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Maximal immune response and cross protection by influenza virus nucleoprotein derived from *E. coli* using an optimized formulation

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ABSTRACT

The highly conserved internal nucleoprotein (NP) is a promising antigen to develop a universal influenza A virus vaccine. In this study, mice were injected intramuscularly with *Escherichia coli*-derived NP protein alone or in combination with adjuvant alum ($Al(OH)_3$), CpG or both. The results showed that the NP protein formulated with adjuvant was effective in inducing a protective immune response. Additionally, the adjuvant efficacy of $Al(OH)_3$ was stronger than that of CpG. Optimal immune responses were observed in BALB/c mice immunized with a combination of NP protein plus $Al(OH)_3$ and CpG. These mice also showed maximal resistance following challenge with influenza A virus PR8 strain. Most importantly, 10 μg NP formulated with $Al(OH)_3$ and CpG induced higher protection than did 90 μg NP. These findings indicated that a combination of $Al(OH)_3$ and CpG may be an efficient adjuvant in the NP formulation.

Introduction

Seasonal influenza epidemics and the inevitable delay between viral identification and production of the specific vaccine have highlighted the urgent need for next-generation influenza vaccines that can preemptively induce broad immunity to different viral strains. Influenza (flu) is a serious hazard to human health, and vaccination is the most effective method for preventing flu. However, the conventional flu vaccine is produced in special-pathogen-free (SPF) chicken eggs, and it takes 4–6 months to obtain subtype-matched vaccine from the vaccine strain. Moreover, conventional flu vaccines induce protective effects depending on antibodies against highly variable hemagglutinin (HA) and neuraminidase (NA) (Gerdil, 2003). In most cases, it is difficult to prevent flu epidemics or pandemics caused by a new type of influenza A virus (Fedson, 2005; Palese, 2006). Therefore, universal flu vaccines based on conserved influenza A virus antigens are required to prevent flu outbreaks.

Nucleoprotein (NP) is a highly conserved internal antigen of the influenza A virus (Altmuller et al., 1989; Shu et al., 1993) and is the major target antigen for cytotoxic T lymphocyte (CTL) responses (Jameson et al., 1998, 1999; McMichael et al., 1983, 1986). At this time, several vaccines based on the NP antigen alone or in combination with other influenza A virus antigens have been developed including peptide vaccines (Adar et al., 2009; Atsmon et al., 2012; Gao et al., 2013; Jeon et al., 2002; Savard et al., 2012), DNA-based vaccines (Kheiri et al., 2012; Lalor et al., 2008; Luo et al., 2012; Price et al., 2009, 2010; Xu et al., 2011), virus vector-based vaccines (Price et al., 2009, 2010; Antrobus et al., 2012, 2014; Barefoot et al., 2009; Berthoud et al., 2011; Brewoo et al., 2013; Hessel et al., 2014; Kim et al., 2013; Lambe et al., 2013; Li et al., 2013; Lillie et al., 2012; Moraes et al., 2011; Mullarkey et al., 2013; Rohde et al., 2013; Sipo et al., 2011; Vitelli et al., 2013), recombinant attenuated Salmonella vaccines (RASVs) (Ashraf et al., 2011) or protein subunit vaccines (Luo et al., 2012; Haynes et al., 2012; MacLeod et al., 2013). The efficacy of these vaccines has been evaluated in animal models. Numerous studies have found that NP-based protein subunit vaccines can protect animals against homologous and heterologous influenza virus. As early as 1986, Wraith et al. (1987) purified NP of influenza A virus X31 (H3N2) and injected BALB/c mice with two 10-μg doses of NP s.c. at 4-week intervals or with one 50-μg dose i.p. They found that NP immunization resulted in significant protection (75%) of mice from a lethal challenge with PR8. However, the protective efficacy of NP protein-based vaccines requires improvement, possibly using an adjuvant. Next, Tamura et al. (1996) expressed rNP of PR8 in insect cells and found that intranasal immunization of mice with 5 μg rNP combined with the adjuvant cholera toxin B subunit (CTB)

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could accelerate viral clearance from the nasal site after a sublethal dose challenge of influenza virus. Moreover, rNP combined with CTB could protect 70–80% of mice against homologous challenge with PR8 (40LD50) and 40–70% of mice against heterologous challenge with B/Ibaraki/2/85 (B/Ibaraki) (40LD50). Later, Guo et al. (2010) used CTB in the formulation of rNP and found that intranasal immunization of mice with 10 μg rNP with CTB resulted in complete protection against the homologous influenza virus (10LD50), and immunization with 100 μg rNP with CTB provided good cross-protection against heterologous H5N1 and H9N2 avian influenza viruses (5LD50). MacLeod et al., (2013) found that NP delivered with adjuvant aluminum completely protected mice against homologous challenge with PR8. In contrast, immunization with NP delivered with alum and the detoxified LPS adjuvant monophosphoryl lipid A (MPL) provided some protection against the homologous viral strain but no protection against infection by influenza expressing a variant NP. Together, these data suggest that the NP protein subunit vaccine is immunogenic in mice and could provide protection against homologous and heterologous influenza viral challenge in the appropriate formulation.

Protein subunits are preferred in vaccine research because of their increased safety, single antigenic component, coverage of more antigen epitopes, and suitability for large-scale production. In our previous studies, the codon-optimized NP protein of the influenza virus A/Beijing/30/95 (H3N2) was expressed in *Escherichia coli* at high levels and administered at a high dose, and it was found to induce protection with high efficiency (Huang et al., 2012). In most cases, protein-based vaccines are poor immunogens and require the addition of adjuvants in the formulation to induce a protective and long-lasting immune response, even though they are advantageous over traditional vaccines considering safety and production cost. Insoluble aluminum salt is a universally used and safe adjuvant (Reed et al., 2009), and CpG is under development as an adjuvant for vaccines against cancer and infectious diseases (Cooper et al., 2004; Gupta and Cooper, 2008). In this study, aluminum and CpG were used to improve the immunogenicity of NP protein derived from *E. coli*. We investigated whether 10 μg NP in combination with adjuvant alum and CpG provides comparable cross protection as 90 μg NP without adjuvant in mice (Fig. 1).

Results

Adjuvant Al(OH)$_3$ and CpG significantly increased the humoral immune response induced by influenza A virus NP subunit vaccine

We explored whether adjuvant Al(OH)$_3$ and CpG increased the immune response elicited by NP protein (Fig. 2). We found that priming vaccination with NP alone or combined with adjuvant induced substantial anti-NP IgG (1.8×10^4–3.2×10^4), and the IgG titers were not significantly different from each other in the NP-immunized groups.

