Influenza A virus nucleoprotein derived from *Escherichia coli* or recombinant vaccinia (Tiantan) virus elicits robust cross-protection in mice

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**Abstract**

**Background:** Immunity to conserved viral antigens is an attractive approach to develop a universal vaccine against epidemic and pandemic influenza. A nucleoprotein (NP)-based vaccine has been explored and preliminary studies have shown promise. However, no study has explored the immunity and cross-protective efficacy of recombinant NP derived from *Escherichia coli* compared with recombinant vaccinia virus (Tiantan).

**Methods:** Recombinant NP protein (rNP) from influenza virus A/Jingke/30/95(H3N2) was obtained from *E. coli* and recombinant vaccinia virus (Tiantan) RVJ1175NP. Purified rNP without adjuvant and RVJ1175NP were used to immunize BALB/c mice intramuscularly. Humoral immune responses were detected by ELISA, while cell-mediated immune responses were measured by *ex vivo* IFN-γ ELISPOT and *in vivo* cytotoxicity assays. The cross-protective efficacy was assessed by a challenge with a heterosubtype of influenza virus A/PR/8/34(H1N1).

**Results:** Our results demonstrate that a high dose (90 μg) of rNP induced NP-specific antibodies and T cell responses that were comparable with those of RVJ1175NP in mice. Importantly, the survival ratio (36, 73, and 78%) of the vaccinated mice after the influenza virus A/PR/8/34(H1N1) challenge was rNP vaccine dose-dependent (10, 30, and 90 μg, respectively), and no significant differences were observed between the rNP- and RVJ1175NP-immunized (91%) mice.

**Conclusions:** Influenza A virus NP derived from *E. coli* or recombinant vaccinia (Tiantan) virus elicited cross-protection against influenza virus in mice, and the immune response and protective efficacy of rNP were comparable to RVJ1175NP. These data provide a basis for the use of prokaryotically expressed NP as a candidate universal influenza vaccine.

**Background**

Influenza virus causes a highly contagious and acute respiratory disease [1]. Vaccination is the primary strategy for preventing and controlling epidemic and pandemic influenza [2,3]. Currently, licensed influenza vaccines are trivalent live attenuated or inactivated killed virus vaccines, consisting of three strains of each virus (influenza A H1N1 and H3N2 and one influenza B) thought to be most prevalent in the upcoming influenza season [4,5]. However, these vaccines elicit neutralizing antibodies against the highly variable hemagglutinin (HA) of influenza virus, providing protection against homologous but non-antigenically distinct heterologous viruses. Thus, these vaccines must be frequently reformulated to match the circulating strains [6,7]. In addition, current commercial influenza vaccines are produced by propagating the virus in embryonated chicken eggs, which is time-consuming and requires one egg per vaccine dose [8,9]. Therefore, the development of a vaccine that induces cross-protection against variant subtypes of influenza A virus and which can be produced quickly at high quantities is desirable.

The highly conserved nucleoprotein (NP) of influenza A virus is an attractive candidate for a broad-spectrum
influenza vaccine [10-13]. NP could generate subtype cross-reactive cytotoxic T lymphocyte (CTL) immunity to accelerate viral clearance in mice and humans [14,15], and the non-neutralization antibodies induced by NP play a role in heterosubtypic immunity in mice [16,17].

Previous studies have demonstrated that NP induces heterosubtypic protection when used as a vaccine component. NP-based vaccines, including DNA vaccines [18,19], viral vector vaccines [20-22], peptide vaccines [23], protein subunit vaccines [24,25], and multiantigenic vaccines [26-28], can generate cross-protection. Recently, a phase I clinical trial was conducted in healthy adults using a modified vaccinia virus Ankara (MVA) vector expressing influenza NP and matrix protein 1 (MVA-NP+M1). In that study, a challenge with influenza H3N2 and H1N1 showed that the MVA-NP+M1 vaccine was safe and immunogenic in humans [29,30]. We previously constructed a recombinant vaccinia virus (Tiantan) RVJ1175NP expressing the NP of influenza virus A/Jingke/30/95(H3N2), which elicited significant protective efficacy in mice [20]. However, the production of this viral vector vaccine was complicated, and the pre-existing vector protein may interfere with vaccination efficacy. Thus, it is important to identify a convenient method for large-scale NP production that does not require embryonated eggs or cell culture.

Escherichia coli expression systems can facilitate the rapid and economical production of recombinant proteins [31,32]. The expression and purification of a single antigenic protein in bacterial culture may be a simple and rapid strategy for generating large quantities of influenza vaccine [33-36]. However, few studies of the immunogenicity and protective efficacy of recombinant NP expressed in E. coli have been performed, and no investigation has compared the efficacy of NP from prokaryotic expression systems with eukaryotic expression systems. To determine whether E. coli-expressed NP could be used as a broad-spectrum influenza vaccine, a comparison of the immunogenicity and protective efficacy of prokaryotic- and eukaryotic-expressed NP is required.

In this study, we purified recombinant NP (rNP) from influenza virus A/Jingke/30/95(H3N2) expressed in E. coli, and constructed a recombinant vaccinia virus (Tiantan) RVJ1175NP expressing the same NP. The immunogenicity and cross-protective efficacy of the rNP was compared with that of RVJ1175NP in BALB/c mice. We found that the E. coli-expressed rNP induced NP-specific antibodies and a T cell response at high doses. Additionally, the cross-protective efficacies of the rNP against a lethal challenge with heterosubtype influenza virus A/PR/8/34(H1N1) were comparable to those of RVJ1175NP. These data provide a basis for the use of E. coli-expressed NP as a potential universal influenza vaccine.

**Results**

**Characterization of rNP purified from E. coli or RVJ1175NP**

To assess the efficacy of rNP expressed in E. coli as a candidate universal influenza vaccine, we constructed an expression plasmid, pET30a-NP, to express rNP of influenza A/Jingke/30/95(H3N2) in E. coli BL21(DE3) (Figure 1A), as well as a recombinant vaccinia virus RVJ1175NP expressing NP (Figure 1D).

