Esteban. 2006. Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. Microbiol Mol Biol Rev **70:**1032-60.

Garcia-Sastre, A. 2001. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology **279:**375-84.

Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology **252:**324-30.

Ge, J., G. Deng, Z. Wen, G. Tian, Y. Wang, J. Shi, X. Wang, Y. Li, S. Hu, Y. Jiang, C. Yang, K. Yu, Z. Bu, and H. Chen. 2007. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J Virol **81:**150-8.

Gerentes, L., N. Kessler, and M. Aymard. 1999. Difficulties in standardizing the neuraminidase content of influenza vaccines. Dev Biol Stand **98:**189-96; discussion 197.

Gherardi, M. M., J. C. Ramirez, and M. Esteban. 2003. IL-12 and IL-18 act in synergy to clear vaccinia virus infection: involvement of innate and adaptive components of the immune system. J Gen Virol **84:**1961-72.

Gherardi, M. M., J. C. Ramirez, D. Rodriguez, J. R. Rodriguez, G. Sano, F. Zavala, and M. Esteban. 1999. IL-12 delivery from recombinant vaccinia virus attenuates the vector and enhances the cellular immune response against HIV-1 Env in a dose-dependent manner. J Immunol **162:**6724-33.

Gill, N., and A. A. Ashkar. 2009. Overexpression of interleukin-15 compromises CD4-dependent adaptive immune responses against herpes simplex virus 2. J Virol **83:**918-26.

Gill, N., G. Paltser, and A. A. Ashkar. 2009. Interleukin-15 expression affects homeostasis and function of B cells through NK cell-derived interferon-gamma. Cell Immunol **258:**59-64.

Grabstein, K. H., J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M. A. Schoenborn, M. Ahdieh, and et al. 1994. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. Science **264:**965-8.

Grant, C. E., M. Z. Vasa, and R. G. Deeley. 1995. cIRF-3, a new member of the interferon regulatory factor (IRF) family that is rapidly and transiently induced by dsRNA. Nucleic Acids Res **23:**2137-46.

Guo, Z., L. M. Chen, H. Zeng, J. A. Gomez, J. Plowden, T. Fujita, J. M. Katz, R. O. Donis, and S. Sambhara. 2007. NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am J Respir Cell Mol Biol **36:**263-9.

Gupta, R. K., E. H. Relyveld, E. B. Lindblad, B. Bizzini, S. Ben-Efraim, and C. K. Gupta. 1993. Adjuvants--a balance between toxicity and adjuvanticity. Vaccine **11:**293-306.

Haghighi, H. R., L. R. Read, S. M. Haeryfar, S. Behboudi, and S. Sharif. 2009. Identification of a dual-specific T cell epitope of the hemagglutinin antigen of an h5 avian influenza virus in chickens. PLoS One **4:**e7772.

Hai, R., M. Schmolke, Z. T. Varga, B. Manicassamy, T. T. Wang, J. A. Belser, M. B. Pearce, A. Garcia-Sastre, T. M. Tumpey, and P. Palese. 2010. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. J Virol **84:**4442-50.

Hale, B. G., D. Jackson, Y. H. Chen, R. A. Lamb, and R. E. Randall. 2006. Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci U S A **103:**14194-9.

Hale, B. G., R. E. Randall, J. Ortin, and D. Jackson. 2008. The multifunctional NS1 protein of influenza A viruses. J Gen Virol **89:**2359-76.

Haller, O., G. Kochs, and F. Weber. 2006. The interferon response circuit: induction and suppression by pathogenic viruses. Virology **344:**119-30.

Haller, O., and F. Weber. 2007. Pathogenic viruses: smart manipulators of the interferon system. Curr Top Microbiol Immunol **316:**315-34.

Hama, Y., M. Kurokawa, M. Imakita, Y. Yoshida, T. Shimizu, W. Watanabe, and K. Shiraki. 2009. Interleukin 12 is a primary cytokine responding to influenza virus infection in the respiratory tract of mice. Acta Virol **53:**233-40.

Hance, K. W., C. J. Rogers, D. A. Zaharoff, D. Canter, J. Schlom, and J. W. Greiner. 2009. The antitumor and immunoadjuvant effects of IFN-alpha in combination with recombinant poxvirus vaccines. Clin Cancer Res **15:**2387-96.

Harris, A., G. Cardone, D. C. Winkler, J. B. Heymann, M. Brecher, J. M. White, and A. C. Steven. 2006. Influenza virus pleiomorphy characterized by cryoelectron tomography. Proc Natl Acad Sci U S A **103:**19123-7.

Hartoonian, C., M. Ebtekar, H. Soleimanjahi, A. Karami, M. Mahdavi, N. Rastgoo, and K. Azadmanesh. 2009. Effect of immunological adjuvants: GM-CSF (granulocyte-monocyte colony stimulating factor) and IL-23 (interleukin-23) on immune responses generated against hepatitis C virus core DNA vaccine. Cytokine **46:**43-50.

Hatta, M., P. Gao, P. Halfmann, and Y. Kawaoka. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-2.

Heiny, A. T., O. Miotto, K. N. Srinivasan, A. M. Khan, G. L. Zhang, V. Brusic, T. W. Tan, and J. T. August. 2007. Evolutionarily conserved protein sequences of influenza a viruses, avian and human, as vaccine targets. PLoS One **2:**e1190.

Hertig, C., B. E. Coupar, A. R. Gould, and D. B. Boyle. 1997. Field and vaccine strains of fowlpox virus carry integrated sequences from the avian retrovirus, reticuloendotheliosis virus. Virology **235:**367-76.

Hghihghi, H. R., L. R. Read, H. Mohammadi, Y. Pei, C. Ursprung, E. Nagy, S. Behboudi, S. M. Haeryfar, and S. Sharif. Characterization of host responses against a recombinant fowlpox virus-vectored vaccine expressing the hemagglutinin antigen of an avian influenza virus. Clin Vaccine Immunol **17:**454-63.

Higgins, D. A., K. F. Shortridge, and P. L. Ng. 1987. Bile immunoglobulin of the duck (Anas platyrhynchos). II. Antibody response in influenza A virus infections. Immunology **62:**499-504.

Hilton, L. S., A. G. Bean, and J. W. Lowenthal. 2002. The emerging role of avian cytokines as immunotherapeutics and vaccine adjuvants. Vet Immunol Immunopathol **85:**119-28.

Hiroi, T., M. Yanagita, N. Ohta, G. Sakaue, and H. Kiyono. 2000. IL-15 and IL-15 receptor selectively regulate differentiation of common mucosal immune system-independent B-1 cells for IgA responses. J Immunol **165:**4329-37.

Hiromoto, Y., Y. Yamazaki, T. Fukushima, T. Saito, S. E. Lindstrom, K. Omoe, R. Nerome, W. Lim, S. Sugita, and K. Nerome. 2000. Evolutionary characterization of the six internal genes of H5N1 human influenza A virus. J Gen Virol **81:**1293-303.

Hoare, M., M. S. Levy, D. G. Bracewell, S. D. Doig, S. Kong, N. Titchener-Hooker, J. M. Ward, and P. Dunnill. 2005. Bioprocess engineering issues that would be faced in producing a DNA vaccine at up to 100 m3 fermentation scale for an influenza pandemic. Biotechnol Prog **21:**1577-92.

Hong, Y. H., H. S. Lillehoj, E. P. Lillehoj, and S. H. Lee. 2006. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following Eimeria maxima infection of chickens. Vet Immunol Immunopathol **114:**259-72.