Boosting immunization performed on day 14 improved anti-NP IgG titers; an NP-specific IgG assay performed on day 28 (Fig. 2, middle) showed that 10 μg NP alone induced higher anti-NP IgG levels (geometric mean: 6.8×10^5). The addition of CpG (G4) induced significantly higher anti-NP IgG titers (geometric mean: 1.8×10^6) than did 10 μg NP alone (G3) (G4 > G3, $p<0.01$), and the addition of Al(OH)$_3$ (G5) induced similar anti-NP IgG titers (geometric mean: 2.8×10^6) as did G4 (G5 ≈ G4, $p>0.05$). Adding Al(OH)$_3$ plus CpG and 10 μg NP (G6) induced higher anti-NP IgG titers (geometric mean: 4.0×10^6) than did G4 alone (G6 > G4, $p<0.05$). However, the anti-NP IgG titers in G5 and G6 were similar ($p>0.05$). Immunization with 90 μg NP (G7) induced similar anti-NP IgG titers (geometric mean: 1.1×10^6) as did G3 and G4 (G7 ≈ G3 and G4, $p>0.05$), but lower titers compared with G5 and G6 (G7 < G5 and G6, $p<0.001$).

After the third immunization, anti-NP IgG titers did not improve in mice immunized with 10 μg NP protein alone (G3), NP protein formulated with CpG (G4) or NP protein formulated with Al(OH)$_3$ and CpG together (G6), compared with after the second immunization. However, anti-NP IgG titers in G5 ($p<0.05$) and G7 ($p<0.05$) improved significantly. As a result, after the third immunization, mice in G5, G6, and G7 showed comparable anti-NP IgG titers (G5 ≈ G6 ≈ G7, $p>0.05$), which were significantly higher than that in G3 (G5, G6 and G7 > G3, $p<0.001$). The anti-NP IgG titer in G4 was significantly higher than that in G3 (G4 > G3, $p<0.01$), comparable to that in G7 (G4 ≈ G7, $p>0.05$) and significantly lower than those in G5 and G6 (G4 < G5 and G6, $p<0.001$).

Antibody subtype analysis (Fig. 3) showed that CpG significantly improved anti-NP IgG2a titer (G4 > G3 and G7, $p<0.01$), but not anti-NP IgG1 titer ($p>0.05$), compared with immunization with NP alone (G3 and G7) (Fig. 3A and B). This decreased the anti-NP IgG1/IgG2a ratio (Fig. 3C), indicative of a potent Th1 response. Al(OH)$_3$ adjuvant significantly increased both anti-NP IgG1 (G5 > G4, $p<0.001$) and IgG2a (G5 > G4, $p<0.05$) titers compared with CpG (Fig. 3A, B), resulting in the highest IgG1/IgG2a ratio (Fig. 3C), which differed from the IgG1/IgG2a pattern in G4 (G5 > G4, $p<0.001$). In G6, lower anti-NP IgG1 (G6 < G5, $p<0.01$) and similar IgG2a (G6 ≈ G5, $p>0.05$) titers were induced, resulting in a lower IgG1/IgG2a ratio compared with group 5 (G6 < G5, $p<0.01$) (Fig. 3C). These results suggest that Al(OH)$_3$ improved the Th2 antibody response against NP protein, which may have been weakened by including CpG in G6. Slightly higher anti-NP IgG1 (G7 > G3, $p<0.05$) and similar IgG2a (G7 ≈ G3, $p>0.05$) levels were induced in G7 compared with G3, resulting in a similar IgG1/IgG2a ratio of these two groups (G7 ≈ G3, $p>0.05$) (Fig. 3).

Adjuvant CpG or Al(OH)$_3$ plus CpG improved the cellular immune response induced by influenza A virus NP subunit vaccine

To characterize the cellular immune responses elicited by NP protein in mice, IFN-γ-, IL-4-, and IL-10-secreting SMNCs after the third immunization were quantified using ELISPOT assays (Fig. 4).

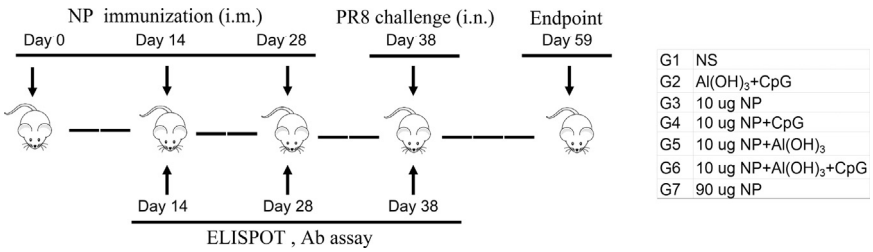


Fig. 1. Experimental schedule of the NP protein. The indicated mice were immunized intramuscularly with NP protein on days 0, 14 and 28. Blood was collected from the mice and analyzed using ELISA on days 14, 28 and 38, after which the mice were sacrificed, and SMNCs were separated and analyzed using the ELISPOT assay. The remaining mice were challenged on day 38 with 20 MLD50 of influenza A virus PR8 and monitored for 3 weeks until day 59. A summary of the mouse groups is provided in the right table.

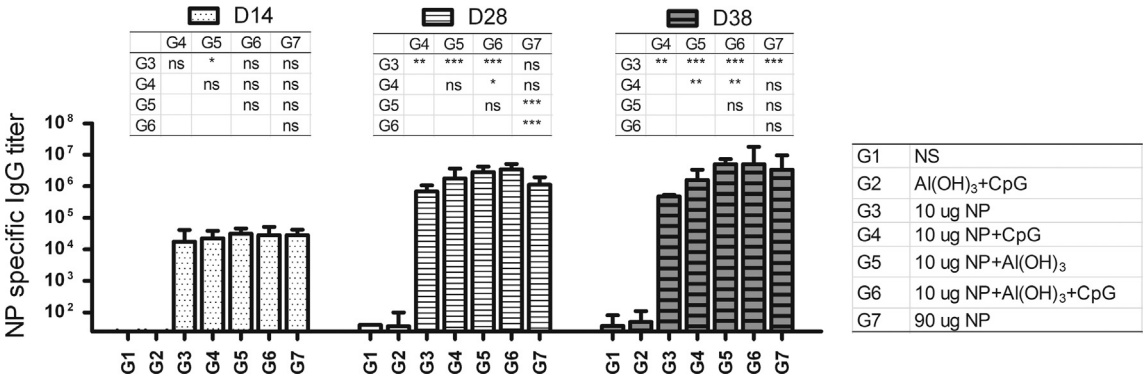


Fig. 2. NP-specific antibody titers in mice sera immunized with NP formulated with adjuvant. 5–6-week-old female BALB/c mice were vaccinated with 10 μg NP, 10 μg NP formulated with adjuvants (CpG, $Al(OH)_3$, $Al(OH)_3$+CpG), or 90 μg NP on days 0, 14 and 28, as described in Fig. 1. A summary of the mouse groups is provided in the lower table. Mice administered NS or $Al(OH)_3$+CpG were treated as negative controls. Sera were collected from six mice in each group on days 14, 28, and 38, respectively. Serum anti-NP IgG titers were determined using ELISA. Sera were individually tested in serial dilutions against purified NP protein. Plots show the geometric mean antibody titers, and bars show the 95% confidence interval (CI) for each treatment group. Log conversion was performed for the mice serum antibody titers before statistical analysis. Tables above the charts show a comparison of the results. ns, not significant; * $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$ based on one-way ANOVA.