The NP gene of influenza virus A/Jingke/30/95(H3N2) was optimized and cloned into pET30a for expression in BL21(DE3) (Figure 1A). Untagged soluble recombinant protein was purified using ion exchange and size exclusion chromatography. SDS-PAGE demonstrated that the rNP was ≥90% pure (Figure 1B). The presence of purified rNP was confirmed by Western blot analysis with mouse anti-NP polyclonal antibodies (55 kDa), whereas the control BL21(ED3) did not produce NP protein (Figure 1C). BHK cells infected with RVJ1175NP or RVJ1175 were analyzed by Western blotting with the same mouse anti-NP polyclonal antibodies (Figure 1E). Our results showed that RVJ1175NP expressed the proteins as expected (55 kDa), whereas the control RVJ1175 did not produce the protein. The above results demonstrate that NP was successfully expressed and purified.

**Comparable NP-specific antibody responses were induced in both rNP- and RVJ1175NP-immunized mice**

The immunization schedule is shown in Figure 2 and Table 1. To analyze NP-specific humoral immunity, serum samples from four mice per group were collected ten days after each priming and boosting event, and purified NP was used to coat a 96-well plate to detect NP-specific IgG antibodies.

As shown in Figure 3, after the priming immunization, the NP-specific antibody titer in each rNP-vaccinated group was slightly lower than that in the RVJ1175NP-vaccinated group (2×10³-4×10⁵ vs. 8×10⁵). Statistically significant differences were observed between 10 μg NP-, 30 μg NP-, and RVJ1175NP-vaccinated groups (P<0.05), as well as between the 10 and 90 μg NP-vaccinated groups (P<0.05). No statistically significant differences were observed between the 90 μg NP- and RVJ1175NP-vaccinated groups. However, after boosting immunization, the NP-specific antibody titer increased markedly (2×10³-4×10⁵ vs. 4×10⁵-1×10⁶, P<0.01), and no significant differences were found between the rNP-vaccinated groups, or between the rNP- and RVJ1175NP-vaccinated groups. The NP-specific antibody titer in each rNP-vaccinated group was slightly higher than that in the RVJ1175NP-vaccinated group (4×10⁵-1×10⁶ vs. 3×10⁵).

These results indicate that rNP elicited comparable NP-specific humoral immunity to RVJ1175NP.
Comparable moderate T cell immune responses were induced in high-dose rNP- and RVJ1175NP-immunized mice

To detect NP-specific T cell-mediated immune responses, five mice from each group were sacrificed ten days after the last immunization, and specific cellular immune responses against the NP147-155 epitope were detected by ex vivo IFN-γ ELISPOT and in vivo cytotoxicity assays, as described.

To identify IFN-γ-positive SFC against the NP147-155 epitope, we performed ex vivo IFN-γ ELISPOT assays. As indicated in Figure 4A, compared with the PBS control group (<5 SFC/10⁶ splenocytes), no significant IFN-γ-positive SFC against the NP147-155 epitope were detected in the 10 or 30 μg NP-immunized mice (<10 SFC/10⁶ splenocytes). However, a significant number of SFC were detected in both the 90 μg NP- and RVJ1175NP-vaccinated groups (19 ± 1 SFC/10⁶ splenocytes, 35 ± 1 SFC/10⁶ splenocytes, respectively), and the immune responses were significantly different when
compared with PBS-immunized mice (P<0.05 and 0.01, respectively) (Figure 4A).

To assess the lytic potential of effector CD8+ T cells in the mice, we examined cell cytotoxicity in vivo by transferring target cells pulsed with NP147-155 into mice. The results of our in vivo cytotoxicity assays were in agreement with those of the ex vivo IFN-γ ELISPOT assays. As shown in Figure 4B, compared with 8% of the NP147-155 peptide-pulsed targets eliminated in PBS control mice, neither the 10 (10%) nor the 30 (4.5%) μg NP-vaccinated groups showed any marked cytotoxic effect. A cytotoxic effect was detected, however, in the 90 μg NP- (17%) and RVJ1175NP-vaccinated groups (45%). Significant differences were observed between the RVJ1175NP- and PBS-vaccinated groups (P<0.05), but not between the RVJ1175NP- and 90 μg NP-vaccinated groups. Taken together, these results indicate that a high dose of rNP elicited a weak T cell response, similar to RVJ1175NP.

Comparable protective efficacies against a lethal challenge with heterosubtype influenza virus A/PR/8/34 (H1N1) were induced in both the rNP- and RVJ1175NP-immunized mice

To assess the cross-protection provided by rNP and RVJ1175NP, we challenged the immunized mice with 10×MLD50 of influenza virus A/PR/8/34 (H1N1) ten days after the last immunization (eleven mice per group) and monitored their weight changes and survival ratios for three weeks.

The observed weight changes are shown in Figure 5A. Body weight decreased to the lowest level in all groups at days 8-9 after the influenza virus A/PR/8/34(H1N1) challenge. Subsequently, body weight decreased continuously in the PBS group until day 17 when the last mouse died. In the RVJ1175NP- and each rNP-vaccinated group, the body weights of the mice returned to baseline at day 9. The body weights were restored to baseline most rapidly in the 90 μg NP-immunized group. The body weights were completely restored in each group of surviving mice at day 21. Statistically significant differences in weight loss were observed between the rNP- and PBS-immunized groups on days 7-21 (P<0.05), between the RVJ1175NP- and PBS-immunized groups, and between the 90 μg NP and other vaccinated mice on days 7-21 (P<0.05). No significant differences were observed between the 10 μg NP-, 30 μg NP-, and RVJ1175NP-vaccinated mice.