Horimoto, T., K. Nakayama, S. P. Smeekens, and Y. Kawaoka. 1994. Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol **68:**6074-8.

Horimoto, T., A. Takada, K. Fujii, H. Goto, M. Hatta, S. Watanabe, K. Iwatsuki-Horimoto, M. Ito, Y. Tagawa-Sakai, S. Yamada, H. Ito, T. Ito, M. Imai, S. Itamura, T. Odagiri, M. Tashiro, W. Lim, Y. Guan, M. Peiris, and Y. Kawaoka. 2006. The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. Vaccine **24:**3669-76.

Horthongkham, N., T. Srihtrakul, N. Athipanyasilp, S. Siritantikorn, W. Kantakamalakul, Y. Poovorawan, and R. Sutthent. 2007. Specific antibody response of mice after immunization with COS-7 cell derived avian influenza virus (H5N1) recombinant proteins. J Immune Based Ther Vaccines **5:**10.

Hu, Y. C., Y. L. Luo, W. T. Ji, J. L. Chulu, P. C. Chang, H. Shieh, C. Y. Wang, and H. J. Liu. 2006. Dual expression of the HA protein of H5N2 avian influenza virus in a baculovirus system. J Virol Methods **135:**43-8.

Hunt, H. D., S. Jadhao, and D. E. Swayne. 2010. Major histocompatibility complex and background genes in chickens influence susceptibility to high pathogenicity avian influenza virus. Avian Dis **54:**572-5.

Institute for Laboratory Animal Research (ILAR). 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington D.C.

Ito, N., M. Takayama, K. Yamada, M. Sugiyama, and N. Minamoto. 2001. Rescue of rabies virus from cloned cDNA and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. J Virol **75:**9121-8.

Ito, T., H. Goto, E. Yamamoto, H. Tanaka, M. Takeuchi, M. Kuwayama, Y. Kawaoka, and K. Otsuki. 2001. Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol **75:**4439-43.

Jackson, D., M. J. Hossain, D. Hickman, D. R. Perez, and R. A. Lamb. 2008. A new influenza virus virulence determinant: the NS1 protein four C-terminal residues modulate pathogenicity. Proc Natl Acad Sci U S A **105:**4381-6.

Jackson, R. J., A. J. Ramsay, C. D. Christensen, S. Beaton, D. F. Hall, and I. A. Ramshaw. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. J Virol **75:**1205-10.

Jeon, W. J., E. K. Lee, Y. J. Lee, O. M. Jeong, Y. J. Kim, J. H. Kwon, and K. S. Choi. 2008. Protective efficacy of commercial inactivated Newcastle disease virus vaccines in chickens against a recent Korean epizootic strain. J Vet Sci **9:**295-300.

Jeshtadi, A., G. Henriquet, S. M. Laidlaw, D. Hot, Y. Zhang, and M. A. Skinner. 2005. In vitro expression and analysis of secreted fowlpox virus CC chemokine-like proteins Fpv060, Fpv061, Fpv116 and Fpv121. Arch Virol **150:**1745-62.

Jiao, P., G. Tian, Y. Li, G. Deng, Y. Jiang, C. Liu, W. Liu, Z. Bu, Y. Kawaoka, and H. Chen. 2008. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol **82:**1146-54.

Jin, M., G. Wang, R. Zhang, S. Zhao, H. Li, Y. Tan, and H. Chen. 2004. Development of enzyme-linked immunosorbent assay with nucleoprotein as antigen for detection of antibodies to avian influenza virus. Avian Dis **48:**870-8.

Johansson, B. E., T. M. Moran, C. A. Bona, S. W. Popple, and E. D. Kilbourne. 1987b. Immunologic response to influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. II. Sequential infection of mice simulates human experience. J Immunol **139:**2010-4.

Johansson, B. E., T. M. Moran, and E. D. Kilbourne. 1987a. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci U S A **84:**6869-73.

Kaiser, P., L. Rothwell, M. Goodchild, and N. Bumstead. 2004. The chicken proinflammatory cytokines interleukin-1beta and interleukin-6: differences in gene structure and genetic location compared with their mammalian orthologues. Anim Genet **35:**169-75.

Kajikawa, A., K. Masuda, M. Katoh, and S. Igimi. 2010. Adjuvant effects for oral immunization provided by recombinant Lactobacillus casei secreting biologically active murine interleukin-1(1). Clin Vaccine Immunol **17:**43-8.

Kalthoff, D., A. Giritch, K. Geisler, U. Bettmann, V. Klimyuk, H. R. Hehnen, Y. Gleba, and M. Beer. 2010. Immunization with plant-expressed hemagglutinin protects chickens from lethal highly pathogenic avian influenza virus H5N1 challenge infection. J Virol **84:**12002-10.

Kanegane, H., and G. Tosato. 1996. Activation of naive and memory T cells by interleukin-15. Blood **88:**230-5.

Karaca, K., J. M. Sharma, B. J. Winslow, D. E. Junker, S. Reddy, M. Cochran, and J. McMillen. 1998. Recombinant fowlpox viruses coexpressing chicken type I IFN and Newcastle disease virus HN and F genes: influence of IFN on protective efficacy and humoral responses of chickens following in ovo or post-hatch administration of recombinant viruses. Vaccine **16:**1496-503.

Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C. R. Willis, K. Brasel, P. J. Morrissey, K. Stocking, J. C. Schuh, S. Joyce, and J. J. Peschon. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med **191:**771-80.

Kilbourne, E. D., W. G. Laver, J. L. Schulman, and R. G. Webster. 1968. Antiviral activity of antiserum specific for an influenza virus neuraminidase. J Virol **2:**281-8.

Kim, S., K. B. Miska, A. P. McElroy, M. C. Jenkins, R. H. Fetterer, C. M. Cox, L. H. Stuard, and R. A. Dalloul. 2009. Molecular cloning and functional characterization of avian interleukin-19. Mol Immunol **47:**476-84.

Kittel, C., B. Ferko, M. Kurz, R. Voglauer, S. Sereinig, J. Romanova, G. Stiegler, H. Katinger, and A. Egorov. 2005. Generation of an influenza A virus vector expressing biologically active human interleukin-2 from the NS gene segment. J Virol **79:**10672-7.

Ko, J. H., H. K. Jin, A. Asano, A. Takada, A. Ninomiya, H. Kida, H. Hokiyama, M. Ohara, M. Tsuzuki, M. Nishibori, M. Mizutani, and T. Watanabe. 2002. Polymorphisms and the differential antiviral activity of the chicken Mx gene. Genome Res **12:**595-601.

Kolibab, K., A. Yang, S. C. Derrick, T. A. Waldmann, L. P. Perera, and S. L. Morris. 2010. Highly persistent and effective prime/boost regimens against tuberculosis that use a multivalent modified vaccine virus Ankara-based tuberculosis vaccine with interleukin-15 as a molecular adjuvant. Clin Vaccine Immunol **17:**793-801.

Kostense, S., W. H. Sun, R. Cottey, S. F. Taylor, S. Harmeling, D. Zander, P. A. Small, Jr., and B. S. Bender. 1998. Interleukin 12 administration enhances Th1 activity but delays recovery from influenza A virus infection in mice. Antiviral Res **38:**117-30.

Ku, C. C., M. Murakami, A. Sakamoto, J. Kappler, and P. Marrack. 2000. Control of homeostasis of CD8+ memory T cells by opposing cytokines. Science **288:**675-8.

