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

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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 105 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.
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 9-10-day-old embryonated eggs and maintained in 2% newborn bovine serum (NBS) 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′-ACCC AATATTTGCTACTCTTTCGTTGTTAC-3′) and N1-R (5′-AGGCAATATTTGCTACTCTTTCGTTGTTAC-3′) primers, with introduction of SspI site as underlined to facilitate blunt-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/NA N1) [27]. Chicken IL-12 gene was inserted downstream of a synthetic/hybrid promoter in vector pEfGpt12S, 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′- CGGAGAC CCATATCGAAGC-3′) and pEFL29-R (5′-CTGAAAG TAGAAATAATTTT-3′); and pPC1.X-F (5′-ATTTTTTT TTGACTCTATTG-3′) and pPC1.X-R (5′-ATCCCGATA CTAGTTATTGTTAC-3′). Cultures that yielded positive PCR amplicons were subjected to plasmid DNA isolation (QIAGEN Miniprep 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 10⁵ 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...
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. 1. Overview of the cloning strategy.](image)

(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.

![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).](image)
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

| 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 ($P \leq 0.05$).
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 6h) 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 at 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.

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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.

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**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 mean±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 (*).
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