The results showed that NP protein alone (10 μg NP or 90 μg NP) did not induce a cellular immune responses in mice. NP protein with adjuvant produced a strong IFN-γ-specific cellular immune response (Fig. 4A) and less potent IL-4- and IL-10-specific immune responses (Fig. 4B and C). NP protein formulated with CpG significantly enhanced the IFN-γ-specific cellular immune responses against $NP_{147-155}$ (72 ± 56 SFC/10^6 SMNC) compared with NP protein alone (G4 > G3, $p < 0.01$). $Al(OH)_3$ had no clear effect on the NP-induced IFN-γ-specific response against NP_{55-69} or $NP_{147-155}$. ELISPOT results also showed that the combination of $Al(OH)_3$ plus CpG significantly enhanced the NP protein-induced IFN-γ-specific response against NP_{55-69} (190 ± 240 SFC/10^6 SMNC, G6 > G3, $p < 0.01$ and G6 > G7, $p < 0.01$), but not against $NP_{147-155}$ (42 ± 33 SFC/10^6 SMNC, G6 ≈ G3 ≈ G7, $p > 0.05$).

Adjuvants $Al(OH)_3$ and CpG improved NP protein-induced cross-protection efficacy

We further investigated the effects of $Al(OH)_3$ and CpG in improving the cross protection induced by NP. On day 38, immunized mice were challenged with 20 MLD50 of influenza A/PR8. All mice immunized with normal saline (NS) (G1) or $Al(OH)_3$ plus CpG (adjuvant control, G2) experienced serious body weight loss, and all died on day 10–12 after challenge (Fig. 5). Mice immunized with NP without any adjuvant in groups 3 (G3) and 7 (G7) also showed significant body weight loss, and some mice did not survive the PR8 challenge. However, mice immunized with NP formulated with CpG (G4), $Al(OH)_3$ (G5), or $Al(OH)_3$ plus CpG (G6) showed less body weight loss than did G3 or G7 (G4, G5 and G6 < G3 and G7). The body weight change curve also suggested that inclusion of CpG (G4), $Al(OH)_3$ (G5) or $Al(OH)_3$ plus CpG (G6) allowed the immunized mice to recover much earlier than did immunization with 10 μg or 90 μg NP protein (G4, G5 and G6 > G3 and G7) (Fig. 5A).

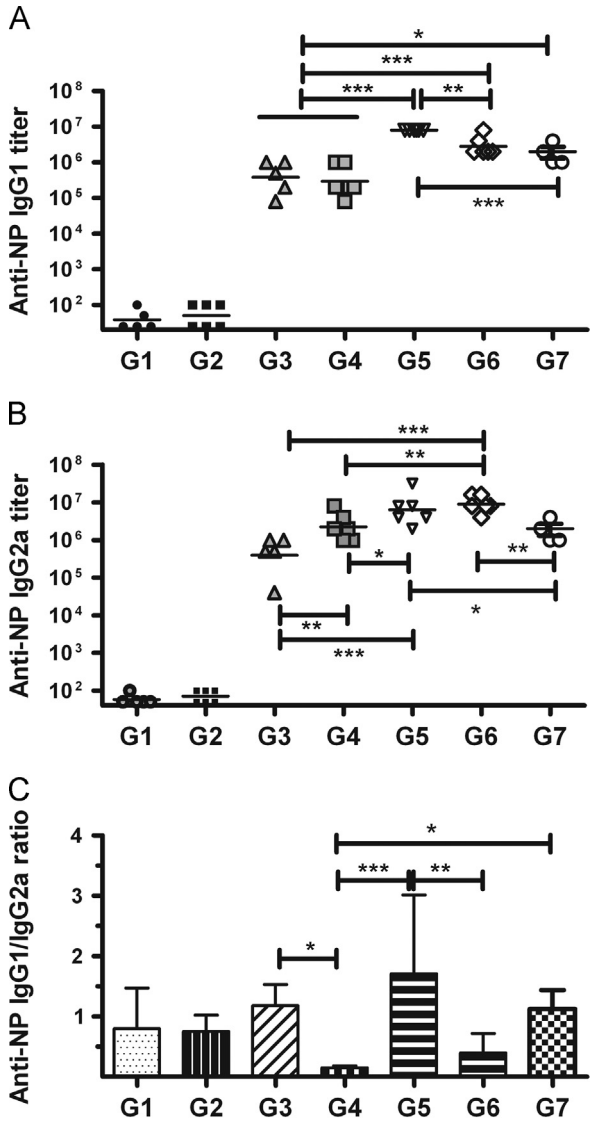
The survival curve showed that including CpG and $Al(OH)_3$ adjuvants with NP protein significantly improved the survival rate to 27% (4/15, G4) and 47% (7/15, G5), respectively, compared with the survival rate of 13% (2/15, G3) induced by 10 μg NP protein alone (G4 > G3, $p \le 0.05$ and G5 > G3, $p \le 0.01$). Including CpG plus $Al(OH)_3$ improved the survival rate to 80% (12/15, G6) (G6 > G3, $p < 0.001$) (Fig. 5B). A total of 43% (6/14, G7) of mice in G7 survived lethal infection (Fig. 5B), which was similar to G5 and significantly lower than G6 (G7 < G6, $p \le 0.05$). Analysis of the time to death showed that mice in G3, G4, G5 and G6 suffered death on days 8–11, 9–12, 9–11 and 10–14 after challenge, respectively, and mice in G7 suffered death on days 10–18 after challenge. All mice in G1 and G2 died (15/15 in G1 and G2) within 12 days after challenge (time of death: days 6–12 after challenge). These results suggested that NP formulated with $Al(OH)_3$ induced a slightly higher protective immunity than did NP formulated with CpG. However, these results were not statistically significant ($p > 0.05$), and NP formulated with CpG plus $Al(OH)_3$ elicited the highest protection among all mouse groups. Moreover, 10 μg NP formulated with $Al(OH)_3$ induced similar survival rates to 90 μg NP (G5 ≈ G7, $p > 0.05$), whereas 10 μg NP formulated with $Al(OH)_3$ plus CpG induced a higher percentage of survival than did 90 μg NP (G6 > G7, $p < 0.05$).