The survival ratios are shown in Figure 5B and Table 2. Compared with the PBS group (0%, 0/11), the survival ratio in the RVJ1175NP group was 91% (10/11, P=0.0001), while the survival ratios in the rNP group at doses of 10, 30, and 90 μg were 36 (4/11, P=0.09), 73 (8/11, P=0.01), and 78% (7/9, P=0.005), respectively. Excluding the 10 μg NP group, statistically significant differences in the survival ratios were observed between the rest of the rNP- and PBS-vaccinated mice (P<0.01). No significant differences were observed between the rNP- and RVJ1175NP-vaccinated mice.

The above results indicate that rNP elicited comparable cross-protection to RVJ1175NP in mice, and the survival ratios tended to increase with a higher dose of rNP.

### Table 1 Immunization program

| Group | Immunogen | Dose | Number of mice | Immunization route |
|-------|-----------|------|----------------|-------------------|
| 1     | PBS       | 0    | 30             | i.m.              |
| 2     | NP        | 10 μg| 30             | i.m.              |
| 3     | NP        | 30 μg| 30             | i.m.              |
| 4     | NP        | 90 μg| 30             | i.m.              |
| 5     | RVJ1175NP | 2×10⁷ PFU | 30 | i.m. |

Note: Amino acid sequences of NP derived from A/Jingke/30/95(H3N2).

**Figure 3** Comparable NP-specific antibody responses were induced in both the rNP- and RVJ1175NP-immunized mice. BALB/c mice were immunized i.m. with 10, 30, or 90 μg of rNP or PBS alone, three times, two weeks apart. Mice immunized with RVJ1175NP (2×10⁷ PFU) twice, four weeks apart, were used as positive controls. Serum samples were collected ten days after priming and boost immunization. NP-specific IgG responses were measured by ELISA as described. All data are shown as the log10 geometric mean titer ± standard deviation of four mice from each group.
An influenza virus A/PR/8/34(H1N1) challenge boosted NP-specific immunity and increased mouse survival

To explore the possible mechanisms of protection, 35 days after influenza virus A/PR/8/34(H1N1) challenge, humoral and cell-mediated immune responses were detected in the surviving mice. An examination of the PBS group was not possible as none of the mice survived.

Based on our ELISA results (Figure 6A), NP-specific humoral immune responses were slightly increased in the surviving mice (P<0.05). The NP-specific IgG titer increased from pre-challenge levels of 4×10^5-1×10^6 to 3×10^6-5×10^6 in each immunized group. No significant differences were found between these groups. The strength of the humoral immune response in the rNP groups was similar to that in the RVJ1175NP group.

Based on our ex vivo IFN-γ ELISPOT assays (Figure 6B), the number of IFN-γ-positive SFC against NP[147-155] was markedly increased in the surviving mice (P<0.01). Compared with pre-challenge (<50 SFC/10^6 splenocytes), the average number of SFC was 756, 802, 1712, and 1080 SFC/10^6 splenocytes in the 10, 30, and 90 μg rNP group and in the RVJ1175NP group, respectively. The number of SFC increased significantly (P<0.01), and tended to increase with higher immunization doses. Significant differences were observed only between the 10 μg NP- and RVJ1175NP-vaccinated surviving mice (P<0.05), and differences were not observed between the rNP-immunized surviving mice at different doses, nor between the rest of the rNP groups and the RVJ1175NP-immunized groups.

As revealed by our in vivo cytotoxicity assays (Figure 6C), NP[147-155] peptide-pulsed target killing was increased markedly in the surviving mice (P<0.01). Compared with the pre-challenged mice (30%), the cytotoxic rates were increased to 93, 96, and 96% with 10, 30, and 90 μg of rNP in the surviving mice, respectively, and increased cytotoxic effects were observed in the surviving RVJ1175NP mice (from 45 to 100%). No differences were observed between each group.
The above results indicate that a challenge with influenza virus increased NP-specific immune responses in the surviving mice, especially NP-specific cell-mediated immune responses.

Discussion

Influenza is a major cause of morbidity and mortality worldwide. Vaccination is the most effective strategy to control influenza epidemic and pandemics. However, currently licensed influenza vaccines are annual vaccines that induce subtype-specific virus-neutralizing antibodies against the highly variable surface antigen HA; thus, they do not protect against new subtypes or antigenic variants. In addition, conventional egg-dependent manufacturing is time-consuming and expensive. Therefore, it is important to produce a vaccine that induces cross-protection and which can be produced rapidly and inexpensively. Immunity to conserved NP antigens is an attractive approach for developing universal influenza vaccines, and a subunit vaccine based on a prokaryotic production system could be rapidly and inexpensively produced. Although many studies have explored the protective capacity of NP as a component of a DNA vaccine or expressed by viral vectors, it remains unclear whether similar results could be

![Graph](image_url) Figure 5 Comparable protective efficacies against a lethal challenge by heterosubtype influenza virus A/PR/8/34(H1N1) were induced in both rNP- and RVJ117SNP-immunized mice. Ten days after the last immunization, mice were anesthetized using a pentobarbital sodium solution and challenged intranasally with a lethal dose (10×MLD50) of influenza virus A/PR/8/34(H1N1). Weight changes and the survival of the mice were monitored for three weeks (n = 11 per group, except n = 9 for the 90 μg rNP group, as two mice were killed while being anesthetized). (A) Weight changes after the viral challenge. The average percent of the initial weight is expressed as a percentage of the examined day relative to the weight prior to the challenge. (B) Percent of mice surviving after the challenge. Survival was analyzed using the Kaplan-Meier log-rank test. Significant differences in survival were compared with the PBS control groups (*, P<0.05; **, P<0.01)

