A CpG 1018 adjuvanted neuraminidase vaccine provides robust protection from influenza virus challenge in mice

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Influenza virus infections pose a significant threat to global health. Vaccination is the main countermeasure against influenza virus spread, however, the effectiveness of vaccines is variable. Current seasonal influenza virus vaccines mostly rely on the immunodominant hemagglutinin (HA) glycoprotein on the viral surface, which usually leads to a narrow and strain-specific immune response. The HA undergoes constant antigenic drift, which can lead to a dramatic loss in vaccine effectiveness, requiring the annual reformulation and readministration of influenza virus vaccines. Recently, it has been demonstrated that the subdominant glycoprotein, neuraminidase (NA), is an attractive target for vaccine development. Here, we tested a newly developed recombinant influenza virus N1 neuraminidase vaccine candidate, named N1-MPP, adjuvanted with CpG 1018, a TLR9 agonist. Additionally, N2-MPP and B-NA-MPP vaccine constructs have been generated to cover the range of influenza viruses that are seasonally circulating in humans. These constructs have been characterized in vitro and in vivo regarding their functionality and protective potential. Furthermore, a trivalent NA-MPP mix was tested. No antigenic competition between the individual NA constructs was detected. By adjuvating the recombinant protein constructs with CpG 1018 it was possible to induce a strong and robust immune response against the NA, which provided full protection against morbidity and mortality after high lethal challenges in vivo. This study provides important insights for the development of a broadly protective NA-based influenza virus vaccine candidate.

INTRODUCTION

Influenza viruses can cause severe respiratory infections in humans and pose a significant threat to global health. According to the World Health Organization, seasonal influenza viruses cause up to five million cases of severe influenza virus infection, including up to 650,000 deaths each year globally. The influenza virus contains two glycoproteins on its viral surface, which include the immunodominant hemagglutinin (HA) and the immunosubdominant neuraminidase (NA). Current available seasonal influenza virus vaccines mostly target the HA as the NA content in the vaccines is not standardized and can vary significantly. While the HA of the influenza virus is known to induce a strong neutralizing antibody response in humans, it is more susceptible to antigenic drift. This can lead to a mismatch between vaccine strains and circulating strains, resulting in a significant loss of effectiveness of the annual vaccines.

Over the past few years, the NA has emerged as an attractive target for vaccine development since it is less prone to undergo antigenic drift and therefore is antigenically more stable. In addition, it has been demonstrated in animal models as well as in humans that anti-NA immunity correlates with protection and reduces viral shedding. The main obstacles to using NA as a vaccine antigen are its lack of standardization in seasonal vaccine preparations as well as its fragile stability. Indeed, the amount of NA in seasonal vaccines usually varies in quality and quantity and it is likely that the structural integrity in current vaccine formulations is suboptimal. Additionally, it has been hypothesized that antigenic competition occurs between HA and NA in vaccine formulations, making the NA immunosubdominant. The anti-NA immunity acquired after vaccination with live attenuated or inactivated vaccines is mediocre at best. Stable, recombinant NA protein has been shown to be immunogenic and protective in animal models and may enhance NA-based immunity in humans by standalone administration or as an admixture to seasonal influenza virus vaccines. Recently, we have developed a recombinant NA vaccine candidate, named N1-MPP10. This vaccine candidate utilized the tetramerization domain of the phosphoprotein of the measles virus to multimerize and stabilize the NA protein. N1-MPP can form fully enzymatically active NA tetramers which are highly protective in vivo in a mouse model and induce high titer of neuraminidase inhibiting (NI) antibodies after vaccination. The tetramerization of the protein is a crucial factor in generating an NA-based vaccine since it has been previously shown that only multimeric but not monomeric protein confers protection in vivo.

In general, the efficiency of a vaccine depends on the magnitude, duration, and quality of the immune response that is induced. However, recombinant protein vaccines usually tend to induce a lower immune response compared to whole virus vaccines due to the lack of molecules that engage innate immune receptors. Here, we tested N1-MPP—as well as novel N2-MPP and B-NA-MPP constructs in a trivalent formulation—adjuvanted with the GMP-produced TLR9 agonist CpG 1018 (ODN1018) in the mouse model. CpG 1018 is currently used in the licensed hepatitis B virus vaccine HEPPLISAV-B. The work described here was performed in preparation for the clinical testing of N1-MPP with CpG 1018.