Kuzuhara, T., D. Kise, H. Yoshida, T. Horita, Y. Murazaki, A. Nishimura, N. Echigo, H. Utsunomiya, and H. Tsuge. 2009. Structural basis of the influenza A virus RNA polymerase PB2 RNA-binding domain containing the pathogenicity-determinant lysine 627 residue. J Biol Chem **284:**6855-60.

Laidlaw, S. M., M. A. Anwar, W. Thomas, P. Green, K. Shaw, and M. A. Skinner. 1998. Fowlpox virus encodes nonessential homologs of cellular alpha-SNAP, PC-1, and an orphan human homolog of a secreted nematode protein. J Virol **72:**6742-51.

Laidlaw, S. M., and M. A. Skinner. 2004. Comparison of the genome sequence of FP9, an attenuated, tissue culture-adapted European strain of Fowlpox virus, with those of virulent American and European viruses. J Gen Virol **85:**305-22.

Langhans, W., and B. Hrupka. 1999. Interleukins and tumor necrosis factor as inhibitors of food intake. Neuropeptides **33:**415-24.

Layton, S. L., D. R. Kapczynski, S. Higgins, J. Higgins, A. D. Wolfenden, K. A. Liljebjelke, W. G. Bottje, D. Swayne, L. R. Berghman, Y. M. Kwon, B. M. Hargis, and K. Cole. 2009. Vaccination of chickens with recombinant Salmonella expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge. Poult Sci **88:**2244-52.

Layton, S. L., D. R. Kapczynski, S. Higgins, J. Higgins, A. D. Wolfenden, K. A. Liljebjelke, W. G. Bottje, D. Swayne, L. R. Berghman, Y. M. Kwon, B. M. Hargis, and K. Cole. 2009. Vaccination of chickens with recombinant Salmonella expressing M2e and CD154 epitopes increases protection and decreases viral shedding after low pathogenic avian influenza challenge. Poult Sci **88:**2244-52.

Le, Q. M., M. Ito, Y. Muramoto, P. V. Hoang, C. D. Vuong, Y. Sakai-Tagawa, M. Kiso, M. Ozawa, R. Takano, and Y. Kawaoka. 2010. Pathogenicity of highly pathogenic avian H5N1 influenza A viruses isolated from humans between 2003 and 2008 in northern Vietnam. J Gen Virol **91:**2485-90.

Lee, L. E., R. L. Witter, S. M. Reddy, P. Wu, N. Yanagida, and S. Yoshida. 2003. Protection and synergism by recombinant fowl pox vaccines expressing multiple genes from Marek's disease virus. Avian Dis **47:**549-58.

Lee, M. S., and J. S. Chen. 2004. Predicting antigenic variants of influenza A/H3N2 viruses. Emerg Infect Dis **10:**1385-90.

Li, J., M. Ishaq, M. Prudence, X. Xi, T. Hu, Q. Liu, and D. Guo. 2009. Single mutation at the amino acid position 627 of PB2 that leads to increased virulence of an H5N1 avian influenza virus during adaptation in mice can be compensated by multiple mutations at other sites of PB2. Virus Res **144:**123-9.

Li, S., J. Schulman, S. Itamura, and P. Palese. 1993. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. J Virol **67:**6667-73.

Li, S. Q., M. Orlich, and R. Rott. 1990. Generation of seal influenza virus variants pathogenic for chickens, because of hemagglutinin cleavage site changes. J Virol **64:**3297-303.

Li, W. X., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. Brydon, K. L. Johnson, A. Garcia-Sastre, L. A. Ball, P. Palese, and S. W. Ding. 2004. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc Natl Acad Sci U S A **101:**1350-5.

Li, Z., H. Chen, P. Jiao, G. Deng, G. Tian, Y. Li, E. Hoffmann, R. G. Webster, Y. Matsuoka, and K. Yu. 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol **79:**12058-64.

Li, Z., Y. Jiang, P. Jiao, A. Wang, F. Zhao, G. Tian, X. Wang, K. Yu, Z. Bu, and H. Chen. 2006. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol **80:**11115-23.

Lillehoj, H. S., X. Ding, M. A. Quiroz, E. Bevensee, and E. P. Lillehoj. 2005. Resistance to intestinal coccidiosis following DNA immunization with the cloned 3-1E Eimeria gene plus IL-2, IL-15, and IFN-gamma. Avian Dis **49:**112-7.

Lillehoj, H. S., W. Min, K. D. Choi, U. S. Babu, J. Burnside, T. Miyamoto, B. M. Rosenthal, and E. P. Lillehoj. 2001. Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-15 and IL-2. Vet Immunol Immunopathol **82:**229-44.

Lipatov, A. S., V. A. Evseenko, H. L. Yen, A. V. Zaykovskaya, A. G. Durimanov, S. I. Zolotykh, S. V. Netesov, I. G. Drozdov, G. G. Onishchenko, R. G. Webster, and A. M. Shestopalov. 2007. Influenza (H5N1) viruses in poultry, Russian Federation, 2005-2006. Emerg Infect Dis **13:**539-46.

Lipatov, A. S., E. A. Govorkova, R. J. Webby, H. Ozaki, M. Peiris, Y. Guan, L. Poon, and R. G. Webster. 2004. Influenza: emergence and control. J Virol **78:**8951-9.

Liu, J., R. Kjeken, I. Mathiesen, and D. H. Barouch. 2008. Recruitment of antigen-presenting cells to the site of inoculation and augmentation of human immunodeficiency virus type 1 DNA vaccine immunogenicity by in vivo electroporation. J Virol **82:**5643-9.

Liu, W., P. Zou, J. Ding, Y. Lu, and Y. H. Chen. 2005. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. Microbes Infect **7:**171-7.

Liu, Y., E. Mundt, A. Mundt, M. Sylte, D. L. Suarez, D. E. Swayne, and M. Garcia. 2010. Development and evaluation of an avian influenza, neuraminidase subtype 1, indirect enzyme-linked immunosorbent assay for poultry using the differentiation of infected from vaccinated animals control strategy. Avian Dis **54:**613-21.

Long, J. X., D. X. Peng, Y. L. Liu, Y. T. Wu, and X. F. Liu. 2008. Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes **36:**471-8.

Lousberg, E. L., C. K. Fraser, M. G. Tovey, K. R. Diener, and J. D. Hayball. 2010. Type I interferons mediate the innate cytokine response to recombinant fowlpox virus but not the induction of plasmacytoid dendritic cell-dependent adaptive immunity. J Virol **84:**6549-63.

Lu, Y., X. Y. Qian, and R. M. Krug. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. Genes Dev **8:**1817-28.

Lu, Y., M. Wambach, M. G. Katze, and R. M. Krug. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. Virology **214:**222-8.

Lucey, D. R., M. Clerici, and G. M. Shearer. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev **9:**532-62.

Ludwig, S., X. Wang, C. Ehrhardt, H. Zheng, N. Donelan, O. Planz, S. Pleschka, A. Garcia-Sastre, G. Heins, and T. Wolff. 2002. The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. J Virol **76:**11166-71.

Lui, V. W., Y. He, L. Falo, and L. Huang. 2002. Systemic administration of naked DNA encoding interleukin 12 for the treatment of human papillomavirus DNA-positive tumor. Hum Gene Ther **13:**177-85.

Lupiani, B., and S. M. Reddy. 2009. The history of avian influenza. Comp Immunol Microbiol Infect Dis **32:**311-23.

Lvov, A., D. Chikvashvili, I. Michaelevski, and I. Lotan. 2008. VAMP2 interacts directly with the N terminus of Kv2.1 to enhance channel inactivation. Pflugers Arch **456:**1121-36.

Lynch, J. M., D. E. Briles, and D. W. Metzger. 2003. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect Immun **71:**4780-8.

Ma, D., C. Ma, L. Pan, G. Li, J. Yang, J. Hong, H. Cai, and X. Ren. 2011. Vaccination of chickens with DNA vaccine encoding Eimeria acervulina 3-1E and chicken IL-15 offers protection against homologous challenge. Exp Parasitol **127:**208-14.

Ma, M., N. Jin, G. Shen, G. Zhu, H. J. Liu, M. Zheng, H. Lu, X. Huo, M. Jin, G. Yin, H. Ma, X. Li, Y. Ji, and K. Jin. 2008. Immune responses of swine inoculated with a recombinant fowlpox virus co-expressing P12A and 3C of FMDV and swine IL-18. Vet Immunol Immunopathol **121:**1-7.

Magdelaine, P., M. P. Spiess, and E. Valceschini. 2008. Poultry meat consumption trends in Europe. World Poultry Sci J Vol. 64.

Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately. 1996. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity **4:**471-81.

Mandel, M., and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J Mol Biol **53:**159-62.

Manetti, R., F. Gerosa, M. G. Giudizi, R. Biagiotti, P. Parronchi, M. P. Piccinni, S. Sampognaro, E. Maggi, S. Romagnani, G. Trinchieri, and et al. 1995. Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. J Exp Med **179:**1273-83.

Marjuki, H., C. Scholtissek, J. Franks, N. J. Negovetich, J. R. Aldridge, R. Salomon, D. Finkelstein, and R. G. Webster. Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks. Arch Virol **155:**925-34.

Markoff, L., B. C. Lin, M. M. Sveda, and C. J. Lai. 1984. Glycosylation and surface expression of the influenza virus neuraminidase requires the N-terminal hydrophobic region. Mol Cell Biol **4:**8-16.

Marks-Konczalik, J., S. Dubois, J. M. Losi, H. Sabzevari, N. Yamada, L. Feigenbaum, T. A. Waldmann, and Y. Tagaya. 2000. IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. Proc Natl Acad Sci U S A **97:**11445-50.

Martinet, W., X. Saelens, T. Deroo, S. Neirynck, R. Contreras, W. Min Jou, and W. Fiers. 1997. Protection of mice against a lethal influenza challenge by immunization with yeast-derived recombinant influenza neuraminidase. Eur J Biochem **247:**332-8.

Matsushita, T., K. Ando, K. Kimura, H. Ohnishi, M. Imawari, Y. Muto, and H. Moriwaki. 1999. IL-12 induces specific cytotoxicity against regenerating hepatocytes in vivo. Int Immunol **11:**657-65.

Mayr, A., V. Hochstein-Mintzel, and H. Stickl. 1975. Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. Infection **3(1):**6-14.

Mayr, A., and K. Malicki. 1966. [Attenuation of virulent fowl pox virus in tissue culture and characteristics of the attenuated virus]. Zentralbl Veterinarmed B **13:**1-13.

Mazur, I., D. Anhlan, D. Mitzner, L. Wixler, U. Schubert, and S. Ludwig. 2008. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell Microbiol **10:**1140-52.

Medrano, G., M. C. Dolan, N. T. Stephens, A. McMickle, G. Erf, D. Radin, and C. L. Cramer. Efficient plant-based production of chicken interleukin-12 yields a strong immunostimulatory cytokine. J Interferon Cytokine Res **30:**143-54.

Metzger, D. W. 2009. IL-12 as an adjuvant for the enhancement of protective humoral immunity. Expert Rev Vaccines **8:**515-8.

Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale, Jr., and A. Garcia-Sastre. 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol **81:**514-24.

Min, J. Y., and R. M. Krug. 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc Natl Acad Sci U S A **103:**7100-5.

Min, J. Y., S. Li, G. C. Sen, and R. M. Krug. 2007. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology **363:**236-43.

Min, W., H. S. Lillehoj, J. Burnside, K. C. Weining, P. Staeheli, and J. J. Zhu. 2001. Adjuvant effects of IL-1beta, IL-2, IL-8, IL-15, IFN-alpha, IFN-gamma TGF-beta4 and lymphotactin on DNA vaccination against Eimeria acervulina. Vaccine **20:**267-74.

Min, W., H. S. Lillehoj, G. Li, E. J. Sohn, and T. Miyamoto. 2002. Development and characterization of monoclonal antibodies to chicken interleukin-15. Vet Immunol Immunopathol **88:**49-56.

Mingxiao, M., J. Ningyi, W. Zhenguo, W. Ruilin, F. Dongliang, Z. Min, Y. Gefen, L. Chang, J. Leili, J. Kuoshi, and Z. Yingjiu. 2006. Construction and immunogenicity of recombinant fowlpox vaccines coexpressing HA of AIV H5N1 and chicken IL18. Vaccine **24:**4304-11.

Mockett, B., M. M. Binns, M. E. Boursnell, and M. A. Skinner. 1992. Comparison of the locations of homologous fowlpox and vaccinia virus genes reveals major genome reorganization. J Gen Virol **73 (Pt 10):**2661-8.

Moore, K. M., J. R. Davis, T. Sato, and A. Yasuda. 2000. Reticuloendotheliosis virus (REV) long terminal repeats incorporated in the genomes of commercial fowl poxvirus vaccines and pigeon poxviruses without indication of the presence of infectious REV. Avian Dis **44:**827-41.

Morse, M. A., T. M. Clay, A. C. Hobeika, T. Osada, S. Khan, S. Chui, D. Niedzwiecki, D. Panicali, J. Schlom, and H. K. Lyerly. 2005. Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. Clin Cancer Res **11:**3017-24.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol **136:**2348-57.

Moss, B. 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A **93:**11341-8.

Mueller, Y. M., C. Petrovas, P. M. Bojczuk, I. D. Dimitriou, B. Beer, P. Silvera, F. Villinger, J. S. Cairns, E. J. Gracely, M. G. Lewis, and P. D. Katsikis. 2005. Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. J Virol **79:**4877-85.

Nazerian, K., L. F. Lee, N. Yanagida, and R. Ogawa. 1992. Protection against Marek's disease by a fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus. J Virol **66:**1409-13.

Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. M. Jou, and W. Fiers. 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med **5:**1157-63.

Nemchinov, L. G., and A. Natilla. 2007. Transient expression of the ectodomain of matrix protein 2 (M2e) of avian influenza A virus in plants. Protein Expr Purif **56:**153-9.

Neumann, G., H. Chen, G. F. Gao, Y. Shu, and Y. Kawaoka. 2010. H5N1 influenza viruses: outbreaks and biological properties. Cell Res **20:**51-61.

Neumann, G., M. T. Hughes, and Y. Kawaoka. 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. Embo J **19:**6751-8.

Nicholls, J. M., A. J. Bourne, H. Chen, Y. Guan, and J. S. Peiris. 2007. Sialic acid receptor detection in the human respiratory tract: evidence for widespread distribution of potential binding sites for human and avian influenza viruses. Respir Res **8:**73.

Niedbala, W., X. Wei, and F. Y. Liew. 2002. IL-15 induces type 1 and type 2 CD4+ and CD8+ T cells proliferation but is unable to drive cytokine production in the absence of TCR activation or IL-12 / IL-4 stimulation in vitro. Eur J Immunol **32:**341-7.