Table 2 Mouse survival calculation after influenza virus A/PR/8/34(H1N1) challenge

| Immunogen   | Number surviving/total | Survival ratio (%) | P (versus PBS) |
|-------------|------------------------|--------------------|---------------|
| PBS         | 0/11                   | 0                  | /             |
| 10 μg of rNP| 4/11                   | 36.4               | 0.09          |
| 30 μg of rNP| 8/11                   | 72.7               | 0.01          |
| 90 μg of rNP| 7/9                    | 77.8               | 0.005         |
| RVJ117SNP   | 10/11                  | 91.0               | 0.0001        |

Note: The immunized mice were challenged with 10×MLD50 of influenza virus A/PR/8/34(H1N1) ten days after the last immunization. N = 11 per group, except n = 9 for the 90 μg rNP group because two mice were killed while being anesthetized. No significant differences were observed between each rNP group, or between the rNP- and RVJ117SNP-immunized groups. The P-values shown here were calculated compared to the PBS control group.
obtained with rNP. In this study, we compared the immunogenicity and cross-protection of rNP from *E. coli* with recombinant vaccinia (Tiantan) virus RVJ1175NP in mice. Our results demonstrate that a high dose of rNP induced comparable anti-NP antibody and T cell responses to RVJ1175NP in mice. Importantly, the protective efficacies of rNP were comparable with that of RVJ1175NP. These data provide a basis for the use of *E. coli*-expressed NP as a candidate universal influenza vaccine for further study.

A wide variety of NP-based vaccine formulations have been evaluated for cross-protection from this highly conserved antigen [18-30]. Recombinant vaccinia viruses are conventionally used to study the immunogenicity of foreign proteins [37-42]. The vaccinia virus Tiantan strain was used as a vaccine against smallpox in China before 1980, and it is now widely used as a vector [43,44]. We previously used RVJ1175 expressing the potential cross-protective antigens of NP, Matrix protein 1 (M1) and Polymerase basic 1 (PB1) of influenza A/Jingke/30/95(H3N2) with the vaccinia virus Tiantan strain to induce cross-protection in Balb/C mice, the results indicated that NP is the most effective antigen among the antigens we tested, and the survival rate of the RVJ1175NP immunized mice could achieved as high as almost 100% against the lethal challenge of influenza virus A/PR/8/34 (H1N1) with a challenge dose ranged from 1LD50 to 5LD50[20]. However, the complicated production process and pre-existing vector immunity may interfere with the vaccine. *Escherichia coli*
coli-based expression systems are the simplest and fastest way to generate large quantities of influenza vaccine. However, no investigation has compared the immunity and cross-protective efficacy of rNP derived from E. coli with recombinant vaccinia virus (Tiantan). To explore the potential of E. coli-expressed NP as a candidate universal influenza vaccine, we compared the immunogenicity and efficacy of three doses of rNP (10, 30, and 90 μg) from E. coli with that of RVJ1175NP.

Antibodies against NP are non-neutralizing, and although viral infection and some replication continues to occur, it can limit viral replication and reduce illness severity [16,17,45,46]. In the present study, the results of NP-specific IgG detection demonstrated that the E. coli-expressed NP without adjuvant could elicit a strong humoral immune response, similar to RVJ1175NP. Recently, Lamere et al. [45] demonstrated that systemic immunization with NP accelerated the clearance of a 2009 pandemic H1N1 influenza virus isolate in an antibody-dependent manner, and that anti-NP IgG specifically promoted influenza virus clearance in mice through a mechanism involving both FcRs and CD8+ T cells [46]. These studies strongly suggest that antibodies induced by immunization with NP can be used to elicit cross-protection.

Currently, NP is thought to play a role in protection mainly through the CTL cross-reaction. Several previous studies have confirmed that NP-based vaccines inducing cell-mediated immunity can provide cross-protection against a heterosubtypic influenza virus challenge [14,15,18-28]. In this study, cell-mediated immune responses were assessed by measuring ex vivo IFN-γ secretion in splenocytes and in vivo cytotoxicity against the CD8+ T cell epitope NP147-155. Although few ex vivo IFN-γ-positive SFC and weak in vivo cytotoxicity were induced at low doses (10 or 30 μg) of NP, a weak cellular immune response could be detected at high doses (90 μg) of NP. Such T cell-mediated immunity was comparable with that induced by RVJ1175NP. These results indicate that the weak cellular immune response was boosted by increasing the immunization dose or by using an adjuvant. Additionally, although the NP-specific CD8+ T cell immunity of NP was weak in the present study, it might establish long-term memory cells. Therefore, a later time point should be investigated.

To assess the cross-protection effect provided by rNP and RVJ1175NP, 10×MLD50 of influenza virus A/PR/8/34 (H1N1) was used to challenge the immunized mice. It should be noted that, according to the amino-acid sequence alignment, the differences in amino-acid sequence between the influenza virus strains of A/Jingke/30/95 (H3N2) and A/PR/8/34 (H1N1) is 40.7% for HA and 92.6% for NP respectively (Data was analyzed by the software of Clustal X (1.8), detailed information would be seen in Figure 7 in the section of supporting file). The observed weight changes were comparable between the rNP-vaccinated mice and the RVJ1175NP-vaccinated mice. Notably, mice immunized with 90 μg of NP showed the earliest and best recovery (better than RVJ1175NP). These results indicate that rNP effectively relieved the symptoms of influenza and reduced the disease severity. In addition, the survival ratios (36, 73, and 78%) were rNP vaccine dose-dependent (10, 30, and 90 μg, respectively), and no statistical differences were observed between the rNP-immunized mice and RVJ1175NP (91%). Consistent with previous studies, the survival protective efficacy in our study was in accordance with the strength of the pre-challenge immune response [47,48]. The above results indicate that E. coli-expressed NP elicited cross-protection in the mice, similar to RVJ1175NP, and that the efficacy was correlated with the magnitude of the immunological response.

To explore the possible mechanisms of protection, immune responses were evaluated in the surviving mice. Compared with pre-challenge, both the NP-specific humoral and cell-mediated immune responses were increased in the surviving mice. These results suggest that NP-specific humoral and cell-mediated immune responses are closely correlated with protection. They also suggest that the protection efficacy might be further enhanced by an outbreak of seasonal influenza [49-51]. However, further support of this hypothesis is important to promote universal flu vaccine development. As under the lethal challenge of influenza virus, the examination of the PBS group was not possible as none of the mice survived, there was lack a proper control in this experiment, so further study should be designed to include an unvaccinated group infected with a sub-lethal dose of influenza virus to quantify the immune response values elicited after infection in animals without previous vaccination.

It also should be noted that, the mechanisms that rNP could induce cross-protection are complex. The general consensus favors the idea that rNP can induce CTL responses that can kill infected cells and help the host recovery from the infection [14,15,18-28], while the non-neutralizing antibodies induced by rNP contribute little to providing protective ability. However, recent studies in mice also demonstrated that antibodies against rNP also contribute to heterosubtypic immunity, and thus can limit viral replication and reduce illness severity, maybe through a mechanism involving both FcRs and CD8+ T cells [16,17,45,46]. In our experiment, in spite of the very low levels of CTL elicited in the rNP and RVJ1175NP vaccinated groups, survival rates were achieved as high as 73%, 78%, and 91% in the 30 μg rNP, 90 μg rNP and RVJ1175NP vaccinated group after the lethal challenge of influenza virus, respectively. The
the mechanism of cross-protection induced by rNP should be investigated by much more studies such as more immune response index should be examined, and passive serum transfer experiment et.al may be helpful to test the role of NP specific serum antibody in providing protection.

In addition, although previous studies have shown that mucosal immunization with NP protein could induce CTL responses, thus generate cross-protective response. And IgG produced by intraperitoneal or intramuscular injection of NP protein was not sufficient to protect mice against heterologous influenza virus, we confirmed the intramuscular injection of NP protein could provide cross-protection in Balb/C mice in this study, such protection would contribute the vaccine form we used, the immune path way of such vaccine, the remnant LPS adjuvants effect in the experiment, the immune responses type induced by the vaccine, and so on. In the future study, much more detailed information should be investigated to learn the structure of the rNP, as the rNP was in soluble form at the end of the fermentation, and the structure of the rNP may be polymer forms, thus would be more immunogenic. In addition, the antibody subtypes induced by the rNP may also be detected in future, as the IgG1, IgG2a subtype may also influence the protective efficacies of the vaccine. And more detailed indicators for the cellular immune response of NP should be investigated to learn the role of both CD8+ and CD4+ T cell immune response in the contribution of the cross-protection.

Conclusions

In summary, our study demonstrates that the immune response and protective efficacy of rNP from *E. coli* were comparable to those of RV1175NP. These data provide a basis for the use of *E. coli*-expressed NP as a candidate universal influenza vaccine for further study. To the best of our knowledge, this is the first study to compare the immunity and protective efficacy of *E. coli*-expressed and vaccinia virus expressed NPs. Further work to improve the cross-protective immunity and efficacy of rNP using adjuvants or by combining it with other protective antigens, and additional influenza virus subtype challenge studies to examine the level of broad-spectrum protection, are required.

Materials and methods

Preparation of rNP and RVJ1175NP

The amino acid of NP protein was based on influenza virus A/Jingke/30/95(H3N2). The NP gene sequence encoding the full-length of NP protein was optimized according to the codon bias of *E. coli* [52]. The recombinant NP expression vector pET30a-NP was
constructed by inserting the NP gene between the NdeI and EcoRI restriction sites. Next, rNP was expressed in E. coli BL21(DE3) cells transformed with pET30a-NP. Briefly, bacteria were grown to log phase at cell concentration of OD600 was 0.6-0.8 in 2×YT medium with 100 μg/mL kanamycin at 37°C, and protein expression was induced by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.1 mmol/L. After 10 h of further incubation at 25°C, the cells were harvested by centrifugation, and SDS-PAGE analysis showed that rNP was mainly in the supernatants of the cell lysis, thus the rNP was expressed in soluble form in E. coli fermentation. Then the untagged soluble recombinant protein was purified using ion exchange exclusion chromatography by DEAE Sepharose Fast Flow column and then size exclusion chromatography by Superdex S200 column. After concentration and filter sterilization, the protein concentration was determined using a commercial bicinchoninic acid (BCA) assay, and rNP concentration was 1 mg/mL in the final purified product. Endotoxin levels were determined using the Tachypleus Amebocyte Lysate assay (Chinese Horsehoe Crab Reagen Manufactory, Xiamen, China) as directed by the manufacturer, and the endotoxin level of the rNP was about 2000 EU/mg. The final purified protein was stored in PBS at -70°C until use.

The original vaccinia virus Tiantan strain and dual-promoter insertion vector pJSA1175 were produced in our laboratory [20]. The NP gene of influenza virus A/Jinke/30/95(H3N2) was inserted into the SmaI site of pJSA1175. Recombinant vaccinia virus was produced by the transfection of pJSA1175-NP into CEF cells that were infected with vaccinia Tiantan strain, and was designated as RVJ1175NP (Figure 1). RVJ1175NP induced marked cross-immune protection in BALB/c mice [20], and was used to immunize mice as a positive control.

Peptide and influenza viruses
The H-2d restricted class I peptide NP147-155 (TYQR-GLGSSA) was synthesized commercially (Beijing Scilight Biotechnology Ltd. Co., Beijing, China). The purity of the peptide was >90% following HPLC and mass spectrum analysis. NP147-155 is the CTL epitope of NP in BALB/c (H-2d) mice and was selected as the optimal peptide by mapping influenza A/PR/8/34(H3N2) NP peptide pools [53]. The peptide was used at 5 μg/mL to analyze NP-specific T cell immune responses using ex vivo gamma interferon enzyme-linked immunospot (IFN-γ ELISPOT) assays and in vivo cytotoxicity assays. Stimulation with PMA (50 ng/mL) and ionomycin (1 μg/mL) was used as a positive control to generate and detect antigen-specific T cells by ELISPOT. Mouse-adapted influenza virus A/PR/8/34(H1N1) was used as the challenge strain. The viruses were propagated in allantoic fluid from nine-day-old embryonated eggs at 34°C for two days. The allantoic fluid was collected, aliquoted, and stored at -70°C until use. The viral 50% lethal dose was measured in BALB/c mice (MLD50) and the TCID50 titer was detected in MDCK cells [54]. All experiments with live influenza virus were performed in a biosafety level 2 containment facility.

SDS-PAGE and Western blotting
rNP purified from E. coli or expressed from RVJ1175NP was analyzed for size and purity by SDS-PAGE and Western blotting using mouse polyclonal antibodies against influenza virus NP. For the rNP, both purified protein and BL21(DE3) cell controls were lysed in SDS-PAGE sample loading buffer and then separated by SDS-PAGE, followed by staining with Coomassie brilliant blue R250. For the RVJ1175NP, BHK cells infected with RVJ1175NP or control RVJ1175 were collected after 48 h, then processed by cell lysis and separated by SDS-PAGE. For Western blot analysis, the lysates separated by SDS-PAGE were transferred by electroblotting to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked for 1 h in 5% skim milk at 37°C and then incubated with polyclonal antibodies in 2% skim milk for 1 h at 37°C. After being washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST), the membrane was subsequently incubated in horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibodies. Binding signals were visualized with 3,3,5,5-tetramethylbenzidine (TMB) as the substrate.

Immunization and challenge
Five- to six-week-old female BALB/c mice were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). All mouse experiments in this study followed the Regulations for Administration of Laboratory Animals of the People’s Republic of China.

The mice were immunized intramuscularly (i.m.) three times, two weeks apart with 10, 30, or 90 μg of rNP or PBS alone, respectively. Age- and sex-matched mice were immunized twice, four weeks apart with 2×10⁵ PFU per mouse of RVJ1175NP as a positive control. Blood samples were collected ten days after each immunization. Ten days after each immunization, five mice per group were sacrificed for cellular immune response assays (ex vivo IFN-γ ELISPOT assays or in vivo cytotoxicity assays). Ten days after the last immunization, eleven mice in each group were lightly anesthetized using a pentobarbital sodium solution and were challenged intranasally with 50 μl of viral suspension containing 10⁴ TCID50 (10×MLD50) of influenza A/
PR/8/34(H1N1). Survival and weight loss were monitored daily for three weeks. The challenge experiment was repeated three times.

Enzyme-linked immunosorbent assay (ELISA)
Costar 96-well plates were coated with purified NP at a concentration of 2 μg/ml at 4°C overnight. The plates were then washed with PBST and blocked with 2% bovine serum albumin (BSA) in PBS. The test samples were serially diluted in PBS containing 1% BSA and incubated at 37°C for 1 h. Diluted HRP-linked goat-anti-mouse IgG antibodies (100 μl) were added to each well, and the plates were incubated at 37°C for 1 h. TMB substrate solution (100 μl) was then added to each well. After a 5-min incubation at room temperature in the dark, the reaction was stopped by adding 50 μl of 2 M H2SO4 per well and the absorbance was measured at 450 nm. The antibody titer was defined as the reciprocal of the highest dilution that yielded an OD450 value ≥2.1 times of the mean value of naïve mouse serum.

IFN-γ ELISPOT assay
The number of NP-specific IFN-γ-secreting cells in mice was counted using commercial ELISPOT assay kits (BD Biosciences) as per the manufacturer’s instructions. Briefly, anti-mouse IFN-γ monoclonal antibodies were coated on multiscreen 96-well plates at 4°C overnight. Next, the plates were washed three times and blocked for 2 h with RPMI 1640 containing 10% FBS (GIBCO) at room temperature. Spleen mononuclear cells (SMNCs) were obtained after the red cells in the spleen cell suspension were lysed. Then the freshly isolated splenocytes (5×10^6) were transferred to each well and NP1471-155 was added at a final concentration of 4 μg/ml. Cells without the peptide were used as a negative control and cells with PMA (50 ng/ml) and ionomycin (1 μg/ml) were used as positive controls. Following incubation for 20-24 h at 37°C in a 5% CO2 incubator, the cell suspensions were aspirated. All wells were washed four times with PBST, biotinylated detection antibody was added, and the plates were incubated for 2 h at room temperature. After four washes, streptavidin horseradish peroxidase antibody was added at 100 μl per well for 1 h at room temperature. Following four more washes, 100 μl of freshly prepared 3-amino-9-ethylicarbazole substrate solution was added for 15-30 min at room temperature in the dark to yield colored spots. Finally, the reaction was stopped by thoroughly rinsing with tap water. The plates were air-dried and stored in the dark until analysis. The number of spots was analyzed with a fully automated computer-assisted video image analysis system (Bioreader 4000; Bio-Sys, Karben, Germany). The average number of spot-forming cells (SFC) was adjusted to 1×10^6 splenocytes for data display.

In vivo cytotoxicity assay
An in vivo cytotoxicity assay was performed as described by Byers et al. [55]. Briefly, to prepare target cells for in vivo cytotoxic activity detection, splenocytes from naïve BALB/c mice were washed and divided into two populations. One population was pulsed with 10 μg/ml NP1471-155, incubated at 37°C for 4 h, and labeled with a high concentration of CFSE (10 μM) (CFSEhigh cells). The second target population was left without peptide and was labeled with a low concentration of CFSE (1.0 μM) (CFSElow cells). For intravenous injection, an equal number of cells from each population were mixed together, such that each mouse received a total of 1×10^7 cells in 100 μl of PBS. The cells were injected into mice vaccinated previously with PBS, rNP, or RVJ1175NP. Specific in vivo cytotoxicity was determined by collecting the spleen from recipient mice 20 h after injection, and labeled fluorescent target cell populations were detected based on their differential CFSE fluorescence intensities by flow cytometry. Decreased numbers of CFSEhigh cells indicated in vivo cytotoxicity. The percentage of specific killing was calculated as follows: Cytotoxicity = [1-(Ratio of naïve group/Ratio of experimental group)] ×100; Ratio = percentage CFSElow/percentage CFSEhigh.