RESULTS

A prime-boost regimen with N1-MPP is required to achieve full protection in a naive mouse model

To assess the adjuvant effect of CpG 1018 on the N1-MPP antigen in a prime-only or prime-boost vaccination regimen, naive female mice...
6–8 week old BALB/c mice (n = 5 per group) were either vaccinated once with 3 μg N1-MPP, 3 μg N1-MPP + 3 μg CpG 1018, or 3 μg of an irrelevant protein, or twice with the same formulations in a 3-week interval (Fig. 1A). Three weeks post boost, mice were then challenged with 10 x the 50% mouse lethal dose (mLD$_{50}$) of A/Singapore/GP1908/2015 H1N1 (IVR-180, this virus is antigenically equivalent to A/Michigan/45/15) virus, and weight loss and survival were monitored over a 14-day period. As shown
in Fig. 1B, mice that only received one vaccination with non-adjuvanted N1-MPP or irrelevant protein all succumbed to infection around day 8 post challenge. Mice vaccinated once with N1-MPP + CpG 1018 experienced high weight loss (approximately 20%) and 4 out of 5 mice succumbed to infection around day 10 post challenge. Nevertheless, mice vaccinated with the adjuvanted formulation survived significantly longer (p = 0.0143). However, groups of mice that received two vaccinations of N1-MPP or N1-MPP + CpG 1018 did not experience any weight loss and all animals in these groups survived the challenge. All mice in the negative control group succumbed to infection around day 8 (Fig. 1B, C). To assess serological characteristics of the serum antibodies induced after vaccination, the serum was tested via an enzyme-linked immunosorbent assay (ELISA) against recombinant Mich15 N1-VASP protein. This protein containing a different tetramerization domain was used to avoid the detection of antibodies induced against the MPP tetramerization domain. After the prime, only a low antibody response against the NA was detectable, with slightly higher levels in the N1-MPP + CpG 1018 group (Fig. 1D). Serum from mice vaccinated with the prime-boost regimen showed a strong increase in N1-specific antibodies, with N1-MPP + CpG 1018 performing the best (Fig. 1D). The same trend was observed in a NI assay using an H7N1Mich15 virus, which contains an irrelevant HA and the N1 of A/Michigan/45/15. Mice vaccinated with the prime-boost regimen showed high levels of NI active antibodies in their serum with the N1-MPP + CpG 1018 group performing best (geometric mean 50% inhibitory dilution (ID$_{50}$) = 7046) indicating that the CpG 1018 adjuvant leads to a more robust immune response to the antigen (Fig. 1E). The NI results also indicated a statistically significant difference between adjuvanted and non-adjuvanted groups in the prime-boost regimen.

Passive serum transfer from vaccinated to naive mice protects from lethal challenge

Serum obtained from mice vaccinated with the prime-boost regimen with N1-MPP, N1-MPP + 3 µg CpG 1018 or an irrelevant protein was used to perform a passive serum transfer into 6–8 week old naive BALB/c mice (n = 5 per group) followed by a challenge with 5xLD$_{50}$ of A/Singapore/GP1908/15 H1N1 (IVR-180). Weight loss and survival were monitored over a 14-day period. Mice which received N1-MPP serum showed ~10% weight loss, with one mouse succumbing to infection on day 6 post challenge. Mice that received N1-MPP + CpG 1018 experienced slightly less weight loss than the N1-MPP group and experienced no mortality (Fig. 2A, B). The negative control group succumbed to infection by day 8 post challenge.

CpG 1018 adjuvantage partially breaks the immunodominance of HA over NA in a QIV + rNA formulation in terms of anti-NA antibody titers

We have previously shown, that – while recombinant NA on its own is immunogenic—admixture to QIV leads to reduced immunogenicity, likely due to the immunodominance of HA over NA. To test if CpG 1018 would improve NA immunogenicity, even in a mixture with QIV, naive female 6–8 week old BALB/c mice (n = 5 per group) were vaccinated IM in a prime-boost regimen. Mice received either 3 µg of N1-MPP, 3 µg N1-MPP + 3 µg CpG 1018, QIV (matched with the challenge virus), QIV + 3 µg CpG 1018, 3 µg N1-MPP admixed with QIV, 3 µg N1-MPP admixed with QIV and 3 µg CpG 1018, 3 µg of N1-MPP given in the right leg and QIV at the same time in the left leg (N1-MPP + QIV (r) + QIV (l)), 3 µg of N1-MPP + 3 µg CpG 1018 given in the right leg and QIV + 3 µg CpG 1018 given in the left leg at the same time (N1-MPP + 3 µg CpG 1018 (r) + QIV + 3 µg CpG 1018 (l)) or 3 µg of irrelevant protein (Fig. 3A). Following vaccination, mice were
Fig. 3 Preclinical assessment of N1-MPP and seasonal QIV in combination with CpG 1018. A Vaccination scheme, mice (n = 5 per group) were vaccinated in a prime/boost regimen and then challenged either with 25xLD₅₀ of A/Singapore/GP1908/15 H1N1 (IVR-180) to monitor weight loss and survival over a 14-day period or with 1xLD₅₀ of A/Singapore/GP1908/15 H1N1 (IVR-180) to determine viral lung titers. B Weight loss curve (mean plus standard deviation) and C survival after viral challenge are shown. Differences in survival were analyzed using a Mantel–Cox log-rank test. N1-MPP vs Neg. Ctrl. p = 0.0404, all other groups vs. Neg. Ctrl. p = 0.0082, all other comparisons were not statistically significant (p > 0.05). D Viral lung titers obtained on day 3 (n = 3 per group) and E day 6 (n = 3 per group) post challenge. Statistical analysis in D and E was performed using an one-way ANOVA corrected for multiple comparisons, shown is mean plus standard deviation.
challenged with 25×10⁶ pfu of A/Singapore/GP1908/2015 H1N1 (IVR-180). Weight loss and survival was monitored over a 14-day period. The non-adjuvanted N1-MPP group showed the highest post challenge weight loss of ~10% and one mouse in the group succumbed to infection on day 6 post infection. All mice in the negative control group succumbed to infection on day 6 post infection. The remaining groups did not experience any morbidity or mortality (Fig. 3B, C).