The protection efficacy induced by NP immunization was closely associated with anti-NP humoral and cellular immune responses

To increase our understanding of the correlation between protective effects and humoral and cellular immune responses induced by NP immunization, correlation coefficients were analyzed. The survival percentage induced by NP immunization was strongly associated with total IgG ($R=0.754$, $p < 0.05$), IgG1 ($R=0.756$, $p < 0.05$), and IgG2a ($R=0.780$, $p < 0.05$) isotypes of Ab against NP protein when the humoral immune response was considered (Fig. 6). Moreover, the survival percentage was significantly associated with the NP_{55-69}-specific, but not with the $NP_{147-155}$-specific, cellular immune response. Particularly, the survival percentage was strongly associated with NP_{55-69}-specific IFN-γ-secreting SMNCs induced by NP immunization ($R=0.872$, $p < 0.01$). These results indicate that the protective efficacy of NP protein vaccine in this study was related to both the Ab response and the cellular immune response.

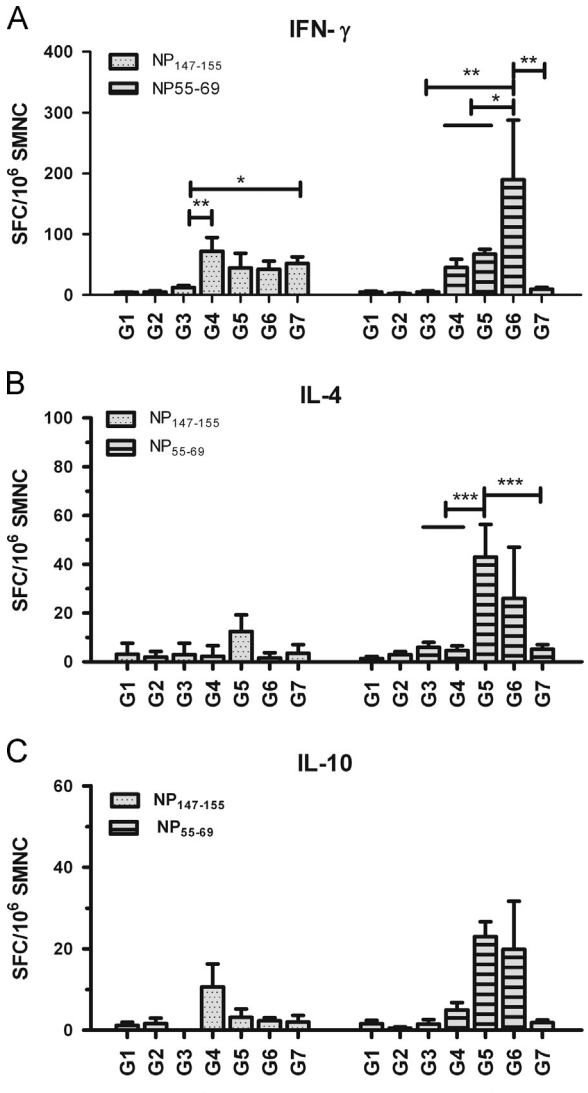
Discussion

The highly conserved internal NP of the influenza A virus is a candidate antigen for the development of a universal influenza vaccine. NP has been expressed in various systems, and its immunogenicity has been tested in animal models. In our previous study, 10 μg, 30 μg, and 90 μg NP derived from *E. coli* showed high immunogenicity in BALB/c mice. A high dose of 90 μg NP induced the highest protection among these groups (Huang et al., 2012). The cross-protection efficacy of NP has been shown in previous reports; however, its immunogenicity requires improvement (Sipo et al., 2011; Jamali et al., 2010; Jimenez et al., 2007). Induction of the maximal immune response with a minimum immune dose remains important in vaccine research. In recent studies, various



G1	NS
G2	Al(OH)$_3$+CpG
G3	10 ug NP
G4	10 ug NP+CpG
G5	10 ug NP+Al(OH)$_3$
G6	10 ug NP+Al(OH)$_3$+CpG
G7	90 ug NP

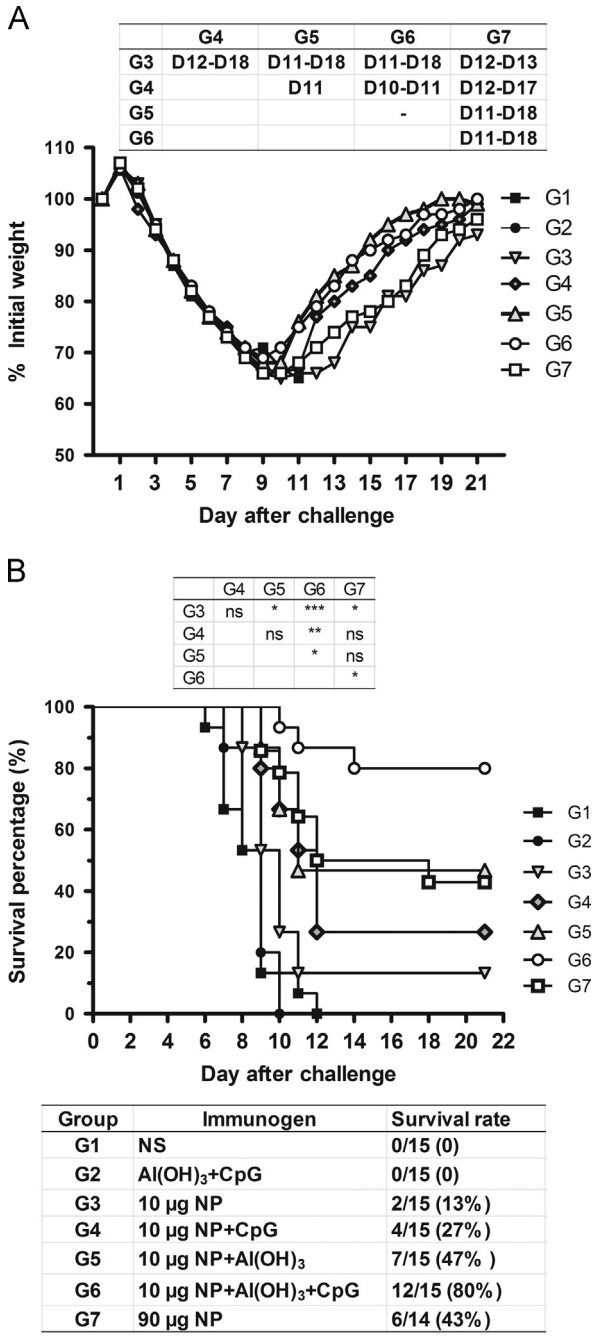
Fig. 3. IgG1 and IgG2a isotypes of serum from NP-immunized mice. Mice sera on day 38 are the same as that in Fig. 2. A summary of the mouse groups is provided in the lower table. NP-specific IgG isotypes on day 38 were analyzed using ELISA. Plots in A and B show the anti-NP IgG1 and IgG2a isotype titers, respectively. Scatter plots show the results each mouse in each group, and bars show the geometric mean for each group. Plots in C represent the anti-NP IgG1/IgG2a ratios in sera collected from mice. Bars show the mean with SD ($n=6$ mice per group, except $n=5$ in the NS- or NP-only-immunized group). Log conversion was performed for the mouse serum antibody titers prior to statistical analysis. Lines above NP and NP+CpG groups in A demonstrate that the two groups have similar results. $^{*}p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$ based on one-way ANOVA.



G1	NS
G2	Al(OH)$_3$+CpG
G3	10 ug NP
G4	10 ug NP+CpG
G5	10 ug NP+Al(OH)$_3$
G6	10 ug NP+Al(OH)$_3$+CpG
G7	90 ug NP

Fig. 4. IFN-γ, IL-4 and IL-10 ELISPOT assays for NP-specific SMNCs were detected using ELISPOT assays. Mice are the same as in Fig. 2. A summary of the mouse groups is provided in the lower table. Spleen lymphocytes were separated from mice on day 38, and 5 μg/ml of NP$_{147-155}$ and NP$_{55-69}$ peptides were used as stimuli in ELISPOT assays. The numbers of SMNC-secreting IFN-γ(A), IL-4(B), or IL-10(C) in response to 40 h stimulation with NP$_{147-155}$ (left) and NP$_{55-69}$ (right) peptides are shown as SFC/10^6 SMNC. $^{*}p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$ based on one-way ANOVA.

adjuvants have been employed using genetic ligation or by addition in the formulation to improve immunogenicity, including nanoparticles derived from papaya mosaic virus capsid protein (PapMV CP) (Savard et al., 2012), T-cell costimulator 4-1BBL (Moraes et al., 2011), aluminum and monophosphoryl lipid A (MPL) (MacLeod et al., 2013), cholera toxin B subunit (CTB) (Guo et al., 2010), Vaxfectin (Jimenez et al., 2007), lipopolysaccharide (LPS) (Lamere et al., 2011) and the tegument protein VP22 of herpes simplex virus 1 (HSV-1) (Saha et al., 2006). We used CpG and Al(OH)$_3$ to improve the immunogenicity of the NM2e fusion protein of the influenza virus in a previous report; the adjuvant showed potential efficacy, strengthened the immune response and elicited cross protection after NM2e immunization in mice. A combination of Al(OH)$_3$ and CpG in the formulation of NM2e induced the highest protection in mice (Wang et al., 2012). In this study, CpG and Al(OH)$_3$ were employed separately or in combination with 10 μg NP expressed in *E. coli* to improve its



Group	Immunogen	Survival rate
G1	NS	0/15 (0)
G2	$Al(OH)_3$+CpG	0/15 (0)
G3	10 μg NP	2/15 (13%)
G4	10 μg NP+CpG	4/15 (27%)
G5	10 μg NP+$Al(OH)_3$	7/15 (47%)
G6	10 μg NP+$Al(OH)_3$+CpG	12/15 (80%)
G7	90 μg NP	6/14 (43%)

Fig. 5. Comparison of NP protein and NP formulated with adjuvant providing protection against influenza A virus PR8 challenge. Mice were immunized as described in Fig. 1. A summary of the mouse groups and the survival rate are provided in the lower table in this chart. Mice were challenged with 20 MLD50 of influenza virus PR8 on day 38. Mice immunized with NS or $Al(OH)_3$+CpG were challenged as negative controls. Body weight loss (A) and survival (B) of the mouse groups were monitored daily for 21 days post-challenge ($n=15$ in all groups, except $n=14$ in G7). The table above graph A displays those days showing differences among groups ($p<0.05$); the table above graph B displays a results comparison among these groups. ns, not significant; $^{*}p\le 0.05$; $^{**}p\le 0.01$; $^{***}p\le 0.001$ based on repeated-measures ANOVA in A and log-rank (Mantel–Cox) test in B.

immunogenicity and protective efficacy. Our results demonstrated that after the third immunization, 10 μg NP alone induced a strong humoral immune response (anti-NP IgG titer: 4.8×10^5) in mice, which was markedly enhanced by the addition of CpG adjuvant and promoted Th1 and CTL responses (Bevan, 2004). In this study, CpG adjuvant markedly improved the anti-NP IgG2a titer, but not IgG1 titer, thus decreasing the IgG1/IgG2a ratio. This was suggestive of a Th1 response. Meanwhile, CpG increased the cellular immune response against NP147–155. Influenza A virus PR8 challenge demonstrated that including CpG in the NP formulation improved the survival rate from 13% to 27%, suggesting that the immune efficacy of CpG was not sufficient to completely protect mice against the challenge. Aluminum adjuvant enhanced the Th2 immune response (Reed et al., 2009; Tritto et al., 2009). In the present study, $Al(OH)_3$ increased anti-NP IgG levels by 10 fold compared with NP protein alone (G3) and enhanced both anti-NP IgG1 and IgG2 titers, which increased the IgG1/IgG2a ratio compared with NP protein alone (G3 and G7). Meanwhile, including $Al(OH)_3$ in the NP formulation had only a minor effect on the cellular immune response. Influenza A virus PR8 challenge showed that including $Al(OH)_3$ in the NP formulation improved the survival rate to 47%, suggestive of stronger immune efficacy of $Al(OH)_3$ than of CpG. Furthermore, $Al(OH)_3$ and CpG showed a synergistic effect in the NP formulation. Our results demonstrated that a combination of $Al(OH)_3$ and CpG increased both anti-NP IgG1 and IgG2a titers and decreased the IgG1/IgG2a ratio compared with $Al(OH)_3$ alone. More importantly, the combination improved the protection and resulted in the highest survival rate of 80% among all mice groups, showing a clear synergistic effect. An immune dose of 90 μg NP was used as a control in this study. The high dose of 90 μg NP induced higher protection in mice than did the low dose of 10 μg NP. Our data demonstrated that the lower protection induced by 10 μg NP alone in mice was enhanced by adjuvant $Al(OH)_3$. Ultimately, including $Al(OH)_3$ in the formulation with 10 μg NP induced similar protection to 90 μg NP alone, and including $Al(OH)_3$ and CpG together in the formulation with 10 μg NP induced higher protection than 90 μg NP. These results suggested that the NP subunit vaccine expressed in *E. coli* is immunogenic in mice, and that the high immune dose of 90 μg NP can be decreased to 10 μg by adding adjuvant. Adjuvant $Al(OH)_3$ or CpG could improve the immunogenicity and protection efficacy of NP, and a combination of $Al(OH)_3$ and CpG in the formulation with NP is a promising strategy for the development of an NP-based universal influenza A virus vaccine to prevent influenza epidemics and pandemics. Guo et al. (2010) reported that immunization with 100 μg recombinant NP formulated with CTB provided good cross-protection in mice, which also supports the immunogenicity of recombinant NP derived from *E. coli*. However, the dose was much higher than that used in this study. MacLeod et al. (2013) showed that vaccination with NP formulated with aluminum provided protection against homologous and heterologous influenza a virus. However, when aluminum and MPL were used together in the formulation, the combination of NP+aluminum+MPL provided some protection against the homologous viral strain but no protection against the heterologous strains. Based on the above results, the addition of adjuvant (especially for aluminum) is important for the development of a universal influenza virus vaccine based on NP.