Nielsen, A. R., P. Hojman, C. Erikstrup, C. P. Fischer, P. Plomgaard, R. Mounier, O. H. Mortensen, C. Broholm, S. Taudorf, R. Krogh-Madsen, B. Lindegaard, A. M. Petersen, J. Gehl, and B. K. Pedersen. 2008. Association between interleukin-15 and obesity: interleukin-15 as a potential regulator of fat mass. J Clin Endocrinol Metab **93:**4486-93.

Nishimura, H., T. Yajima, Y. Naiki, H. Tsunobuchi, M. Umemura, K. Itano, T. Matsuguchi, M. Suzuki, P. S. Ohashi, and Y. Yoshikai. 2000. Differential roles of interleukin 15 mRNA isoforms generated by alternative splicing in immune responses in vivo. J Exp Med **191:**157-70.

Obenauer, J. C., J. Denson, P. K. Mehta, X. Su, S. Mukatira, D. B. Finkelstein, X. Xu, J. Wang, J. Ma, Y. Fan, K. M. Rakestraw, R. G. Webster, E. Hoffmann, S. Krauss, J. Zheng, Z. Zhang, and C. W. Naeve. 2006. Large-scale sequence analysis of avian influenza isolates. Science **311:**1576-80.

Oh, S., G. T. Belz, and M. C. Eichelberger. 2001. Viral neuraminidase treatment of dendritic cells enhances antigen-specific CD8(+) T cell proliferation, but does not account for the CD4(+) T cell independence of the CD8(+) T cell response during influenza virus infection. Virology **286:**403-11.

Oh, S., J. A. Berzofsky, D. S. Burke, T. A. Waldmann, and L. P. Perera. 2003. Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity. Proc Natl Acad Sci U S A **100:**3392-7.

Oh, S., and M. C. Eichelberger. 1999. Influenza virus neuraminidase alters allogeneic T cell proliferation. Virology **264:**427-35.

Oh, S., L. P. Perera, D. S. Burke, T. A. Waldmann, and J. A. Berzofsky. 2004. IL-15/IL-15Ralpha-mediated avidity maturation of memory CD8+ T cells. Proc Natl Acad Sci U S A **101:**15154-9.

Ohuchi, M., N. Asaoka, T. Sakai, and R. Ohuchi. 2006. Roles of neuraminidase in the initial stage of influenza virus infection. Microbes Infect **8:**1287-93.

Omar, A. R., K. A. Schat, L. F. Lee, and H. D. Hunt. 1998. Cytotoxic T lymphocyte response in chickens immunized with a recombinant fowlpox virus expressing Marek's disease herpesvirus glycoprotein B. Vet Immunol Immunopathol **62:**73-82.

O'Neill, R. E., J. Talon, and P. Palese. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. Embo J **17:**288-96.

Opitz, B., A. Rejaibi, B. Dauber, J. Eckhard, M. Vinzing, B. Schmeck, S. Hippenstiel, N. Suttorp, and T. Wolff. 2007. IFNbeta induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell Microbiol **9:**930-8.

Orange, J. S., T. P. Salazar-Mather, S. M. Opal, R. L. Spencer, A. H. Miller, B. S. McEwen, and C. A. Biron. 1995. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. J Exp Med **181:**901-14.

Orange, J. S., S. F. Wolf, and C. A. Biron. 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. J Immunol **152:**1253-64.

Paganin, C., I. Frank, and G. Trinchieri. 1995. Priming for high interferon-gamma production induced by interleukin-12 in both CD4+ and CD8+ T cell clones from HIV-infected patients. J Clin Invest **96:**1677-82.

Park, S. S., H. S. Lillehoj, P. C. Allen, D. W. Park, S. FitzCoy, D. A. Bautista, and E. P. Lillehoje. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with Eimeria maxima and Clostridium perfringens with the use of an animal model of necrotic enteritis. Avian Dis **52:**14-22.

Peiris, J. S., W. C. Yu, C. W. Leung, C. Y. Cheung, W. F. Ng, J. M. Nicholls, T. K. Ng, K. H. Chan, S. T. Lai, W. L. Lim, K. Y. Yuen, and Y. Guan. 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet **363:**617-9.

Perdue, M. L., M. Garcia, D. Senne, and M. Fraire. 1998. Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. Virus Res **49:**173-86.

Perera, L. P., C. K. Goldman, and T. A. Waldmann. 2001. Comparative assessment of virulence of recombinant vaccinia viruses expressing IL-2 and IL-15 in immunodeficient mice. Proc Natl Acad Sci U S A **98:**5146-51.

Perera, L. P., T. A. Waldmann, J. D. Mosca, N. Baldwin, J. A. Berzofsky, and S. K. Oh. 2007. Development of smallpox vaccine candidates with integrated interleukin-15 that demonstrate superior immunogenicity, efficacy, and safety in mice. J Virol **81:**8774-83.

Picker, L. J., E. F. Reed-Inderbitzin, S. I. Hagen, J. B. Edgar, S. G. Hansen, A. Legasse, S. Planer, M. Piatak, Jr., J. D. Lifson, V. C. Maino, M. K. Axthelm, and F. Villinger. 2006. IL-15 induces CD4 effector memory T cell production and tissue emigration in nonhuman primates. J Clin Invest **116:**1514-24.

Poon, L. L., Y. H. Leung, J. M. Nicholls, P. Y. Perera, J. H. Lichy, M. Yamamoto, T. A. Waldmann, J. S. Peiris, and L. P. Perera. 2009. Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice. J Immunol **182:**3063-71.

Potter, C. W. 2001. A history of influenza. J Appl Microbiol **91:**572-9.

Qiao, C., K. Yu, Y. Jiang, C. Li, G. Tian, X. Wang, and H. Chen. 2006. Development of a recombinant fowlpox virus vector-based vaccine of H5N1 subtype avian influenza. Dev Biol (Basel) **124:**127-32.

Qiao, C. L., K. Z. Yu, Y. P. Jiang, Y. Q. Jia, G. B. Tian, M. Liu, G. H. Deng, X. R. Wang, Q. W. Meng, and X. Y. Tang. 2003. Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathol **32:**25-32.

Qingzhong, Y., T. Barrett, T. D. Brown, J. K. Cook, P. Green, M. A. Skinner, and D. Cavanagh. 1994. Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). Vaccine **12:**569-73.

Qu, D., S. Wang, W. Cai, and A. Du. 2008. Protective effect of a DNA vaccine delivered in attenuated Salmonella typhimurium against Toxoplasma gondii infection in mice. Vaccine **26:**4541-8.

Quinn, L. S., B. G. Anderson, R. H. Drivdahl, B. Alvarez, and J. M. Argiles. 2002. Overexpression of interleukin-15 induces skeletal muscle hypertrophy in vitro: implications for treatment of muscle wasting disorders. Exp Cell Res **280:**55-63.

Rao, S., W. P. Kong, C. J. Wei, Z. Y. Yang, M. Nason, D. Styles, L. J. DeTolla, A. Panda, E. M. Sorrell, H. Song, H. Wan, G. C. Ramirez-Nieto, D. Perez, and G. J. Nabel. 2008. Multivalent HA DNA vaccination protects against highly pathogenic H5N1 avian influenza infection in chickens and mice. PLoS One **3:**e2432.

Rautenschlein, S., J. M. Sharma, B. J. Winslow, J. McMillen, D. Junker, and M. Cochran. 2000. Embryo vaccination of turkeys against Newcastle disease infection with recombinant fowlpox virus constructs containing interferons as adjuvants. Vaccine **18:**426-33.

Ridge, J. P., F. Di Rosa, and P. Matzinger. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature **393:**474-8.

Roberts, P. C., W. Garten, and H. D. Klenk. 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J Virol **67:**3048-60.

Rogge, L., A. Papi, D. H. Presky, M. Biffi, L. J. Minetti, D. Miotto, C. Agostini, G. Semenzato, L. M. Fabbri, and F. Sinigaglia. 1999. Antibodies to the IL-12 receptor beta 2 chain mark human Th1 but not Th2 cells in vitro and in vivo. J Immunol **162:**3926-32.

Rohm, C., T. Horimoto, Y. Kawaoka, J. Suss, and R. G. Webster. 1995. Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? Virology **209:**664-70.

Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. J Immunol **173:**2675-82.

Rott, R. 1979. Molecular basis of infectivity and pathogenicity of myxovirus. Brief review. Arch Virol **59:**285-98.

Rott, R. 1992. The pathogenic determinant of influenza virus. Vet Microbiol **33:**303-10.

Ryman, K. D., W. B. Klimstra, K. B. Nguyen, C. A. Biron, and R. E. Johnston. 2000. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. J Virol **74:**3366-78.

Saelens, X., P. Vanlandschoot, W. Martinet, M. Maras, S. Neirynck, R. Contreras, W. Fiers, and W. M. Jou. 1999. Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin. Eur J Biochem **260:**166-75.

Salomon, R., J. Franks, E. A. Govorkova, N. A. Ilyushina, H. L. Yen, D. J. Hulse-Post, J. Humberd, M. Trichet, J. E. Rehg, R. J. Webby, R. G. Webster, and E. Hoffmann. 2007. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med **203:**689-97.

Sarmento, L., C. L. Afonso, C. Estevez, J. Wasilenko, and M. Pantin-Jackwood. 2008. Differential host gene expression in cells infected with highly pathogenic H5N1 avian influenza viruses. Vet Immunol Immunopathol **125:**291-302.

Schaefer-Klein, J., I. Givol, E. V. Barsov, J. M. Whitcomb, M. VanBrocklin, D. N. Foster, M. J. Federspiel, and S. H. Hughes. 1998. The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis-sarcoma viruses and vectors. Virology **248:**305-11.

Schneider, K., F. Puehler, D. Baeuerle, S. Elvers, P. Staeheli, B. Kaspers, and K. C. Weining. 2000. cDNA cloning of biologically active chicken interleukin-18. J Interferon Cytokine Res **20:**879-83.

Schoenhaut, D. S., A. O. Chua, A. G. Wolitzky, P. M. Quinn, C. M. Dwyer, W. McComas, P. C. Familletti, M. K. Gately, and U. Gubler. 1992. Cloning and expression of murine IL-12. J Immunol **148:**3433-40.

Scull, M. A., L. Gillim-Ross, C. Santos, K. L. Roberts, E. Bordonali, K. Subbarao, W. S. Barclay, and R. J. Pickles. 2009. Avian Influenza virus glycoproteins restrict virus replication and spread through human airway epithelium at temperatures of the proximal airways. PLoS Pathog **5:**e1000424.

Seder, R. A. 1996. High-dose IL-2 and IL-15 enhance the in vitro priming of naive CD4+ T cells for IFN-gamma but have differential effects on priming for IL-4. J Immunol **156:**2413-22.

Sen, G. C. 2001. Viruses and interferons. Annu Rev Microbiol **55:**255-81.

Senne, D. A., B. Panigrahy, Y. Kawaoka, J. E. Pearson, J. Suss, M. Lipkind, H. Kida, and R. G. Webster. 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7

avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis **40:**425-37.

Sharma, J. M., Y. Zhang, D. Jensen, S. Rautenschlein, and H. Y. Yeh. 2002. Field trial in commercial broilers with a multivalent in ovo vaccine comprising a mixture of live viral vaccines against Marek's disease, infectious bursal disease, Newcastle disease, and fowl pox. Avian Dis **46:**613-22.

Shaw, I., and T. F. Davison. 2000. Protection from IBDV-induced bursal damage by a recombinant fowlpox vaccine, fpIBD1, is dependent on the titre of challenge virus and chicken genotype. Vaccine **18:**3230-41.

Shearn-Bochsler, V., D. Earl Green, K. A. Converse, D. E. Docherty, T. Thiel, H. N. Geisz, W. R. Fraser and D. L. Patterson-Fraser. 2008. Cutaneous and diphtheritic avian poxvirus infection in a nestling Southern Giant Petrel (Macronectes giganteus) from Antarctica. Polar Biol **31:**569-573.

Shen, G., N. Jin, M. Ma, K. Jin, M. Zheng, T. Zhuang, H. Lu, G. Zhu, H. Jin, M. Jin, X. Huo, X. Qin, R. Yin, C. Li, H. Li, Y. Li, Z. Han, Y. Chen, and M. Jin. 2007. Immune responses of pigs inoculated with a recombinant fowlpox virus coexpressing GP5/GP3 of porcine reproductive and respiratory syndrome virus and swine IL-18. Vaccine **25:**4193-202.

Shin, Y. K., Q. Liu, S. K. Tikoo, L. A. Babiuk, and Y. Zhou. 2007. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. J Gen Virol **88:**13-8.

Singh, P., T. J. Kim, and D. N. Tripathy. 2000. Re-emerging fowlpox: evaluation of isolates from vaccinated flocks. Avian Pathol **29:**449-55.

Singh, P., and D. N. Tripathy. 2003. Fowlpox virus infection causes a lymphoproliferative response in chickens. Viral Immunol **16:**223-7.

Singh, V., U. Gowthaman, S. Jain, P. Parihar, S. Banskar, P. Gupta, U. D. Gupta, and J. N. Agrewala. 2010. Coadministration of interleukins 7 and 15 with bacille Calmette-Guerin mounts enduring T cell memory response against Mycobacterium tuberculosis. J Infect Dis **202:**480-9.

Skinner, M. A., S. M. Laidlaw, I. Eldaghayes, P. Kaiser, and M. G. Cottingham. 2005. Fowlpox virus as a recombinant vaccine vector for use in mammals and poultry. Expert Rev Vaccines **4:**63-76.

Skok, J., J. Poudrier, and D. Gray. 1999. Dendritic cell-derived IL-12 promotes B cell induction of Th2 differentiation: a feedback regulation of Th1 development. J Immunol **163:**4284-91.

Smits, H. H., J. G. van Rietschoten, C. M. Hilkens, R. Sayilir, F. Stiekema, M. L. Kapsenberg, and E. A. Wierenga. 2001. IL-12-induced reversal of human Th2 cells is accompanied by full restoration of IL-12 responsiveness and loss of GATA-3 expression. Eur J Immunol **31:**1055-65.

Springer, W. T., and R. W. Truman. 1981. Effect of subcutaneous pox vaccination of young chicks on immune responses and weight gains. Poult Sci **60:**1213-20.

Steding, C. E., S. T. Wu, Y. Zhang, M. H. Jeng, B. D. Elzey, and C. Kao. 2011. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. Immunology.

Steel, J., S. V. Burmakina, C. Thomas, E. Spackman, A. Garcia-Sastre, D. E. Swayne, and P. Palese. 2008. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine **26:**522-31.

Steinhauer, D. A., and J. J. Skehel. 2002. Genetics of influenza viruses. Annu Rev Genet **36:**305-32.

Stevceva, L., M. Moniuszko, and M. G. Ferrari. 2006. Utilizing IL-12, IL-15 and IL-7 as Mucosal Vaccine Adjuvants. Lett Drug Des Discov **3:**586-592.

Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. Embo J **11:**2407-14.

Su, B. S., H. H. Chiu, C. C. Lin, J. H. Shien, H. S. Yin, and L. H. Lee. 2011. Adjuvant activity of chicken interleukin-12 co-administered with infectious bursal disease virus recombinant VP2 antigen in chickens. Vet Immunol Immunopathol **139:**167-75.

Su, B. S., H. S. Yin, H. H. Chiu, L. H. Hung, J. P. Huang, J. H. Shien, and L. H. Lee. 2010. Immunoadjuvant activities of a recombinant chicken IL-12 in chickens vaccinated with Newcastle disease virus recombinant HN protein. Vet Microbiol.

Suarez, D. L. 2005. Overview of avian influenza DIVA test strategies. Biologicals **33:**221-6.

Suarez, D. L., and S. Schultz-Cherry. 2000b. The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis **44:**861-8.

Suarez, D. L., and S. Schultz-Cherry. 2000a. Immunology of avian influenza virus: a review. Dev Comp Immunol **24:**269-83.

Subbarao, E. K., W. London, and B. R. Murphy. 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol **67:**1761-4.

Subbarao, K., and J. Katz. 2000. Avian influenza viruses infecting humans. Cell Mol Life Sci **57:**1770-84.

Subbarao, K., A. Klimov, J. Katz, H. Regnery, W. Lim, H. Hall, M. Perdue, D. Swayne, C. Bender, J. Huang, M. Hemphill, T. Rowe, M. Shaw, X. Xu, K. Fukuda, and N. Cox. 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science **279:**393-6.

Sun, H. L., Y. F. Wang, D. Y. Miao, P. J. Zhang, H. D. Zhi, L. L. Xu, M. Wang, G. Z. Tong, and M. Wang. 2006. [Construction and characterization of a recombinant fowlpox virus co-expressing F, HN genes of Newcastle disease virus and gB gene of infectious laryngnotracheitis virus]. Sheng Wu Gong Cheng Xue Bao **22:**931-9.

Sundick, R. S., and C. Gill-Dixon. 1997. A cloned chicken lymphokine homologous to both mammalian IL-2 and IL-15. J Immunol **159:**720-5.

Swain, S. L., J. N. Agrewala, D. M. Brown, D. M. Jelley-Gibbs, S. Golech, G. Huston, S. C. Jones, C. Kamperschroer, W. H. Lee, K. K. McKinstry, E. Roman, T. Strutt, and N. P. Weng. 2006. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. Immunol Rev **211:**8-22.

Swayne, D. E. 2008. Avian influenza vaccines and therapies for poultry. Comp Immunol Microbiol Infect Dis **32:**351-63.

Swayne, D. E., J. R. Beck, and N. Kinney. 2000. Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. Avian Dis **44:**132-7.

Swayne, D. E., J. R. Beck, and T. R. Mickle. 1997. Efficacy of recombinant fowl poxvirus vaccine in protecting chickens against a highly pathogenic Mexican-origin H5N2 avian influenza virus. Avian Dis **41:**910-22.

Swayne, D. E., C. W. Lee, and E. Spackman. 2006. Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Pathol **35:**141-6.

Sylte, M. J., B. Hubby, and D. L. Suarez. 2007. Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine **25:**3763-72.

Symons, J. A., A. Alcami, and G. L. Smith. 1995. Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. Cell **81:**551-60.

Szabo, S. J., N. G. Jacobson, A. S. Dighe, U. Gubler, and K. M. Murphy. 1995. Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. Immunity **2:**665-75.

Szretter, K. J., S. Gangappa, X. Lu, C. Smith, W. J. Shieh, S. R. Zaki, S. Sambhara, T. M. Tumpey, and J. M. Katz. 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. J Virol **81:**2736-44.

Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre. 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. J Virol **74:**7989-96.

Tan, J. T., B. Ernst, W. C. Kieper, E. LeRoy, J. Sprent, and C. D. Surh. 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. J Exp Med **195:**1523-32.

Tanaka, S., F. Isoda, Y. Ishihara, M. Kimura, and T. Yamakawa. 2001. T lymphopaenia in relation to body mass index and TNF-alpha in human obesity: adequate weight reduction can be corrective. Clin Endocrinol (Oxf) **54:**347-54.

Tang, C., H. Yamada, K. Shibata, S. Yoshida, W. Wajjwalku, and Y. Yoshikai. 2009. IL-15 protects antigen-specific CD8+ T cell contraction after Mycobacterium bovis bacillus Calmette-Guerin infection. J Leukoc Biol **86:**187-94.

Tanimoto, T., R. Nakatsu, I. Fuke, T. Ishikawa, M. Ishibashi, K. Yamanishi, M. Takahashi, and S. Tamura. 2005. Estimation of the neuraminidase content of influenza viruses and split-product vaccines by immunochromatography. Vaccine **23:**4598-609.

Taubenberger, J. K. 2006. The origin and virulence of the 1918 "Spanish" influenza virus. Proc Am Philos Soc **150:**86-112.

Taylor, J., L. Christensen, R. Gettig, J. Goebel, J. F. Bouquet, T. R. Mickle, and E. Paoletti. 1996. Efficacy of a recombinant fowl pox-based Newcastle disease virus vaccine candidate against velogenic and respiratory challenge. Avian Dis **40:**173-80.

Taylor, J., and E. Paoletti. 1988. Fowlpox virus as a vector in non-avian species. Vaccine **6:**466-8.

Taylor, J., R. Weinberg, B. Languet, P. Desmettre, and E. Paoletti. 1988. Recombinant fowlpox virus inducing protective immunity in non-avian species. Vaccine **6:**497-503.

Thomas, J. D., K. R. Morris, D. I. Godfrey, J. W. Lowenthal, and A. G. Bean. 2008. Expression, purification and characterisation of recombinant Escherichia coli derived chicken interleukin-12. Vet Immunol Immunopathol **126:**403-6.

Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol **3:**133-46.

Tsurita, M., M. Kurokawa, M. Imakita, Y. Fukuda, Y. Watanabe, and K. Shiraki. 2001. Early augmentation of interleukin (IL)-12 level in the airway of mice administered orally with clarithromycin or intranasally with IL-12 results in alleviation of influenza infection. J Pharmacol Exp Ther **298:**362-8.

van den Berg, T., B. Lambrecht, S. Marche, M. Steensels, S. Van Borm, and M. Bublot. 2008. Influenza vaccines and vaccination strategies in birds. Comp Immunol Microbiol Infect Dis **31:**121-65.

Van Der Sluijs, K. F., L. J. Van Elden, R. Arens, M. Nijhuis, R. Schuurman, S. Florquin, J. Kwakkel, S. Akira, H. M. Jansen, R. Lutter, and T. Van Der Polls. 2005. Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus. Immunology **114:**112-20.

van der Sluijs, K. F., L. J. van Elden, Y. Xiao, R. Arens, M. Nijhuis, R. Schuurman, S. Florquin, H. M. Jansen, R. Lutter, and T. van der Poll. 2006. IL-12 deficiency transiently improves viral clearance during the late phase of respiratory tract infection with influenza A virus in mice. Antiviral Res **70:**75-84.

Vecino, W. H., N. M. Quanquin, L. Martinez-Sobrido, A. Fernandez-Sesma, A. Garcia-Sastre, W. R. Jacobs, Jr., and G. J. Fennelly. 2004. Mucosal immunization with attenuated Shigella flexneri harboring an influenza hemagglutinin DNA vaccine protects mice against a lethal influenza challenge. Virology **325:**192-9.

Veits, J., A. Romer-Oberdorfer, D. Helferich, M. Durban, Y. Suezer, G. Sutter, and T. C. Mettenleiter. 2008. Protective efficacy of several vaccines against highly pathogenic H5N1 avian influenza virus under experimental conditions. Vaccine **26:**1688-96.

Vey, M., M. Orlich, S. Adler, H. D. Klenk, R. Rott, and W. Garten. 1992. Hemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-K/R-R. Virology **188:**408-13.

Vliegenthart, J. F. G., and J. Montreuil. 1995. Primary structure of glycoprotein glycans. *In* Glycoproteins, New Comprehensive Biochemistry, vol. 29a. Elsevier, Amsterdam.

Vogel, L. A., L. C. Showe, T. L. Lester, R. M. McNutt, V. H. Van Cleave, and D. W. Metzger. 1996. Direct binding of IL-12 to human and murine B lymphocytes. Int Immunol **8:**1955-62.

Vogelstein, B., and D. Gillespie. 1979. Preparative and analytical purification of DNA from agarose. Proc Natl Acad Sci U S A **76:**615-9.

Wagner, R., T. Wolff, A. Herwig, S. Pleschka, and H. D. Klenk. 2000. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J Virol **74:**6316-23.

Wambura, P. N., and S. K. Godfrey. 2010. Protective immune response of chickens to oral vaccination with thermostable live Fowlpox virus vaccine (strain TPV-1) coated on oiled rice. Trop Anim Health Prod **42:**451-6.

Wang, J., D. Vijaykrishna, L. Duan, J. Bahl, J. X. Zhang, R. G. Webster, J. S. Peiris, H. Chen, G. J. Smith, and Y. Guan. 2008. Identification of the progenitors of Indonesian and Vietnamese avian influenza A (H5N1) viruses from southern China. J Virol **82:**3405-14.

Wang, X., M. Li, H. Zheng, T. Muster, P. Palese, A. A. Beg, and A. Garcia-Sastre. 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol **74:**11566-73.

Wang, Y. F., Y. K. Sun, Z. C. Tian, X. M. Shi, G. Z. Tong, S. W. Liu, H. D. Zhi, X. G. Kong, and M. Wang. 2009. Protection of chickens against infectious bronchitis by a recombinant fowlpox virus co-expressing IBV-S1 and chicken IFNgamma. Vaccine **27:**7046-52.

Webster, D. P., S. Dunachie, S. McConkey, I. Poulton, A. C. Moore, M. Walther, S. M. Laidlaw, T. Peto, M. A. Skinner, S. C. Gilbert, and A. V. Hill. 2006b. Safety of recombinant fowlpox strain FP9 and modified vaccinia virus Ankara vaccines against liver-stage P. falciparum malaria in non-immune volunteers. Vaccine **24:**3026-34.

Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. Microbiol Rev **56:**152-79.

Webster, R. G., Y. Kawaoka, J. Taylor, R. Weinberg, and E. Paoletti. 1991. Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. Vaccine **9:**303-8.

Webster, R. G., M. Peiris, H. Chen, and Y. Guan. 2006a. H5N1 outbreaks and enzootic influenza. Emerg Infect Dis **12:**3-8.

Webster, R. G., and H. G. Pereira. 1968. A common surface antigen in influenza viruses from human and avian sources. J Gen Virol **3:**201-8.

Webster, R. G., P. A. Reay, and W. G. Laver. 1988. Protection against lethal influenza with neuraminidase. Virology **164:**230-7.

Wei, F., Q. Liu, Y. Zhai, Z. Fu, W. Liu, L. Shang, J. Men, S. Gao, H. Lian, H. Jin, C. Chen, J. Lin, Y. Shi, Z. Xia, and X. Q. Zhu. 2009. IL-18 enhances protective effect in mice immunized with a Schistosoma japonicum FABP DNA vaccine. Acta Trop **111:**284-8.

Wood, G. W., J. Banks, J. W. McCauley, and D. J. Alexander. 1994. Deduced amino acid sequences of the haemagglutinin of H5N1 avian influenza virus isolates from an outbreak in turkeys in Norfolk, England. Arch Virol **134:**185-94.

Wood, G. W., J. W. McCauley, J. B. Bashiruddin, and D. J. Alexander. 1993. Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Arch Virol **130:**209-17.

World-Health-Organization, W. H. O. 2008. H5N1 avian influenza: Timeline of major events. http://www.who.int/csr/disease/avian_influenza/ai_timeline/en/index.html.

Wu, W. L., Y. Chen, P. Wang, W. Song, S. Y. Lau, J. M. Rayner, G. J. Smith, R. G. Webster, J. S. Peiris, T. Lin, N. Xia, Y. Guan, and H. Chen. 2008. Antigenic profile of avian H5N1 viruses in Asia from 2002 to 2007. J Virol **82:**1798-807.

Xiang, Y., and B. Moss. 1999. IL-18 binding and inhibition of interferon gamma induction by human poxvirus-encoded proteins. Proc Natl Acad Sci U S A **96:**11537-42.

Yang, Y., D. Leggat, A. Herbert, P. C. Roberts, and R. S. Sundick. 2009. A novel method to incorporate bioactive cytokines as adjuvants on the surface of virus particles. J Interferon Cytokine Res **29:**9-22.

Yin, X., X. Yan, Q. Yang, H. Cao, and H. Liang. 2011. Antitumor mechanism of recombinant murine interleukin-12 vaccine. Cancer Biother Radiopharm **25:**263-8.

Zamarin, D., A. Garcia-Sastre, X. Xiao, R. Wang, and P. Palese. 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog **1:**e4.

Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. Immunity **8:**591-9.

Zhou, J. J., J. Fu, D. Y. Fang, H. J. Yan, J. Tian, J. M. Zhou, J. P. Tao, Y. Liang, and L. F. Jiang. 2007. Molecular characterization of the surface glycoprotein genes of an H5N1 influenza virus isolated from a human in Guangdong, China. Arch Virol **152:**1515-21.

Zhou, J. Y., J. Y. Wang, J. G. Chen, J. X. Wu, H. Gong, Q. Y. Teng, J. Q. Guo, and H. G. Shen. 2005. Cloning, in vitro expression and bioactivity of duck interleukin-2. Mol Immunol **42:**589-98.

Zhu, H., J. Wang, P. Wang, W. Song, Z. Zheng, R. Chen, K. Guo, T. Zhang, J. S. Peiris, H. Chen, and Y. Guan. 2010. Substitution of lysine at 627 position in PB2 protein does not change virulence of the 2009 pandemic H1N1 virus in mice. Virology **401:**1-5.

Zhu, Q., H. Yang, W. Chen, W. Cao, G. Zhong, P. Jiao, G. Deng, K. Yu, C. Yang, Z. Bu, Y. Kawaoka, and H. Chen. 2008. A naturally occurring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens. J Virol **82:**220-8.

Zielinski, R. J., J. V. Smedley, P. Y. Perera, P. M. Silvera, T. A. Waldmann, J. Capala, and L. P. Perera. 2010. Smallpox vaccine with integrated IL-15 demonstrates enhanced in vivo viral clearance in immunodeficient mice and confers long term protection against a lethal monkeypox challenge in cynomolgus monkeys. Vaccine **28:**7081-91.