To observe the effect of CpG 1018 on the reduction of viral load in the lungs, a subset of mice was infected with a lower challenge dose of 1×10⁶ pfu A/Singapore/GP1908/15 H1N1 (IVR-180). Lungs were extracted on day 3 (Fig. 3D) and day 6 (Fig. 3E) post infection and the viral titers were determined. On day 3, the negative control group showed the highest viral titer (7.8×10⁵ pfu/ml), followed by the N1-MPP group (5.1×10⁵ pfu/ml), the N1-MPP+CpG 1018 group (3.0×10⁵ pfu/ml), and the QIV groups (1.0×10⁵ pfu/ml). In the remaining groups, no virus was detectable. On day 6, the virus cleared out in the N1-MPP + CpG 1018 group as well as in the QIV group. The viral titer remained detectable in the N1-MPP group (8.5×10⁵ pfu/ml) but was lower than in the negative control group (2.4×10⁵ pfu/ml).

Since it is known that high titers of NA antibodies correlate with reduction of viral replication as well as less severe disease outcome, we wanted to assess the level of NA active antibodies induced after vaccination with N1-MPP and QIV in combination with CpG 1018. NA assays were performed with H7N1_{Mich15} virus, which contains the matching NA component to the vaccine antigen, and with H7N1_{Cal09} virus to observe if cross-reactive NA antibodies can be induced. Against H7N1_{Mich15}, N1-MPP + CpG 1018 induced the highest level of NA antibodies (ID₅₀ = 62090), followed by N1-MPP + CpG 1018 (r) and QIV + CpG 1018 (l) (ID₅₀ = 7602). N1-MPP + QIV + CpG 1018 was also shown to be effective in the adjuvanted system (ID₅₀ = 15954) and unadjuvanted N1-MPP (ID₅₀ = 15409) (Fig. 4A). Of note, the N1-MPP + CpG 1018, N1-MPP + CpG 1018 (r) and QIV + CpG 1018 (l) and N1-MPP + QIV + CpG 1018 group NA activity were not significantly different suggesting that both administration of HA and NA containing vaccine in different limbs as well as co-administration in the presence of adjuvant may induce similar immune responses while this is not the case when recombinant NA is admixed with QIV without adjuvant. In case of the heterologous H7N1_{Cal09} virus, N1-MPP + CpG 1018 had the highest NA inhibition potential (ID₅₀ = 4585), followed by the unadjuvanted N1-MPP group (ID₅₀ = 1479) (Fig. 4B).

We also wanted to test if antibodies induced through vaccination with N1-MPP would induce N1 subtype cross-reactive antibodies. The serum was tested in ELISAs against recombinant Mich15 N1-VASP protein (Fig. 4C) matching the vaccine antigen strain, and against NC99 N1 (pre-pandemic N1, Fig. 4D), N1 (prototype N1, Fig. 4E), Cal09 N1 (Fig. 4F), Bd18 N1 (Fig. 4G) and Vn04 N1 (avian N1, Fig. 4H). Overall, the same trend was seen as in the NA assay, with N1-MPP + CpG 1018 performing the best, followed by N1-MPP + ODN1018 (r) and QIV + CpG 1018 (l) and N1-MPP + QIV + CpG 1018 admixed. This underscores that CpG 1018 is beneficial in inducing a strong and robust antibody response, which appears to be cross-reactive within the N1 subgroup. N1-MPP + QIV admixed induced only a low N1-specific antibody response as well as lower titers of NA active antibodies, underlining the antigenic competition between HA and NA.