Understanding the immune protection mechanism of NP is important for the development of a universal vaccine based on NP. Once we understand the immune protection mechanism, we can develop vaccine research strategies and improve the immunogenicity of the NP vaccine by applying the appropriate adjuvant or delivery technique.

Most studies consider NP protein the primary target antigen against CTL in influenza virus. NP elicited protective immunity mainly through $CD4^+$T- and $CD8^+$T-cellular immune responses (Lalor et al., 2008; Brewoo et al., 2013; Jimenez et al., 2007; Lo et al., 2008; Epstein et al., 2005, 2000; Grant et al., 2013; Ulmer et al., 1998). NP-induced CTL response may be mediated by cytokines, secreted by Th, and implemented by Tc killing (Jameson et al., 1998, 1999). In some studies, NP vaccine-immunized T-cell-depleted mice could not survive influenza virus challenge (Epstein et al., 2005),

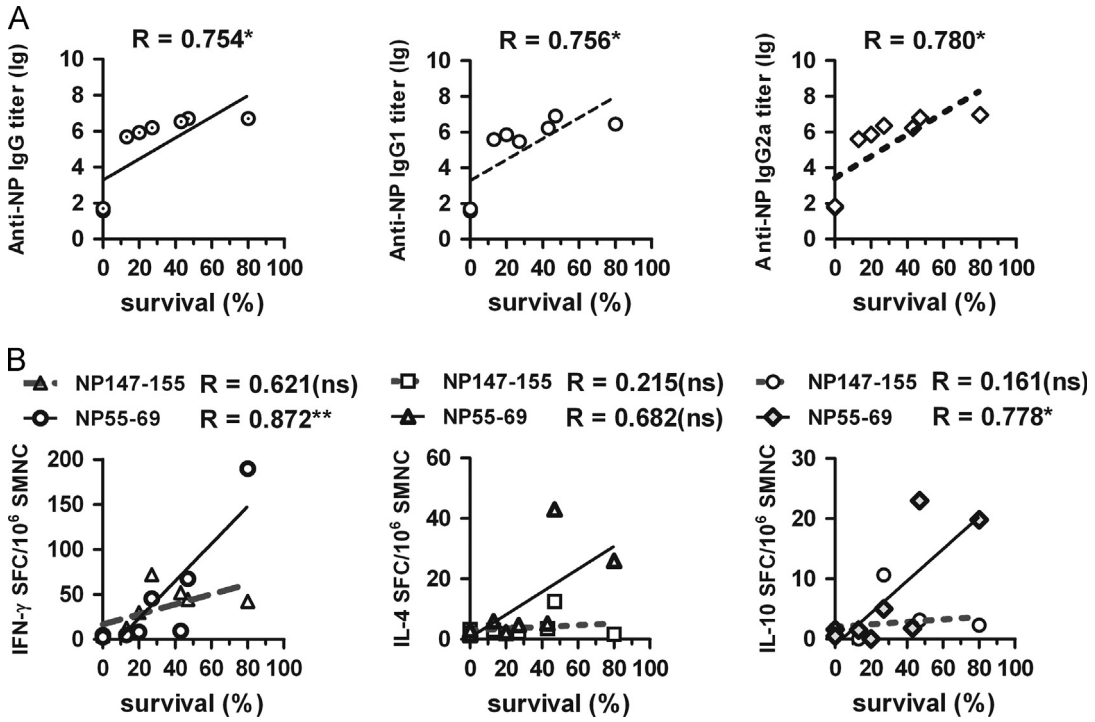


Fig. 6. Correlation analysis between survival percentage and immune responses in mice. (A) Correlation analysis was conducted to determine the relationships between survival percentage data from Fig. 5 and the NP-specific IgG on day 38 data (left) from Fig. 2 and IgG1 (middle) and IgG2a (right) ELISA data in Fig. 3A and B. Log conversion was performed for the mouse serum antibody titers. (B) Correlation analysis was performed to explore relationships of the survival percentage data in Fig. 5 with the IFN-γ- (left), IL-4- (middle), and IL-10-secreting (right) SMNCs stimulated with NP147–155 or NP55–69 peptide based on the ELISPOT data in Fig. 4. ns, not significant; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ based on correlation analysis.

whereas naïve mice could survive with adoptive transfer of T cells from NP vaccine-immunized mice (Ulmer et al., 1998; Fu et al., 1999). In this study, the survival percentage of mice challenged with PR8 was strongly associated with the cellular response induced by the NP protein. To further explore the protective mechanism induced by NP immunization, we compared the Ab response and cellular immune response induced by 10 μg NP formulated with Al$(OH)_3$ and CpG together (G6) and 90 μg NP alone (G7). Mice in G6 experienced less weight loss than those in G7. Meanwhile, challenge results demonstrated that the survival percentage of 80% in G6 was significantly higher than that in G7 (43%). Immune response analysis demonstrated that the antibody response against NP was comparable in G6 and G7, but mice in G6 showed a higher cellular response against NP, and those in G7 showed a lower cellular response. Based on these results, the cellular response induced by NP immunization improved mouse survival; however, more definitive results such as adoptive transfer of T cells are required to support this hypothesis. In this study, we did not observe a correlation between the survival percentage and the $NP_{147-155}$-specific cellular response, possibly because the $NP_{147-155}$-related response was not efficiently induced by the formulation in BALB/c mice.

Although $CD4^+$ and $CD8^+$ T cells can be used for protection after NP immunization, some studies have shown that T cells are not required for protection induced by NP (Epstein et al., 2000), which suggests that other mechanisms, such as antibodies, may contribute to the protective effects of NP vaccine. Ab against NP may be induced by influenza virus infection and NP vaccine immunization (Rangel-Moreno et al., 2008); however, NP is an internal influenza virus protein, and Ab against NP could not neutralize virus. Further results demonstrated that adoptive transfer of NP Ab to immune deficient scid mice could not protect the mice against influenza virus challenge. Therefore, Ab against NP could not offer protection (Gerhard et al., 1997). In fact, immune complexes formed by MAb against NP promoted DC maturation, Th1-type cytokine production, and $CD8^+$ CTL response after influenza virus infection in normal individuals (Zheng et al., 2007). Additionally, NP is expressed on the cell surface of P815 infected with influenza virus NP, which can be lysed by the complement system stimulated by NP-specific IgG *in vitro* (Yewdell et al., 1981). Passive immunization of anti-NP serum promoted clearance, decreased the severity of the disease, and played an important role in cross protection against influenza virus (Rangel-Moreno et al., 2008; Carragher et al., 2008; Nguyen et al., 2007; Sealy et al., 2003). These results suggest that antibody may be an important protective mechanism of NP (Rangel-Moreno et al., 2008; Carragher et al., 2008). Next, Lamere et al. (2011) demonstrated that anti-NP IgG played a protective role via a mechanism involving both FcRs and $CD8^+$ T cells. In this study, NP protein expressed in *E. coli* induced a strong antibody response in mice with or without adjuvant. Especially, 90 μg NP without any adjuvant protected against influenza virus. Furthermore, correlation analysis suggested that the survival percentage of mice immunized with NP was significantly associated with NP-specific IgG, IgG1, and IgG2a titers.

Overall, given that both T lymphocytes and Ab against NP contribute to cross-protection (Lamere et al., 2011; Rangel-Moreno et al., 2008; Carragher et al., 2008), appropriate adjuvants should be used to induce NP-related protection, similar to Al$(OH)_3$ and CpG adjuvants. In this study, a combination of Al$(OH)_3$ and CpG in the NP formulation could be used for the development of an NP-based universal influenza A virus vaccine to prevent influenza epidemics and pandemics. However, several issues need to be addressed, as follows:

1) The protective durability induced by NP immunization. In this study, the immunized mice were challenged 10 days after the last immunization. It is unknown whether they will remain protected once the response decreases to a constant “memory”

level. We immunized mice with a recombinant fusion protein of NP and M2e (NM2e) formulated with $Al(OH)_3$ three times at 2-week intervals in a previous study. Long-term monitoring data showed that immunization induced high levels of IgG, which were maintained for at least 7 months, against NP and M2e (Wang et al., 2008), Afterwards, mice immunized with NM2e+$Al(OH)_3$ were challenged with influenza A virus PR8 2 weeks, 2 months or 6 months after the last immunization, respectively. Our results showed that challenge at 2 or 6 months after the last immunization induced only a slightly lower survival rate; however, they were not significantly different from the challenge results at 2 weeks after the last immunization (data not shown). Based on these results, NP+Al$(OH)_3$+CpG immunization in this study may induce long-term protection at least 6 months after the last immunization, after which boosting immunization may be required for higher protective efficacy.

2) The practicality of a three-dose NP-based vaccine administered at biweekly intervals. It was important to confirm the immunogenicity of NP in this preliminary study, although the three-dose delivery strategy was inconvenient (Savard et al., 2012; Guo et al., 2010). The delivery strategy, such as the immunization dosage, immunization times and delivery interval, should be optimized for convenience. Previously, the immunization times and intervals were evaluated using the NM2e vaccine in our lab. Mice were immunized with NM2e+$Al(OH)_3$ three times at 2-week intervals or two times at 4-week intervals, respectively, and the two groups showed comparable survival percentages (data not shown). The two-dose immunization strategy would be preferred if the NP-based vaccine performance is similar to that of NM2e. Further studies are required to optimize the immunization strategy of NP+$Al(OH)_3$+CpG vaccine in this study.
3) Relationship between Th epitope-directed responses and Ab titers. This study showed that NP immunization induced high levels of antibody against NP in G3-G7; however, the Th epitope (NP_{55-69})-directed response was only observed in G4, G5 and especially G6. Thus, the Th epitope-directed response did not correlate with ELISA titers. A helper T-cell response of NP may play a role in secreting cytokines, such as IFN-γ and IL-2, which facilitated switching of the immunoglobulin isotype and lysing MHC class II cells that express the target antigens and priming of a memory CTL response *in vivo* (Ulmer et al., 1998). Based on the results in G6, specific cytokines, such as IFN-γ, but little or no IL-4 or IL-10 were secreted into the culture supernatants of restimulated cells during NP_{55-69} restimulation *in vitro* (Fig. 4), which was indicative of a Th1 type of helper T-cell response (Ulmer et al., 1998). As may be expected from this Th1 type of response, the immunoglobulin subtype profile of anti-NP antibodies was predominated by IgG2a, with lesser amounts of IgG1 (Fig. 3). Notably, only one Th epitope NP_{55-69}, but not the entire NP protein or peptide pool of NP, was used in this study. Moreover, ELISPOT only showed the cell spots that reacted with specific Ab, but the magnitude of the role they play in the active, composite response of animals remains unknown. The Th-epitope directed response should be further characterized, such as analysis of cytokine concentrations in the supernatant, to increase our understanding of the Th response elicited by NP immunization.

Conclusions

NP formulated with $Al(OH)_3$ and CpG induced optimal immune responses and protection against the influenza A virus PR8 strain in BALB/c mice, which indicates that a combination of $Al(OH)_3$ and CpG may be an efficient adjuvant in the NP formulation. In addition, the high immune dose of NP can be reduced by the use of $Al(OH)_3$ and CpG.

Materials and methods

Protein and peptides

The expression and purification of NP protein in *E. coli* were performed as described previously (Huang et al., 2012). Briefly, the optimized NP gene from influenza A virus A/Beijing/30/95 (H3N2) was synthesized and ligated into the pET-30a(+) vector (Merck-Novagen, Darmstadt, Germany) between the *Nde*I and *Eco*RI sites to form pET30a-NP, which was used to transform *E. coli* BL21(DE3) (Merck-Novagen). The expressed NP was purified using ion-exchange chromatography (DEAE-Sepharose Fast Flow, Amersham Biosciences Corp., Piscataway, NJ, USA) followed by gel chromatography (Superdex 200, Amersham) with purity > 90%. The purified NP was concentrated, quantified, and stored at −70 °C.

In our previous study, NP peptide pools were used to screen the ELISPOT epitopes of NP in BALB/c (H-2^d) mice infected with influenza A virus A/PR8. We identified some ELISPOT epitopes of NP including the CTL epitope $NP_{147-155}$-(TYQRTRALV) and two potential Th epitopes, NP_{53-67}-(EGRLIQNSLTIERMV) and NP_{57-71}-(IQNSLTIERMVLSAF), both of which contain a portion of the H-2^d restricted Th epitope sequence NP_{55-69}-(RLIQNSLTIERMVLS) (Wang et al., 2008). Since $NP_{147-155}$-(TYQRTRALV) and NP_{55-69}-(RLIQNSLTIERMVLS) have been reported in previous studies, they were used to identify the ELISPOT response induced by the NP-based vaccine in this study.

Influenza virus

The influenza virus A/Puerto Rico/8/34 (H1N1) (A/PR8) was inoculated into the allantoic cavity of 9-day-old chick embryos and cultured for 2 days at 34 °C. Viral allantoic fluid was then harvested and stored at −70 °C. The MLD50 value of PR8 virus was determined in BALB/c mice.

Immunization and virus challenge

5–6-week-old SPF female BALB/c mice were obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences, and raised in an animal house in the Institute for Occupational Health and Poison Control (IOHPC), Chinese Center for Disease Control and Prevention (China CDC). This mouse study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of China CDC. The protocol was approved by the Committee on the Ethics of Animal Experiments of IOHPC (Permit number: EAWE-2010-029). Aluminum hydroxide gel (Alhydrogel, Brenntag Biosector, DK-3600 Frederikssund, Denmark) ($Al(OH)_3$) and CpG 1826 (5′-TCCATGACGTTCCTGACGTT-3′) (synthesized in Takara Biotechnology (Dalian) Co., Ltd., Japan) were used as adjuvants, separately or in combination, with NP protein. The mice were injected intramuscularly with NP protein alone or with adjuvant (Table 1). Mice immunized with placebo normal saline (NS) or $Al(OH)_3$ plus CpG were used as controls. Immunization was performed three times at 2-week intervals (Fig. 1). Blood samples were collected from six mice in each group on days 14, 28 and 38, after which mice were sacrificed, and spleens were aseptically removed and ground through a 200-mesh sieve. Spleen mononuclear cells (SMNCs) were obtained after erythrocytes were depleted using Ammonium-Chloride–Potassium (ACK) lysis buffer (0.15 mol/L NH_4Cl, 0.01 mol/L $KHCO_3$ and 0.1 mol/L Na2-EDTA.$2H_2O$, pH 7.2–7.4). On day 10 after the final immunization, 15 mice in each group were anesthetized with sodium pentobarbital (10 mg/ml) at a dose of 60 mg/kg body weight and challenged with 50 μl of 20-MLD50 PR8 by intranasal infection. The daily body weight loss and mortality were monitored for 3 weeks after challenge. Mice that lost 30% of their initial weight were euthanized and scored as dead (Wang et al., 2014; Tompkins et al., 2007; Patterson et al., 2007). All experiments were performed twice.

Table 1
Summary of the mouse groups immunized with NP formulated with adjuvant.

Group	Antigen/dose	Adjuvant/dose	Immune route
G1	NS placebo	None	i.m.
G2	NS placebo	$Al(OH)_3$/100 μg+CpG/10 μg	i.m.
G3	NP/10 μg	None	i.m.
G4	NP/10 μg	CpG/10 μg	i.m.
G5	NP/10 μg	$Al(OH)_3$/100 μg	i.m.
G6	NP/10 μg	$Al(OH)_3$/100 μg+CpG/10 μg	i.m.
G7	NP/90 μg	None	i.m.

ELISA protocol

NP protein-specific antibody titers were measured using ELISA. The 96-well ELISA plates were pre-coated with 100 μl of NP protein (2 μg/ml) in 50 mM sodium bicarbonate buffer (pH 9.6) overnight at 4 °C. Washed wells were blocked by incubation with PBS containing 2% BSA (Amresco, Solon, OH, USA) for 2 h at 37 °C. A total of 100 μl of serially diluted serum samples in PBS containing 1% BSA were added to each well and incubated for 1.5 h at 37 °C, followed by the addition of 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) (Sigma-Aldrich, St. Louis, MO, USA), IgG1 or IgG2a (Southern Biotech, Birmingham, AL, USA) (1:5000 dilution), respectively, for 1.5 h at 37 °C. After washing, 100 μl of tetramethylbenzidine (TMB) substrate was added to each well. Plates were incubated for 5 min at room temperature in darkness. The reaction was stopped by adding 50 μl of 1 M H_2SO_4. The absorbance at 450 nm was determined using an ELISA reader (Thermo Labsystems Dragon, Wellscan MK3, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibody levels in serum were expressed as endpoint titers and defined as the highest sample dilution with an OD 450 nm reading greater than 2.1-fold the mean value of the naïve samples (unvaccinated mice serum).

ELISPOT assay

The number of peptide NP_{55-69}-(RLIQNSLTIERMVLS, Th epitope in BALB/c (H-2^d) mice) and $NP_{147-155}$-(TYQRTRALV, CTL epitope in BALB/c (H-2^d) mice) specific SMNCs that secreted IFN-γ, IL-4, or IL-10 from immunized mice was determined using the murine ELISPOT kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, ELISPOT plates were coated with anti-murine IFN-γ, IL-4, and IL-10 antibodies (5 μg/ml) overnight at 4 °C. RPMI-1640 medium containing 10% fetal bovine serum (FBS, GIBCO, Langley, OK, USA) (R-10) was added to block nonspecific sites for 2 h at room temperature. SMNCs were aseptically isolated, and 5×10^5 SMNCs suspended in 100 μl of R-10 containing 10 μg/ml of NP_{55-69} or $NP_{147-155}$ were added to each well. After incubation for 40 h in a 5% CO_2 incubator at 37 °C, 100 μl detection antibody (biotinylated anti-mouse IFN-γ, IL-4, or IL-10; 2 μg/ml) was added to each well and incubated for 2 h at room temperature. Then, 100 μl enzyme complex solution (100-fold dilution) was added, followed by incubation for 1 h at room temperature. Substrate solution (100 μl) was added to each well, and the reaction was allowed to proceed for 20 min at room temperature in darkness. The ELISPOT plate was rinsed with flowing water to terminate the reaction. An ELISPOT image analyzer (Bio-SYS GmbH, Bioreader 4000 PRO-X, Karben, Germany) was used to determine the number of spot-forming cells (SFCs).

Statistical analysis

SPSS (version 17.0) and Prism (version 5.0a) softwares were used for statistical analysis. Antibody titers were converted by $\log_{10}$ before statistical analysis. Statistical analysis of the antibody titers and ELISPOT results among groups was performed using one-way ANOVA. The weight changes on each day were analyzed using repeated-measures ANOVA. Survival-rate curves were analyzed using the Log-rank (Mantel−Cox) test. Differences with $p \le 0.05$ were considered significant.

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