Statistical analysis
Statistical analyses were performed with GraphPad Prism version 5.01 (GraphPad Software, Inc., 2007) and the SPSS software package (release 12.1; SPSS Inc., Chicago, IL). Comparisons of the mean immune responses among the mouse groups were performed using analyses of variance with an unpaired t-test. Comparisons of antibody titers among the treatment groups were performed using Student’s t-test. Comparisons of the percentage of specific killing were performed with Fisher’s exact test. Comparisons of the loss of body weight and survival curves were calculated by t-tests and the log-rank (Mantel-Cox) test. All reported P-values were two-sided; values <0.05 were considered to be statistically significant.

Animal ethics statement
This mouse study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Chinese Center for Disease control and prevention. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute for Occupational Health and Poison Control (Permit Number: EAWE-2010-029). Serum was obtained by orbital sinus puncture. In the ELISPOT assay and in vivo CTL assay, mice were sacrificed by cervical dislocation. Challenge experiment was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. After influenza virus challenge, mice were monitored closely for
three weeks for signs of illness. Any animals in a moribund condition were euthanized.

Supporting data file

Figure 7. The amino-acid sequence alignment between the HA and NP of the influenza virus strains of A/Jingke/30/95 (H3N2) and A/PR/8/34 (H1N1). According to the amino-acid sequences alignment by the software of Clustal X (1.8), the results demonstrated that the differences in amino-acid sequence between the influenza virus strains of A/Jingke/30/95 (H3N2) (referred as JK3 in the graph) and A/PR/8/34 (H1N1) (referred as PR8 in the graph) is 40.7% for HA and 92.6% for NP respectively. The data sets supporting the results of this article is included within the article.

Abbreviations

BSA: Bovine serum albumin; CTL: Cytotoxic T lymphocyte; ELISA: Enzyme-linked immunosorbent assay; ELISPOT: Enzyme-linked immunospot; HA: Hemagglutinin; M1: Matrix protein 1; MLD50: 50% lethal dose in mice; NP: Nucleoprotein; PBS: Phosphate buffered saline; SFC: Spot Forming Cell; TCID50: 50% tissue culture infection dose.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

BH and WW generated the E. coli expression plasmids, expressed and purified the recombinant NP protein, performed immunogenicity studies in mice, and drafted the manuscript. RL participated in generating the viral construct and detection. XW, TJ, XQ and YG participated in performing the immunogenicity studies in mice. WT participated in designing the study and construct and detection. XW, TJ, XQ and YG participated in performing the immunogenicity studies in mice. All authors read and approved the manuscript.

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References

1. Cox NJ, Subbarao K: Influenza. Lancet 1999, 354:1277–82.
2. Nichol KL, Treanor JJ: Vaccines for seasonal and pandemic influenza. J infect Dis 2006, 194(Suppl 2):S111–S118.
3. Lambert LC, Fauci AS: Influenza vaccines for the future. N Engl J Med 2010, 363(21):2036–44.
4. Sambhara S, Stephenson I: Moving influenza vaccines forward. Expert Rev Vaccines 2009, 8(4):375–7.
5. Centers for Disease Control and Prevention (CDC): Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). 2011, Morb Mortal Wkly Rep 2011, 60(33):1128–32.
6. Carrat F, Flahault A: Influenza vaccine: the challenge of antigenic drift. Vaccine 2007, 25(39–40):6852–62.
7. Russell CJ, Webster RG: The genesis of a pandemic Influenza virus. Cell 2005, 123(3):368–71.
8. Palese P: Making better influenza virus vaccines? Emerg Infect Dis 2006, 12(1):61–65.
9. Fedson DS: Preparing for pandemic vaccination: an international policy agenda for vaccine development. J Public Health Policy 2005, 26(1):4–29.
10. Shu LL, Bean WJ, Webster RG: Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. J Virol 1993, 67(5):2723–9.
11. Portela A, Digard P: The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J Gen Virol 2002, 83(Pt 4):723–34.
12. Heini AT, Miotto O, Srinivasan KN, Khan AM, Zhang GL, Brusic V, Tan TW, August JT: Evolutionarily conserved protein sequences of influenza A viruses, avian and human, as vaccine targets. PLoS One 2007, 2(11):e1190.
13. Price GE, Sobolevsky MR, Lo CY, Misplon JA, Pappas C, Houser KV, Tumpey TM, Epstein SL: Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. Vaccine 2009, 27(47):6512–21.
14. McMichael AJ, Gojch FM, Noble GR, Beare PA: Cytotoxic T-cell immunity to influenza. N Engl J Med 1983, 309(11):13–17.
15. Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caullfield MJ: Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J Virol 1998, 72(7):5646–53.
16. Caraglieri DM, Karninski DA, Moquin A, Harkton L, Randall TD: A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J Immunol 2008, 180(4):1168–76.
17. Rangel-Moreno J, Caraglieri DM, Misra RS, Kusser K, Harkton L, Moquin A, Lund FE, Randall TD: B cells promote resistance to heterosubtypic strains of influenza via multiple mechanisms [J]. J Immunol 2008, 180(1):454–63.
18. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dvarki UJ, Gromkowski SH, Deck RR, Dewitt CM, Friedman A, et al: Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1991, 250(5012):1745–9.
19. Epstein SL, Kong WP, Misplon JA, Lo CY, Xu L, Nabel GJ: Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 2005, 23(46–47):5401–10.
20. Li RQ: Immunologic characteristics of non-major protective antigens for influenza A virus. Beijing: Institute for Virus Disease Control and Prevention, Chinese Center for Disease Control and Prevention; 2006.
21. Attebo AD, Girelman AK, Smirnov YA, Piskaeva LM, Zakhova LG, Pathyvkina GV, Shmarov MM, Zhimov OP, Varich NP, Ilyinskii PO, Shneider AM: Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. Arch Virol 2006, 151(Suppl 2):921–31.
22. Roy S, Kobinger GP, Lin L, Figueredo J, Calcedo R, Kobasa D, Wilson JM: Partial protection against H5N1 influenza in mice with a single dose of a chimpanzee adenovirus vector expressing nucleoprotein. Vaccine 2007, 25(39–40):6845–51.
23. Jeon SH, Yedidia TB, Amon R: Intranasal immunization with synthetic recombinant vaccine containing multiple epitopes of influenza virus. Vaccine 2002, 20(21–22):2772–80.
24. Wrath DC, Verssey AE, Askonas BA: Purified influenza virus nucleoprotein protects mice from lethal infection [J]. J Gen Virol 1987, 68(2):433–40.
25. Hampson AW, Osterhaus AD, Pervikov Y, Kyen MP: Report of the second meeting on the development of influenza vaccines that induce broad-spectrum and long-lasting immune responses, World Health Organization, Geneva, Switzerland, 6–7 December 2005. Vaccine 2006, 24(23):4897–900.
26. Zhimov OP, Iseeva EI, Konakova TE, Thidios G, Piskaeva LM, Akopova II, Kartashov A, Attebo AD, Ilyinskii PO, Shneider AM: Protection against mouse and avian influenza A strains via vaccination with a combination of conserved proteins NP, M1 and NS1. Influenza Other Respi Viruses 2007, 1:71–79.
27. Jimenez GS, Planchon R, Wei Q, Rusakov D, Geall A, Enas J, Laker P, Leary V, Vahle R, Cui LJ, Roland A, Kaslow DC, Smith LB: Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. Hum Vaccine 2007, 3(5):157–64.
28. Lo CY, Wu Z, Misplon JA, Price GE, Pappas C, Kong WP, Tumpey TM, Epstein SL: Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: Cold-adapted vaccine versus DNA prime-adenovirus boost strategies. Vaccine 2008, 26(17):2062–72.
29. Berthoud TK, Hamill M, Lillie PJ, Hvenda L, Collins KA, Ewer KJ, Milicic A, Poyntz HC, Lambe T, Fletcher HA, Hill AV, Gilbert SC. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. Clin Infect Dis 2011, 52(1):1–7.