Antigen dose de-escalation shows a clear adjugnet effect of CpG 1018

In the previous animal experiments described above, a standard amount of 3 μg N1-MPP protein per vaccine dose was used in a prime/boost regimen. Using this quantity of NA protein, it was observed that mice vaccinated with unadjuvanted N1-MPP, experienced approximately 10% weight loss (Fig. 5A), with one mouse which succumbing to infection on day 7 post challenge (Fig. 5B). However, mice which received N1-MPP + CpG 1018 did not experience any significant morbidity or mortality. To assess if this trend could be maintained with a reduced amount of N1-MPP, we performed a dose de-escalation study. Mice (n = 4–5 per group) were vaccinated IM in a prime/boost regimen with different amounts of antigen and challenged three weeks after the boost with 25×10⁶ pfu of A/Singapore/GP1908/15 H1N1 (IVR-180). Mice were vaccinated with 1 μg, 0.3 μg, or 0.1 μg of N1-MPP, N1-MPP + CpG 1018 or an irrelevant protein. Mice which received 1 μg of N1-MPP experienced around 20% weight loss (Fig. 5C), with two mice succumbing to infection on day 6 and 7 post challenge (Fig. 5D). However, the 1 μg N1-MPP + CpG 1018 group experienced only 5% weight loss and showed full protection. When vaccinated with 0.3 μg of non-adjuvanted N1-MPP, all mice succumbed to infection by day 7 similar to the negative control group (Fig. 5E, F). The 0.3 μg N1-MPP + CpG 1018 group experienced high weight loss of almost 20% and three mice succumbed to infection by day 8. Vaccination with 0.1 μg of antigen, no matter if adjuvanted or non-adjuvanted, failed to protect mice against viral challenge and all mice succumbed to infection by day 7 (Fig. 5G, H).

N2-MPP and B-NA-MPP form stable tetramers and exhibit full enzymatic activity

Current circulating influenza viruses in humans include H1N1, H3N2 and influenza B viruses. Therefore, we generated N2-MPP and B-NA-MPP constructs in addition to the N1-MPP construct. We cloned the sequences encoding for N2 of A/Kansas/14/17 (H3N2) and influenza B-NA of B/Colorado/6/17 into a pFastBac Dual vector containing a measles virus phosphoprotein tetramORIZATION domain (BSA) served as monomeric control. To confirm that the NA-MPP proteins present the correct antigenic epitopes, an ELISA was performed using a broad panel of N2 (Fig. 6C) and B-NA (Fig. 6D) specific human monoclonal antibodies (mAbs). An irrelevant anti-Lassa antibody KL-AV-1A12 was included as negative control. All mAbs showed strong binding to N2-MPP and B-NA-MPP suggesting that the probed epitopes are presented in a native-like
Fig. 4 Crossreactivity of serum antibodies against different N1 proteins in NI and ELISA. A NI using a reassortant H7N1\textsubscript{Mich15} virus containing the N1 of A/Michigan/45/2015 or B containing the N1 of A/California/04/09. Geometric mean plus standard deviation is shown. Statistical analysis in A and B was performed using an one-way ANOVA corrected for multiple comparisons, shown is mean plus standard deviation. ELISA crossreactivity testing against C Mich15 N1-VASP protein, D NC99 N1-VASP protein, E PR8 N1-VASP protein, F Cal09 N1-VASP protein, G Bris18 N1-VASP protein, and H Vn04 N1-VASP protein. For C to H, mean and standard deviation are shown. N = 5 per group.
conformation. Next, we wanted to determine if the proteins are enzymatically active. For this, a standard NA-Star assay was performed. N1-MPP was included as a positive control. It was observed that N2-MPP and B-NA-MPP had high enzymatic activity comparable to the already established N1-MPP construct (Fig. 6E). However, the NA activity significantly varied between N1, N2, and B-NA-MPP which is not surprising since the different NA subtypes are known to have varying enzymatic activity based on the strain of origin17.

Vaccination with recombinant N2-MPP and B-NA-MPP provides full protection against lethal influenza virus challenge in the mouse model

To test if the recombinant N2-MPP and B-NA-MPP could induce a protective immune response, we vaccinated mice IM with 3 µg of the respective antigen (n = 5 per group) in a prime/boost regimen. The protein was either given non-adjuvanted or supplemented with CpG 1018. Irrelevant protein was administered as negative control. Vaccination with N1-MPP was included as a positive control. Following vaccination, mice were challenged either with 25xL50 of A/Singapore/GP1908/15 (H1N1), A/Switzerland/9715293/13 (H3N2, mouse adapted) or B/New York/PV01181/18. Mice vaccinated with N1-MPP alone experienced a weight loss of around 10%, whereas the N1-MPP + CpG 1018 group did not show any morbidity or mortality (Fig. 7A, B). In case of N2-MPP vaccination, both the non-adjuvanted and adjuvanted group showed ~10% weight loss (Fig. 7D). However, the N2-MPP + CpG 1018 group did not experience any mortality, whereas in the N2-MPP group one mouse succumbed to infection on day 8 post challenge (Fig. 7E). For the B-NA-MPP vaccination, the unadjuvanted group experienced 10% weight loss, with two mice succumbing to infection on day 3 and 5 post challenge (Fig. 7G, H). However, the B-NA-MPP + CpG 1018 group did not show any morbidity or mortality. The antibody response against the individual NA-MPP antigens was tested via ELISA. In general, non-adjuvanted groups induced a lower immune response compared to the groups receiving NA protein with CpG 1018 (Fig. 7C, F, I).

A trivalent NA-MPP vaccine formulation does not induce antigenic competition between the individual NAs and is capable of inducing a strong immune response

Since H1N1, H3N2 and influenza B viruses are all circulating in humans, a trivalent vaccine formulation containing all three NAs...
For this reason, mice ($n=5$ per group) were vaccinated either with N1-MPP, N1-MPP + CpG 1018, N1 + N2 + B-NA-MPP, N1 + N2 + B-NA-MPP + CpG 1018 or influenza B virus HA protein (negative control) in a prime-boost regimen. After vaccination, mice were challenged with 25xMLD50 of A/Singapore/GP1908/15 (H1N1). The group which received N1-MPP alone experienced ~10% weight loss with one mouse which succumbed to infection on day 6 (Fig. 8A, B). The other groups did not show any morbidity or mortality. To test if vaccination would also protect against challenge with a heterologous N1 virus, another subset of mice was challenged with 25xMLD50 of A/Vietnam/1203/04 (H5N1, 6:2 A/Puerto Rico/8/34 reassortant, HA polybasic cleavage site removed). While all groups experienced weight loss of ~20%, partial protection was observed in all vaccinated groups with the trivalent, adjuvanted formulation performing best (Fig. 8C, D). In terms of serology, mice which had been vaccinated with N1 + N2 + B-NA-MPP + CpG 1018 had the strongest antibody response in ELISA against recombinant Mich15-VASP protein (Fig. 8E). In an NI assay using H7N1Mel15, the inhibition potential of groups N1-MPP + CpG 1018 and N1 + N2 + B-NA-MPP + CpG 1018 was the highest (Fig. 8F).

DISCUSSION

Influenza virus vaccines provide significant protection against influenza virus infections but the current vaccines are impacted by antigenic drift\textsuperscript{18–22}. In addition, seasonal influenza virus vaccines induce an unbalanced immune response mostly targeting the HA but not the NA of the virus\textsuperscript{6,15}. However, NA undergoes somewhat slower antigenic drift\textsuperscript{8} and anti-NA immunity can be highly protective in animal models and humans\textsuperscript{1–6,9,11,23–28}. Several factors may influence this lack of immunogenicity of NA in seasonal vaccines, including non-standardized amounts of NA in the vaccine preparations, instability of conformational epitopes on the NA in the formulations, and HA immunodominance over NA. Vaccination with stable, tetrameric recombinant NA could overcome these issues, either when used as a standalone vaccine or when admixed to current seasonal vaccines. We have previously reported recombinant NA constructs based on N1 NA fused to a measles virus phosphoprotein tetramerization domain\textsuperscript{10}. Here we have expanded this work to N2 and influenza B virus NA and have shown that these proteins are also immunogenic and protective in the mouse model, especially when used with a TLR9 adjuvant, CpG 1018. Importantly, mixing these three components into a trivalent formulation did not decrease the immune response to the N1 component or protection.
from challenge with H1N1 or H5N1 viruses. Through passive transfer experiments we also demonstrated that the induced humoral immune response is sufficient for protection, even though cellular immune responses to NA may contribute to protection as well. For initial experiments we used a 3 µg dose of recombinant NA. The rationale behind this was that split vaccine with 1 µg of HA per dose is often used by us and others in the mouse model. The human dose is 15 µg, so 1/15th of the human dose is used. Recombinant HA-based vaccines for humans contain 45 µg of HA per subtype and since the vaccine tested here is a recombinant protein vaccine, we used 1/15th of 45 µg for the recombinant NA as well, resulting in a 3 µg dose. In a dose de-escalation study performed here the 3 µg dose worked indeed better than lower doses. However, it is unclear if increasing the dose further would lead to an improvement in terms of protection. For humans, we are planning to give only one dose since we do not think that higher amounts of NA per shot would be commercially feasible.

