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RESEARCH ARTICLE

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Evolutionary conservation and positive selection of influenza A nucleoprotein CTL epitopes for universal vaccination

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Abstract

Influenza (flu) infection is a leading cause of respiratory diseases and death worldwide. Although seasonal flu vaccines are effective at reducing morbidity and mortality, such effects rely on the odds of successful prediction of the upcoming viral strains. Additional threats from emerging flu viruses that we cannot predict and avian flu viruses that can be directly transmitted to humans urge the strategic development of universal vaccination that can protect against flu viruses of different subtypes and across species. Annual flu vaccines elicit mainly humoral responses. Under circumstances when antibodies induced by vaccination fail to recognize and neutralize the emerging virus adequately, virus-specific cytotoxic T lymphocytes (CTLs) are the major contributors to the control of viral replication and elimination of infected cells. Our studies exploited the evolutionary conservation of influenza A nucleoprotein (NP) and the fact that NP-specific CTL responses pose a constant selecting pressure on functional CTL epitopes to screen for NP epitopes that are highly conserved among heterosubtypes but are subjected to positive selection historically. We identified a region on NP that is evolutionarily conserved and historically positively selected ($NP_{137-182}$) and validated that it contains an epitope that is functional in eliciting NP-specific CTL responses and immunity that can partially protect immunized mice against lethal dose infection of a heterosubtypic influenza A virus. Our proof-of-concept study supports the hypothesis that evolutionary conservation and positive selection of influenza NP can be exploited to identify functional CTL epitope to elicit cross-protection against different heterosubtypes, therefore, to help develop strategies to modify flu vaccine formula for a broader and more durable protective immunity.

KEYWORDS

conserved epitope, CTL immunity, influenza, positive selection, universal vaccine

1 | INTRODUCTION

Influenza (flu) is a highly contagious, airborne respiratory tract infection associated with a significant disease burden. The annual "mild" influenza epidemic caused by antigenic drift of the virus affects 10%–20% of the world's population with up to 5 million cases of serious illness and 500,000 deaths.[1,2] Although seasonal flu vaccines are effective at reducing morbidity and mortality, such effects rely on the odds of successful prediction of the circulating strains during the upcoming flu season. With the realization that avian flu viruses can be directly transmitted to humans, flu is now considered a major global health threat.[3] There is an urgent need for the strategic development of universal vaccination that can protect the immunized population against flu viruses of different subtypes and across species.

Viral clearance relies on the development of a strong immune response. This includes the induction of antibodies and cytotoxic T lymphocyte (CTL) responses, which are required for killing infected cells and clearance of the virus.[4–7] In the face of flu viral antigenic drift and different subtypes emerging in different flu seasons, annual flu vaccines that elicited mainly humoral responses failed to provide robust and durable protection. When antibodies induced by previous infection or vaccination fail to recognize and neutralize the virus adequately, virus-specific CTLs are the major contributors to the control of viral replication and elimination of infected cells. Antigenic sites of the influenza A virus (IAV) hemagglutinin (HA) recognized by vaccine-elicited neutralizing antibodies display a high degree of variability by time and across IAV heterosubtypes; therefore, HA tends to nurture evolution that would allow the viruses to escape from the humoral immune pressure.[8] In contrast, most known IAV epitopes that are recognized by CTLs were relatively more conserved.[9,10]

The conserved internal nucleoprotein (NP) of IAV is a major target of immunodominant $CD8^+$ CTLs.[11] NP is critical for influenza viral genome function and is highly conserved across different heterosubtypes within influenza type A, B, C, and D, respectively, with several conserved motifs shared by all types,[12–14] despite stable mutations associated with viral genetic evolution and/or due to positive selection by host immunity.[15,16] Our preliminary computational studies utilized the combined features of evolutionary conservation and the evoluted variants due to positive selection of the IAV NP and identified a CTL epitope within the longest conserved fragment of the NP amino acid (AA) sequence. Experimentally, we validated that this epitope is effective in eliciting CTL immune responses and can partially protect immunized mice against a lethal dose heterosubtypic IAV infection. A better understanding of the flu genomic evolution and host biological correlates regarding the conserved NP and functional CTL responses against IAVs is aligned with a better ability to utilize the genomic and genetic features of influenza to better design flu vaccines that could be more effective, broad, and durable in protection. Our proof-of-concept research supports that it is worth exploring the potential of CTL epitopes that are within the conserved and positively selected regions of the IAV NP protein in driving a broad and durable anti–flu immunity as a universal vaccination strategy.

2 | MATERIALS AND METHODS

2.1 | Mice

All mice were on the BALB/c background. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee and Institutional Review Board at Louisiana State University.

2.2 | Mouse-adapted IAV

Mouse-adapted influenza viruses, A/Purto Rico/8/34 (PR8, H1N1),[17] were a kind gift from Dr. Rong Hai (University of California, Riverside) and was propagated in allantoic fluids of 10-day-old embryonated chicken eggs at 37°C for 3 days. The median tissue culture infectious dose (TCID50) of PR8 viruses were determined in MDBK cells as previously described.[18] The lethal doses of PR8 viruses in mice were titrated on BALB/c mice and calculated by the Reed–Muench method.[19]

2.3 | Conservation scoring of influenza A heterosubtype nucleocapsid proteins

To determine the conserved regions of nucleocapsid proteins (at AA sequence level) in different influenza A heterosubtype viruses, reference sequences were obtained from the NCBI gene database, including those for A/Puerto Rico/8/34(H1N1) (NP_040982.1), A/New York/392/2004(H3N2) (YP_308843.1), A/Goose/Guangdong/1/96(H5N1) (YP_308667.1), and A/Hong Kong/1073/99(H9N2) (YP_581749.1). ClustalX[20,21] was used to process the AA sequences and assess the quality of the alignment to generate the conservation scores to indicate the structure/function-based similarity of each AA.

2.4 | Determination of historically positively selected residuals on nucleocapsid protein

To determine the historically positively selected regions of influenza A NP, we selected viral isolates obtained from New York during 1993–2007, because multiple H3N2 isolates from the same population in this area were sequenced and during the selected period, there was no dramatic change in circulating viral strains or pandemic that could disrupt the model of stable immune pressure-mediated historical positive selection. We randomly selected four isolates from each year, with an accumulative number of 60 NP sequences of IAV H3N2 subtypes. These NP sequences were analyzed in ClustalX and a phylogenetic tree was generated using TreeView, followed by

analyses in phylogenetic analysis by maximum likelihood (PAML)[22] to determine IAV H3N2 NP AA residual sites that are positively selected ($dN/dS > 1$) by a steady immune pressure.

2.5 | Peptides

NPCTL (N-TYQRTRALV, influenza A $NP_{147-155}$), M2e (N-KSLLT EVETPIRNEWGCRCNDSSD, in influenza A M2),[23] and (2F5)2 (N-ELDKWA-ELDKWA, from HIV GP41 epitope for monoclonal antibody 2F5)[24] peptides were synthesized at Genemed Synthesis Inc. All the peptides were purified by high-performance liquid chromatography and analyzed by mass spectrometry. The purity of the peptides was above 90%.

2.6 | Immunization

Female BALB/c mice (6–8 weeks old) were intraperitoneally immunized with NPCTL peptide (10 μg per mouse), in Freund's incomplete adjuvant (Thermo Fisher Scientific, 77145, referred to as "F" hereafter), Alum adjuvant (Thermo Fisher Scientific, 77161, referred to as "Al" hereafter), oil-in-water emulsion adjuvant (as previously described by us and others,[25,26] referred to as "MF59"), CpG-ODN 1926 (InvivoGen, referred to as "CpG"), or combined adjuvant as indicated, or in phosphate-buffered saline (PBS) at a final volume of 200 μl. Boosting immunization was given with the same vaccine formula 2 weeks post the first immunization. To determine the immunogenicity of the vaccine formulas, three mice per group were immunized. For antibody titer analysis, sera were collected 7 days after the booster. