JOURNAL OF GENERAL VIROLOGY

SHORT COMMUNICATION

Majid *et al.*, *Journal of General Virology* 2020;101:772–777

DOI 10.1099/jgv.0.001428

MICROBIOLOGY SOCIETY

OPEN MICROBIOLOGY

Negligible effect of chicken cytokine IL-12 integration into recombinant fowlpox viruses expressing avian influenza virus neuraminidase N1 on host cellular immune responses

Nadzreeq Nor Majid[1,2], Abdul Rahman Omar[3,4] and Abdul Razak Mariatulqabtiah[2,3,*]

Abstract

In comparison to the extensive characterization of haemagglutinin antibodies of avian influenza virus (AIV), the role of neuraminidase (NA) as an immunogen is less well understood. This study describes the construction and cellular responses of recombinant fowlpox viruses (rFWPV) strain FP9, co-expressing NA N1 gene of AIV A/Chicken/Malaysia/5858/2004, and chicken IL-12 gene. Our data shows that the N1 and IL-12 proteins were successfully expressed from the recombinants with 48 kD and 70 kD molecular weights, respectively. Upon inoculation into specific-pathogen-free (SPF) chickens at 10^5 p.f.u. ml^{-1}, levels of CD3+/CD4+ and CD3+/CD8+ populations were higher in the wild-type fowlpox virus FP9 strain, compared to those of rFWPV-N1 and rFWPV-N1-IL-12 at weeks 2 and 5 time points. Furthermore, rFWPV-N1-IL-12 showed a suppressive effect on chicken body weight within 4 weeks after inoculation. We suggest that co-expression of N1 with or without IL-12 offers undesirable quality as a potential AIV vaccine candidate.

Avian influenza viruses (AIV) infecting poultry can be divided into two distinct groups (pathotypes) based on their ability to cause disease (pathogenicity). The two surface glycoproteins of the virus, haemagglutinin (HA) and neuraminidase (NA) are the most important antigens for inducing protective immunity in the host and, therefore, preferred to be manipulated in AIV vaccine development. 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 [1]. The NA activity also helps to prevent virus from self-aggregating or remaining in cell membranes [2]. In comparison to the extensive characterization of HA antibodies to protect against AIV infection, the role of NA-specific antibodies as protective agents is less well understood [3]. 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 [4], six or even seven [5] times higher than those of NA, with a consequently skewed levels of serum antibodies (humoral response) towards HA after influenza virus infection [6]. Secondly, the quantity of NA in licensed inactivated vaccines is not standardized due to the different relative ratio of HA/NA in a given vaccine preparation [7], as the quantity of HA/NA is influenced by the specific genetic background and subtype of the virus [8]. Vaccination with purified recombinant NA protein [8, 9], NA-encoding DNA [10] or NA virus-like particles [3] 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 vaccinia virus [11] and adenovirus [12]. A recombinant fowlpox virus (rFWPV) co-expressing HA and NA of AIV has been shown to offer complete protection upon lethal homologous challenge in poultry [13]. No NA-only rFWPV was included in the study, making it difficult to evaluate the specific role of NA-specific antibodies. rFWPV against AIV has been licensed and commercially used since the 1990s [14]. The attenuated European fowlpox virus (FWPV) vaccine strain, FP9, was shown to be immunogenic for CD8+T cells and

Received 11 March 2020; Accepted 17 April 2020; Published 19 May 2020

Author affiliations: [1]Office of Deputy Vice Chancellor (Research and Innovation), Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia; [2]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; [3]Laboratory of Vaccines and Immunotherapeutic, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; [4]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

***Correspondence:** Abdul Razak Mariatulqabtiah, mariatulqabtiah@upm.edu.my

Keywords: recombinant fowlpox virus; avian influenza virus; neuraminidase; cytokine; IL-12.

Abbreviations: AIV, avian influenza virus; FWPV, fowlpox virus; NA, neuraminidase; PBMC, peripheral blood mononuclear cells; rFWPV, recombinant fowlpox virus.

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 [15]. FP9 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. More discoveries and characterization of avian cytokines have triggered their development as vaccine co-immunostimulators.

IL-12 possesses a significant role as a pro-inflammatory molecule, in regulating cell-mediated immunity response of the immune machinery (NK, NKT, CD4+ and CD8+T cell), to combat against intracellular pathogens. Although IL-12 is predicted to direct the early host immune defence against influenza A virus infection [16], reports from studies of influenza virus infection offer contradictory results, as administration of recombinant IL-12 to influenza virus-infected mice either enhanced [17] or delayed [18, 19] the virus clearance. 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 [20, 21], IL-10 mRNA expression and opsonic activity [21]. Similar observations were made when murine IL-12 was used as adjuvant for conjugate vaccines against *Neisseria meningitidis* meningococcus [20], 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 [22]. In limited avian model studies, 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 [23]. They also showed higher levels of IFN-γ production and enhancement of protection rate upon challenge (83%) from their rFWPV expressing HN gene of NDV constructs, in the presence of recombinant chicken IL-12 [24]. Co-expression of NA N1 protein of AIV H5N1 and chicken IL-12 in rFWPV and its characterization upon inoculation into chickens as described here has not previously been studied.

The initial stock of parental FWPV FP9 (WT FP9) was from Dr Mike Skinner laboratory (Imperial College London). Chicken embryonic fibroblasts (CEF) were prepared from 9- to 10-day-old embryonated eggs and maintained in 2% newborn bovine serum (NBBS) in DMEM media (Gibco) [25]. Previously cloned and sequenced cDNAs encoding full-length NA N1 of influenza strain A/Ch/Malaysia/5744/2004 gene [26] were amplified by PCR using N1-F (5′-ACCG AATATTATGAATCCAAATAAGAAG-3′) and N1-R (5′-AGGCAATATTCTACTTGTCAATGGTG-3′) primers, with introduction of *SspI* site as underlined to facilitate blund-end ligation. The amplicon was inserted into recombination vector pEFL29, which contained a copy of the vaccinia virus p7.5 early/late promoter, at the *SmaI* site (pEFL29/N1) [27]. Chicken IL-12 gene was inserted downstream of a synthetic/hybrid promoter in vector pEF*gpt*12S, before being subcloned into vector pPC1.X (pPC1.X/IL-12). Selection of positive transformants using analytical PCR [28] was performed using primer sets: pEFL29-F (5′- CGGAGACCATATCCATACGC-3′) and pEFL29-R (5′-CGTAAAAG TAGAAAATATATTC-3′); and pPC1.X-F (5′-ATGAAAAA TAGTACCACTATGG-3′) and pPC1.X-R (5′-ATCCGATA CTAGTATTAGGTTAGC-3′). Cultures that yielded positive PCR amplicons were subjected to plasmid DNA isolation (QIAGEN Minipreparation Kit), restriction enzyme digestion and sequencing (data not shown). The detailed protocol for recombination/transfection has been described elsewhere [28, 29]. Stocks of recombinant viruses were titrated using plaque assay prior to use. The recombinant protein lysates were prepared by infecting CEFs, with rFWPV-N1 and rFWPV-N1-IL-12 at an m.o.i. of 3, for 48 h. The cell pellet was subjected to 15% SDS-PAGE. The electro-transferred nitrocellulose membrane (GE Healthcare) was incubated with incubated with a rabbit polyclonal primary antibody against AIV NA of A/H5N1/Vietnam/1203/2004 (Cat. No. ab70759, Abcam, USA) with the final concentration of 1 μg μl^{-1}, for 1 h. The membrane was developed using a commercial kit using the chromogenic substance, WesternBreeze (Invitrogen). Monoclonal antibody HC8, specific for the chicken IL-12 p70 heterodimer, was used to monitor for the presence of the soluble secreted chicken IL-12 protein. This membrane was developed using Amersham ECL Western-blotting detection kit (GE Healthcare) and observed under an autoradiograph machine.

One-day-old specific pathogen-free (SPF) chickens were inoculated subcutaneously with PBS (negative control) or 10^5 p.f.u. of WT FP9, rFWPV-N1 and rFWPV-N1-IL-12, diluted in PBS to a total volume of 100 μl. Nine chickens were assigned for each group. At weeks 2 and 5, whole blood (0.2 ml) of each chicken was sampled and pooled into three groups (0.6 ml in total for each tube). Isolation of peripheral blood mononuclear cells (PBMC) was done by following the standard protocol (GE Healthcare) and the pelleted cells were transferred into flow tubes for CD4+ and CD8+ immunophenotyping analysis using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [28]. All chickens were weighed at week 1 until week 4. Differences between groups of chicken were analysed by one-way ANOVA paired-sample *t*-test using SPSS (version 15) software. Results were expressed as the mean±SEM. *P*-values less than 0.05 were considered statistically significant in all cases.

Schematic diagram of the cloning processes was shown (Fig. 1). Recombination of NA N1 gene into FWPV was verified using PCR after genomic DNA extraction with primer set pEFL29-F and pEFL29-R, producing an amplicon of 1350 bp (result not shown). The chicken IL-12 expression cassette was homologously recombined into rFWPV already carrying the AIV N1 gene. 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 IL-12 insert) [30], so, PCR analysis of their viral DNA genomes was carried

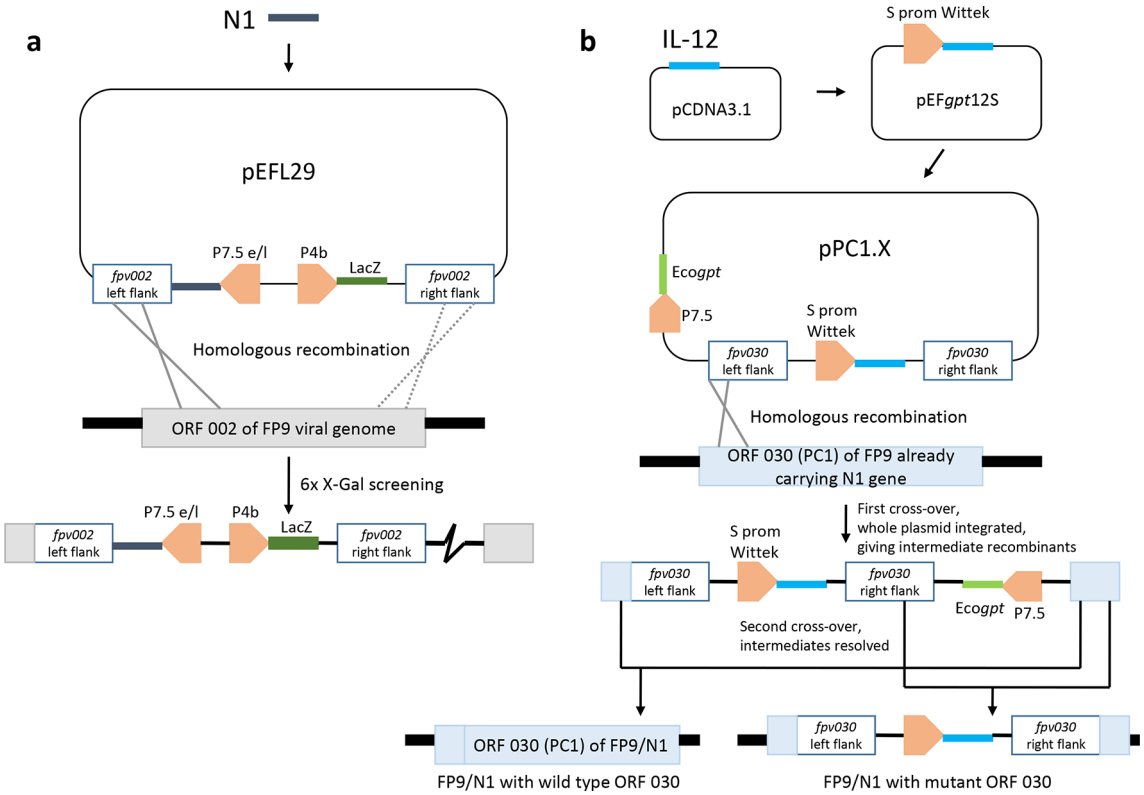

Fig. 1. Overview of the cloning strategy. (a) Vector pEFL29 carrying N1 gene was recombined into FP9 genome. (b) Cytokine gene IL-12 expression cassette was recombined into rFWPV already carrying the N1 gene.

out after every stage of plaque-purification, to assay for retention of the cytokine gene. A flanking primers pPC1.X-F and pPC1.XR was applied to screen the IL-12-recombinant clones. rFWPV-N1 showed a 984 bp PCR product, while integration of IL-12 into the genome (rFWPV-N1-IL-12) generated a 1934 bp product (Fig. 2). Using Western blotting, a prominent band was observed at ~48 kD, which corresponding to the predicted unglycosylated size of N1 protein, 49 kD (Fig. 3a).

This is the first report on the size of N1 protein from strain A/Ch/Malaysia/5744/2004. The presence of the soluble secreted chicken IL-12 p70 heterodimer (fusion protein consisting of p35 and p40 subunits) in supernatants from infected and uninfected CEF was analysed. A doublet of bands (the upper band being fainter) was observed at about 70 kD for five recombinants (four intermediate clones and one resolved

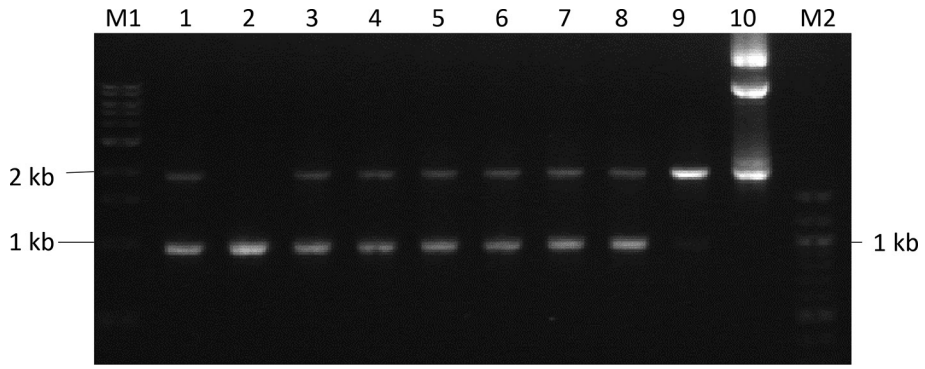

Fig. 2. Recombination of IL-12 gene into rFWPV-N1 was verified using PCR after genomic DNA extraction using primer set pPC1.X-F and pPC1.X. Lanes 1, 3–8: intermediate viruses, which produced amplicons of 984 and 1934 bp; lane 2: rFWPV-N1, which has lost the IL-12 gene; lane 9: rFWPV-N1 carrying the IL-12 gene; lane 10: pPC1.X-IL-12 plasmid as positive control. M1 and M2 are 1 kb and 100 bp ladder markers, respectively (New England Biolabs).

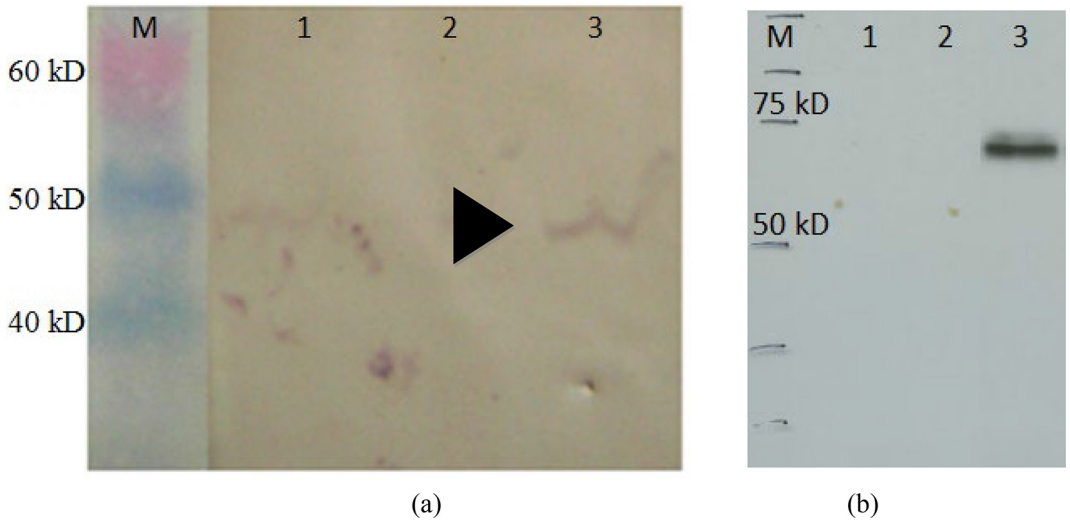

Fig. 3. Detection of recombinant proteins N1 from rFWPV-N1 at 48 kD (a) and IL-12 from rFWPV-N1-IL-12 at 70 kD (b). Lane 1: uninfected CEF as negative control; lane 2: CEF infected with WT FP9; lane 3: CEF infected with rFWPV-N1 (a) or rFWPV-N1-IL-12 (b). M is a protein ladder marker.

rFWPV-N1-IL-12) but not for uninfected CEF nor for WT FP9 (Fig. 3b).

The levels of CD4+T cells in the control and rFWPV-N1 groups remained relatively constant at weeks 2 and 5. Samples from groups vaccinated with WT FP9 demonstrated increases in CD4+T-cell population levels over time, of 2.06 point percentages. The rFWPV-N1-IL-12 vaccinated group showed a higher CD4+T-cell population relative to control at week 2 but had decreased by 3.95 point percentages over time and returned to control levels by week 5. The rFWPV-N1 and rFWPV-N1-IL-12 vaccinated groups showed significantly lower CD4+T-cell populations relative to WT FP9 at week 5 ($P \leq 0.05$). No statistically significant difference in CD4+T-cell levels was observed for other groups at either sampling point. Animal experiments also revealed a relatively constant CD8+T population for control and WT FP9 vaccinated chickens. The CD8+T-cell level was significantly higher for the WT FP9 vaccinated group relative to the control group at both sampling points. The rFWPV-N1 and rFWPV-N1-IL-12 vaccinated groups showed comparable CD4+T-cell population relative to control at weeks 2 and 5. Both recombinant vaccines had also shown significant decreases in CD8+T-cell population over time ($P \leq 0.05$) (Table 1).

Body weights of chickens in WT FP9 and rFWPV-N1 decreased 10.6 and 2.7%, respectively, compared to control chickens, with rFWPV-N1-IL-12 having the highest weight loss of 25.9% or 27.2 g, after 1 week post-vaccination. At week 2, the relative weight reduction was irreversible for WT FP9, rFWPV-N1 and rFWPV-N1-IL-12. This weight pattern was consistent for all groups until week 4, with WT FP9, rFWPV-N1 and rFWPV-N1-IL-12 having a statistically significant weight loss compared to the control. Relative to WT FP9, rFWPV-N1 did not show any significant difference. However, integration of IL-12 into the recombinants showed a detrimental effect to the weight of the chickens (Table 2).

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. Furthermore, lack of standardization of NA protein

Table 1. Immunophenotyping of CD3+/CD4 and CD3+/CD8+lymphocytes from chickens after mock-treatment with PBS (control), or immunization with WT FP9, rFWPV-N1 or rFWPV-N1-IL-12

Vaccine groups	Weeks post-inoculation			
	CD3+/CD4+		CD3+/CD8+	
	Week 2	Week 5	Week 2	Week 5
Control	15.64±1.75	16.54±3.33	8.54±0.55	8.17±1.15
WT FP9	18.75±2.53	20.81±0.84	12.71±0.82†	13.28±1.06†
rFWPV-N1	14.92±1.06	14.09±0.82*	9.49±1.93	8.56±1.43‡
rFWPV-N1-IL-12	16.36±1.97	12.41±1.29*	9.91±1.89	8.73±0.71‡

Each value represents the mean percentages of T lymphocytes sub-population ±SEM, from PBMC samples of nine chickens pooled in threes (n=3), sampled at weeks 2 and 5. Significant differences between control and other groups (†) and WT FP9 and other groups (*), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different time points (‡) were determined by paired-sample t-test ($P \leq 0.05$).

Table 2. Effect of WT FP9 or rFWPV inoculation of 1-day-old chicks on mean body weight (g) at weeks 1, 2, 3 and 4

Vaccine	Mean body weight in g 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-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*

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≤0.05) and indicated by an asterisk (*).

content might hinder the advancement of characterization of NA vaccine-induced protection [3]. In this study, rFWPV co-expressing NA N1 and chicken IL-12 was constructed and its immunogenicity was evaluated by assessing the cellular response of the host upon inoculation.

IL-12 has been demonstrated to induce priming for high production of IFN-γ in both CD4+ and CD8+T-cell clones [31]. However, the cytokine has a short life-span (5 to 6 h) in blood circulation, thus leading to a dramatic decrease of IFN-γ levels after peak release [32]. 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.

Our findings suggest that co-expression of IL-12 in rFWPV-N1 does not influence persistence of total CD4+T cells in PBMC. In fact, vaccination using rFWPV-N1 or rFWPV-N1-IL-12 reduced the level of CD4+T-cell populations, although not at a significant level. The 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 other *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 [23]. However, 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 [23]. 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.

No significant augmentation of CD8+T-cell level was observed for rFWPV-N1 and rFWPV-N1-IL-12. An increase over time in the CD8+T-cell population 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 [33, 34]; (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 [33]. It is not known whether the immediate CD8+T-cell responses against N1 after vaccination suppress further proliferation of CD8+T.

The negligible cellular immune response augmentation and weight loss in chickens upon IL-12 integration as reported in this study have not been reported elsewhere. However, it has been noted that IL-12-deficient mice have normal size and weight, despite having defective IFN-γ production and type 1 cytokine responses [35]. *In vivo* study in mice also suggested that administration of high dose IL-12 can increase the level of TNF-α [36], a cytokine that can regulate physiological and pathological changes, and can promote weight loss or anorexia. Unfortunately, the levels of cytokines were not measured during the course of the experiment, so it is not possible to propose whether or not the weight loss was IFN-γ or TNF-α-dependent. There is also a possibility that the effects of IL-12 in our study involved the FWPV or AIV antigen-specific interaction. As we did not manage to construct rFWPV co-expressing H5 gene of AIV and IL-12, or rFWPV expressing IL-12 alone, which can serve as a comparison to rFWPV-N1-IL-12, the results could not be explained mechanistically.

In conclusion, the study showed that IL-12 co-expression from a recombinant poultry vaccine against AIV would require careful consideration due to the detrimental effect on body weight and inconsequential activation of host cellular responses.

Funding information
This study was supported by the Ministry of Higher Education, Government of Malaysia, through Universiti Putra Malaysia's Geran Putra (grant no. 9623200) and Institute of Bioscience's Higher Institution Centre of Excellence (IBS HICoE) grant (no. 6369101).

Acknowledgement
We thank the late Professor Dr Pete Kaiser of then Institute of Animal Health, Compton, UK, and the late Dr Rico Wittek of World Health Organization, for contributions of vector plasmids and antibodies. We acknowledge Dr Mike Skinner of Imperial College London for his guidance and support during the construction of the recombinants.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The *in vivo* experiments performed in this study were conducted in an Animal Biosafety Level 2 facility and in accordance with the policies and ethical standards of the local Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) with reference number UPM/FPV/PS/3.2.1.551/AUP-R72.

References

1. Ohuchi M, Asaoka N, Sakai T, Ohuchi R. Roles of neuraminidase in the initial stage of influenza virus infection. *Microbes Infect* 2006;8:1287–1293.
2. Li S, Schulman J, Itamura S, Palese P. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J Virol* 1993;67:6667–6673.
3. Smith GE, Sun X, Bai Y, Liu YV, Massare MJ *et al*. Neuraminidase-based recombinant virus-like particles protect against lethal avian influenza A(H5N1) virus infection in ferrets. *Virology* 2017;509:90–97.
4. Webster RG, Pereira HG. A common surface antigen in influenza viruses from human and avian sources. *J Gen Virol* 1968;3:201–208.
5. Harris A, Cardone G, Winkler DC, Heymann JB, Brecher M *et al*. Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proc Natl Acad Sci U S A* 2006;103:19123–19127.
6. Johansson BE, Moran TM, Kilbourne ED. 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* 1987;84:6869–6873.
7. Getie-Kebtie M, Sultana I, Eichelberger M, Alterman M. Label-Free mass spectrometry-based quantification of hemagglutinin and neuraminidase in influenza virus preparations and vaccines. *Influenza Other Respi Viruses* 2013;7:521–530.
8. Wohlbold TJ, Nachbagauer R, Xu H, Tan GS, Hirsh A *et al*. Vaccination with adjuvanted recombinant neuraminidase induces broad heterologous, but not heterosubtypic, cross-protection against influenza virus infection in mice. *mBio* 2015;6:e02556–14.
9. Deroo T, Jou WM, Fiers W. Recombinant neuraminidase vaccine protects against lethal influenza. *Vaccine* 1996;14:561–569.
10. Chen Z, Kadowaki S, Hagiwara Y, Yoshikawa T, Matsuo K *et al*. Cross-Protection against a lethal influenza virus infection by DNA vaccine to neuraminidase. *Vaccine* 2000;18:3214–3222.
11. Webster RG, Reay PA, Laver WG. Protection against lethal influenza with neuraminidase. *Virology* 1988;164:230–237.
12. Guo Z, Chen L-mei, Zeng H, Gomez JA, Plowden J *et al*. Ns1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. *Am J Respir Cell Mol Biol* 2007;36:263–269.
13. Qiao C-L, Yu K-Z, Jiang Y-P, Jia Y-Q, Tian G-B *et al*. 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* 2003;32:25–31.
14. Skinner MA, Laidlaw SM, Eldaghayes I, Kaiser P, Cottingham MG. Fowlpox virus as a recombinant vaccine vector for use in mammals and poultry. *Expert Rev Vaccines* 2005;4:63–76.
15. Webster DP, Dunachie S, McConkey S, Poulton I, Moore AC *et al*. Safety of recombinant fowlpox strain FP9 and modified vaccinia virus Ankara vaccines against liver-stage P. falciparum malaria in non-immune volunteers. *Vaccine* 2006;24:3026–3034.
16. Hama Y, Kurokawa M, Imakita M, Yoshida Y, Shimizu T *et al*. Interleukin 12 is a primary cytokine responding to influenza virus infection in the respiratory tract of mice. *Acta Virol* 2009;53:233–240.
17. Tsurita M, Kurokawa M, Imakita M, Fukuda Y, Watanabe Y *et al*. 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* 2001;298:362–368.
18. Kostense S, Sun WH, Cottey R, Taylor SF, Harmeling S *et al*. Interleukin 12 administration enhances Th1 activity but delays recovery from influenza A virus infection in mice. *Antiviral Res* 1998;38:117–130.
19. van der Sluijs KF, van Elden LJR, Xiao Y, Arens R, Nijhuis M *et al*. Il-12 deficiency transiently improves viral clearance during the late phase of respiratory tract infection with influenza A virus in mice. *Antiviral Res* 2006;70:75–84.
20. Buchanan RM, Briles DE, Arulanandam BP, Westerink MA, Raeder RH *et al*. IL-12-mediated increases in protection elicited by pneumococcal and meningococcal conjugate vaccines. *Vaccine* 2001;19:2020–2028.
21. Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. *Infect Immun* 2003;71:4780–4788.
22. Baron SD, Singh R, Metzger DW. Inactivated Francisella tularensis live vaccine strain protects against respiratory tularemia by intranasal vaccination in an immunoglobulin A-dependent fashion. *Infect Immun* 2007;75:2152–2162.
23. Su BS, Chiu HH, Lin CC, Shien JH, Yin HS *et al*. Adjuvant activity of chicken interleukin-12 co-administered with infectious bursal disease virus recombinant VP2 antigen in chickens. *Vet Immunol Immunopathol* 2011;139:167–175.
24. Su BS, Yin HS, Chiu HH, Hung LH, Huang JP *et al*. Immunoadjuvant activities of a recombinant chicken IL-12 in chickens vaccinated with Newcastle disease virus recombinant HN protein. *Vet Microbiol* 2011;151:220–228.
25. Goldman A. Isolation of fibroblasts from chicken embryos. *CSH Protoc* 2006;2006:pdb.prot4475.
26. Balasubramaniam VR, Hassan SS, Omar AR, Mohamed M, Noor SM *et al*. Cellular transcripts regulated during infections with highly pathogenic H5N1 avian influenza virus in 3 host systems. *Virol J* 2011;8:196.
27. Qingzhong Y, Barrett T, Brown TD, Cook JK, Green P *et al*. Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). *Vaccine* 1994;12:569–573.
28. Mariatulqabtiah AR, Nor Majid N, Giotis ES, Omar AR, Skinner MA. Inoculation of fowlpox viruses coexpressing avian influenza H5 and chicken IL-15 cytokine gene stimulates diverse host immune responses. *APJMBB* 2019;27:84–94.
29. Laidlaw SM, Skinner MA. 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* 2004;85:305–322.
30. Falkner FG, Moss B. Transient dominant selection of recombinant vaccinia viruses. *J Virol* 1990;64:3108–3111.
31. Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P *et al*. 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* 1994;179:1273–1283.
32. Lui VWY, He Y, Falo L, Huang L. Systemic administration of naked DNA encoding interleukin 12 for the treatment of human papillomavirus DNA-positive tumor. *Hum Gene Ther* 2002;13:177–185.
33. Anderson RJ, Hannan CM, Gilbert SC, Laidlaw SM, Sheu EG *et al*. 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* 2004;172:3094–3100.
34. Cottingham MG, van Maurik A, Zago M, Newton AT, Anderson RJ *et al*. 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* 2006;13:747–757.
35. Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY *et al*. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* 1996;4:471–481.
36. Orange JS, Salazar-Mather TP, Opal SM, Spencer RL, Miller AH *et al*. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J Exp Med* 1995;181:901–914.