The main focus of this study was to explore the combination of an adjuvant with our recombinant NA constructs. The TLR9 agonist CpG 1018 was selected as adjuvant since it has an extensive safety record and is currently used in a licensed hepatitis B virus vaccine13,14 which are important pragmatic reasons to include it as adjuvant and which facilitate clinical development significantly. Here we show, that CpG 1018 has a significant adjuvant effect in terms of the induced immune response, protection from the challenge as well as in terms of antigen sparing. In addition, we detected strong cross-reactivity to heterologous N1 NAs, especially in the adjuvanted groups, and the adjuvanted trivalent NA vaccine showed the highest degree of protection against H5N1 challenge. The adjuvanted recombinant NA vaccine consistently outperformed the non-adjuvanted recombinant NA vaccine in our study. Previously, we have shown that even when recombinant NA is admixed to seasonal vaccine preparations, HA dominates over NA and suppresses a robust anti-NA response10. By administering seasonal vaccine into one leg of the mice and recombinant NA into another leg this could be partially circumvented and led to a strong NA response. However, this is of course less practical for a vaccine product. Here we observed that the addition of CpG 1018 to the seasonal vaccine/recombinant NA admixture at least partially broke the immune dominance of the HA, making it possible to just administer one shot that still resulted in robust anti-NA immunity. The mechanism behind this is so far unclear but could be as simple as attracting more immune cells to the injection side and providing an innate immune trigger that enhances the immune response in general. If this effect is unique to CpG 1018 remains to be determined. In the future, we plan to explore this and also conduct an analysis of innate and T-cell immune responses after vaccination with adjuvanted recombinant NA immunogens.
An important caveat of our study which needs to be pointed out is, that it was performed in naive mice and that so far we have not tested the vaccine candidate in other animal models like hamsters and ferrets. Humans are already immunologically primed for NA and will therefore likely respond differently than naive mice.

In summary, we show that the combination of recombinant NA with CpG 1018 is inducing robust anti-NA immunity and protection in the mouse model. This warrants further clinical development of the combination and will hopefully result in a next-generation seasonal influenza virus vaccine with more resistance to antigenic drift, which may also partially protect from emerging pandemic influenza virus subtypes.

**METHODS**

**Cells and viruses**

Madin-Darby canine kidney (MDCK) cells (ATCC #CCL-34) were maintained in Dulbecco's Modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin antibiotics mix (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco) and 1% hydroxyethylpiperazine ethane sulfonic acid (HEPES; Gibco). BTN-TN-SB1-4 (Trichoplusia ni, High Five) cells were maintained in Express Five media (Gibco) containing 1% L-glutamine (Gibco) and 1% penicillin/streptomycin antibiotics mix. SF9 (Spodoptera frugiperda) cells were maintained in Trichoplusia ni medium – Fred Hink (TNM-FH; Gemini Bioproducts) containing 10% FBS, 1% penicillin/streptomycin antibiotics mix, and 1%...
Pluronic F-68 (Sigma Aldrich). For passaging of baculovirus stocks in SF9 cells, the medium was switched to TNE-FH containing 3% FBS, 1% Pluronic F-68 and 1% penicillin/streptomycin antibiotics mix. The reasortant viruses used in this study were grown in 10-day-old embryonated chicken eggs (Charles River Laboratories). The H7N1 viruses used in NA inhibition assay, contain the internal genes of A/Puerto Rico/8/34 H1N1 an exotic H7 HA of A/mallard/Alberta/24/01 H7N3 and either the N1 of A/Michigan/45/2015 H1N1 (H7N1M2bac) or A/California/04/09 H1N1 (H7N1CaG4). The challenge virus A/Singapore/1908/15 (H1N1, Ivr-180 strain) possesses the internal proteins of A/Texas/1/77 (H3N2) and the surface glycoproteins of A/Singapore/1908/15 (pH1N1). A/Scotland/97/15293/13 (H3N2) and B/New York/1/1118/18 are based on wild type backbones but are mouse-adapted; A/Vietnam/1203/04 (H5N1) is a reassortant virus with the internal genes of A/Puerto Rico/6/34 H1N1 and has a deleted polybasic cleavage site.

Recombinant proteins

Recombinant proteins used in this study were generated by using the baculovirus expression system. Briefly, coding sequences for N1-MPP, N2-MPP and B-MPP were cloned into a modified pFastBac vector. The vectors where then transformed into DH10Bac, appropriate clones were picked based on blue/white screening, the clones were grown and mid-prepped and the resulting bacmids were transfected into SF9 cells for baculovirus rescue. Rescued baculovirus was then propagated in SF9 cells and used to infect High Five cells for protein expression at a multiplicity of infection of 10. Three days post infection, the High Five cell supernatant was harvested and recombinant NA proteins were purified using Ni⁺⁺ chelate chromatography. The recombinant N1-MPP, N2-MPP and B-MPP proteins used for animal vaccination studies are structured into an N-terminal signal peptide, a Lassa glycoprotein domain instead of a MPP domain. Protein concentrations were measured using Quick Start™ Bradford 1x Dye Reagent (BioRad). The proteins were stored at −80 °C.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To confirm protein integrity, an SDS-PAGE was performed under reducing conditions and by using a bis-sulfosuccinimidyl suberate (BS3; Thermo-Fisher) crosslinker. For the BS3 crosslinker SDS-PAGE, the proteins were treated with the crosslinker according to the manufacturer’s instruction. For the SDS-PAGE 1.5 μg of the respective NA protein was mixed 1:1 with 2× Laemmli loading buffer (BioRad) supplemented with 5% beta-mercaptoethanol. The samples were then heated for 10 minutes at 95 °C prior to loading them on a sodium dodecyl-sulfate polyacrylamide gel (4–20% Mini-PROTEAN TGX™ Precast Protein Gels, BioRad). Afterwards, the gels were stained with Coomassie blue (ThermoFisher) for 1 hour at room temperature and destained with distilled water to visualize the proteins. BSA was used as a monomeric control.

Animal work

All animal experiments were performed under protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee. For all animal experiments conducted, female 6–8 week old BALB/c or DBA2 mice (Jackson laboratories, n = 5 per group) were used, unless otherwise mentioned. The adjuvant, CpG 1018 (TLR9 agonist), used in this study was provided by Dynavax Technologies and administered at a dose of 3 µg per mouse intramuscularly in the vaccine groups. The positive control Flucelvax (LOT 252380, season 2017/18). To observe if two vaccinations are required to induce a robust protective immune response, mice were primed intramuscularly (IM) or primed and boosted (with a 3-week interval) with 3 µg N1-MPP, 3 µg N1-MPP + 3 µg CpG 1018 or 3 µg of an irrelevant protein (B-Mal-HA). Six weeks after the prime, mice were intranasally challenged with 10x the 50% mouse lethal dose (mLD₅₀) of A/Singapore/GP1908/15 H1N1 (Ivr-180). Weight loss and survival were monitored over a period of 14 days. Mice were euthanized if they lost more than 25% of their initial body weight. Blood was taken from each mouse on day 0 and day 42 post prime plus one challenge dose.

In addition, a passive serum transfer was performed using serum from mice previously vaccinated with N1-MPP, N1-MPP + CpG 1018 and B-Mal-HA. Naïve mice received 200 µl of serum intraperitoneally. After 2 hours, the mice were intranasally challenged with 5xLD₅₀ of A/Singapore/ GP1908/15 H1N1 (Ivr-180) and weight loss and survival were monitored over 14 days.

To assess the effect of CpG 1018 in more detail, mice were vaccinated IM in a follow up experiment in a prime/boost regimen separated into the following groups: 3 µg N1-MPP, 3 µg N1-MPP + 3 µg CpG 1018, QIV (1 µg of each HA), QIV + 3 µg CpG 1018, 3 µg N1-MPP admixed with QIV, 3 µg N1-MPP admixed with QIV and 3 µg CpG 1018, 3 µg N1-MPP given in the right leg and QIV given in the left leg at the same time (N1-MPP (r)/QIV (l)), N1-MPP + 3 µg CpG 1018 (r)/QIV + 3 µg CpG 1018 (l) or 3 µg B-Mal-HA. Mice were then challenged intranasally with 25xLD₅₀ of A/Singapore/ GP1908/15 H1N1 (Ivr-180). Weight loss and survival were monitored over 14 days. Blood was obtained on day 21 and day 42 after prime. Mice were euthanized if they lost 25% or more of their initial body weight.

To determine the viral load in murine lung tissues, mice were vaccinated using the same regimen but with the difference that they were challenged with a reduced challenge dose of 1xLD₅₀ of A/Singapore/GP1908/15 H1N1 (Ivr-180) to gain a better resolution. Lungs were taken on day 3 and day 6 post challenge, homogenized and the viral titer determined via standard plaque assay.

To assess if the amount of N1-MPP protein could be potentially reduced yet still induce a protective immune response, mice were vaccinated IM in a prime-boost regimen with different doses of N1-MPP. Mice received either 3 µg, 1 µg, 0.3 µg, or 0.1 µg of N1-MPP, N1-MPP + the matching amount of CpG 1018 or irrelevant B-Mal-HA protein. Mice were then challenged with 25xLD₅₀ of A/Singapore/GP1908/15 H1N1 (Ivr-180) and weight loss and survival were monitored over 14 days. Mice were euthanized if they lost more than 25% of their initial body weight.

To observe the protective potential of N2-MPP and B-NA-MPP, mice were vaccinated IM in a prime-boost regimen with 3 µg N2/B-NA-MPP, 3 µg N2/B-NA-MPP + CpG 1018, or 3 µg B-Mal-HA (or Lassa glycoprotein for mice challenged with influenza B virus). Vaccination with N1-MPP in the same setup was included as a positive control. Mice were then either challenged with 25xLD₅₀ of A/Singapore/GP1908/15 H1N1 (Ivr-180) or 25xLD₅₀ of B/New York/PV0118/18. For the challenge with 25xLD₅₀ of A/Switzerland/97/15293/13 (H3N2) female DBA/2J mice were used. Weight loss and survival were monitored over 14 days. Mice were euthanized if they lost more than 25% of their initial body weight. Blood was obtained on day 21 and day 42 after prime.