30. Lillie PJ, Berthoud TK, Powell TJ, Lambe T, Mullarkey C, Spencer AJ, Hamill M, Peng Y, Blais ME, Duncan CJ, Sheehy SH, Haddecock T, Faust SN, Williams RL, Gilbert A, Oxford J, Dong T, Hill AV, Gilbert SC. A preliminary assessment of the efficacy of a T cell-based influenza vaccine, MVA-NP+M1, in humans. Clin Infect Dis 2012, 51(9):119–25.

31. Levine MM, Szelin MB. Vaccine development strategies for improving immunization: the role of modern immunology. Nature Medicine 2004, 5(5):460–6.

32. Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, Jacobs A, Liu G, Huang Y, Desai P, Maksymik G, Takahashi V, Umlauf S, Reiseroa L, Bell R, Li H, Zhang Y, McDonald WF, Powell TJ, Tussely L. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One 2008, 3(5):e257.

33. Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 2001, 98(19):10170–5.

34. Aguilar-Yáñez JM, Portillo-Lara R, Mendoza-Ochoa GI, García-Echauri SA, Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 2001, 98(19):10170–5.

35. Thueng-in K, Maneewatch S, Srimanote P, Songserm T, Tapchaisri P, Moss B. Candidate influenza vaccines based on influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. JJ. PLoS One 2008, 3(5):e257.

36. Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, Jacobs A, Liu G, Huang Y, Desai P, Maksymik G, Takahashi V, Umlauf S, Reiseroa L, Bell R, Li H, Zhang Y, McDonald WF, Powell TJ, Tussely L. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One 2008, 3(5):e257.

37. Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 2001, 98(19):10170–5.

38. Aguilar-Yáñez JM, Portillo-Lara R, Mendoza-Ochoa GI, García-Echauri SA, Biesova Z, Miller MA, Schneerson R, Shiloach J, Green KY. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 2001, 98(19):10170–5.

39. Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci U S A 1996, 93(21):11341–8.

40. Smith GL, Levin JZ, Palese P, Moss B. Synthesis and cellular location of the influenza virus nucleoprotein in protection: analysis using vaccinia virus recombinants. Scand J Immunol 1985, 15(1):765–75.

41. Andrew ME, Coupar BE, Boyle DB, Ada GL. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. J Immunol 2011, 186(7):4331–9.

42. Rimmelzwaan GF. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T lymphocyte populations directed to human influenza A virus. J Virol 2008, 82(11):5161–6.

43. Huang BY, Wang WL, Wang XP, Jiang T, Tan WJ, Ruan L. Efficient soluble expression and purification of influenza A nucleoprotein in Escherichia coli. Bior. Dig Xue Bao 2011, 27(1):50–7.

44. Wang WL, Huang BY, Wang XP, Tan WJ, Ruan L. Epitope screening of influenza virus nucleocapsid protein in BALB/c mice by enzyme-linked immuno-blot and its correlation with CTL epitope. Letters in Biotechnology 2008, 19(3):1–5.

45. Guo YJ, Chen XW. Influenza virus and its experimental techniques. Beijing: China Three Gorges Press; 1997:1–70.

46. Byers AM, Kemball CC, Moser JM, Lukacher AE. Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells. J Immunol 2003, 171(1):17–21.

47. Huang et al. Virology Journal 2012, 9:322
http://www.virologyj.com/content/9/1/322