To determine if there would be antigenic competition between the individual NA-MPP proteins when combined in one vaccination, mice were vaccinated IM in a prime-boost regimen either with 3 µg N1-MPP, 3 µg N1-MPP + CpG 1018, 3 µg of each N1, N2 and B-NA-MPP, 3 µg of each N1, N2 and B-NA-MPP + CpG 1018 or 3 µg of B-Mal-HA. Mice were then challenged either with 25xLD₅₀ of A/Singapore/GP1908/15 H1N1 (Ivr-180) or 5xLD₅₀ of A/Vietnam/1203/04 (H5N1, 6:2 A/Puerto Rico/8/34 reassortant, HA with deleted F-68 and 1% penicillin/streptomycin antibiotics mix. The reassortant virus (A/Singapore/GP1908/15 (H1N1, Ivr-180 strain) possesses the internal proteins of A/Texas/1/77 (H3N2) and the surface glycoproteins of A/Singapore/GP1908/15 (pH1N1). A/Scotland/97/15293/13 (H3N2) and B/New York/1/1118/18 are based on wild type backbones but are mouse-adapted; A/Vietnam/1203/04 (H5N1) is a reassortant virus with the internal genes of A/Puerto Rico/6/34 H1N1 and has a deleted polybasic cleavage site.

NA-star assay

The NA enzymatic activity of the NA-MPP proteins was determined by using the NA-Star™ Influenza NA Inhibitor Resistance Detection Kit (ThermoFisher) following the manufacturer’s instructions. As a starting control (Ivr-180), 10µg/ml of the respective protein was used and then serially diluted 1:3 across the plate. The signal was based on luminescence read-out and was measured using a Synergy H1 hybrid multimode microplate reader (BioTek). The data were analyzed using GraphPad Prism 8.

Enzyme-linked immunosorbant assay (ELISA)

ELISAs were conducted as described previously. Briefly, flat-bottom 96-well plates (Ismunlon 4 HBX plates, ThermoFisher) were coated overnight...
then developed by adding KPL TrueBlue Peroxidase Substrate (SeraCare). For ELISAs which were performed using mAbs, the antibodies were diluted to a start concentration of 30 µg/ml and then serially diluted 1:3 across the plate. The antibodies were incubated on the plate for 1 h at RT.

Neuraminidase inhibition (NI) assay

The NI assay was conducted as described previously12. Briefly, flat-bottom 96-well plates (Immulon 4 HBX plates, ThermoFisher) were coated overnight at 4 °C with 150 µl/well of fetuin (50 µg/ml; Sigma Aldrich). The next day, serum samples were heat inactivated for 1 h at 56 °C and then diluted to a starting concentration of 1:100 in PBS. The samples were diluted 1:2 across a fresh 96-well plate. The reagent viruses used in this assay, H7N1Cadeo and H7N1Michi15, were diluted in PBS and then added to the serum dilution at 2× the 50% effective concentration (EC50) for 1 h at 37 °C. The reaction was stopped by adding 50 µl/well of 3 M hydrochloric acid (HCl). The signal was read using a Synergy H1 hybrid multimode microplate reader (BioTek) at an optical density of 490 nm. The data were analyzed using GraphPad Prism 8 software and values were expressed as the area under the curve (AUC). The cutoff was defined as the average of all blank wells plus three times the standard deviation of the blank wells.

Lung titers

Plaque assays for virus titration have been conducted as described previously. Briefly, confluent MDCK monolayers were infected with different sample dilutions (1:10 to 1:1000000) of homogenized lung tissues diluted in 1X minimum essential medium (MEM) (11% penicillin/streptomycin antibiotics mix, 1% HEPE, 1% L-glutamine and 1% sodium-bicarbonate (Gibco)) for 1 h at 37 °C. Afterwards, the virus dilution was removed and an overlay consisting of 2% Oxoid agar (ThermoFisher), H2O, 2X MEM, diethylaminoethanol (0.1% wt/vol) DEAE) dextran and N-p-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (1 µg/ml) was added to the cells. Plates were incubated for 2 days at 37 °C and then fixed using 10% paraformaldehyde overnight at 4 °C. Afterwards, the agar overlay was carefully removed, and the plaques visualized by immunostaining. Plates were blocked for 1 h at RT with 3% milk/PBS. The blocking solution was discarded, and the plates incubated with primary antibody (anti-N1 mAb 4A5) diluted 1:3000 in 1% milk/PBS for 1 h at RT. The plates were washed three times with PBS and then developed by adding KPL TrueBlue Peroxidase Substrate (SeraCare).

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COMPETING INTERESTS
F.K. is listed as an inventor on patent applications regarding NA-based influenza vaccines filed by the Icahn School of Medicine at Mount Sinai. J.D.C., P.T., and R.L.C. are present or former employees of Dynavax Technologies and may hold stock or stock options.

ADDITIONAL INFORMATION

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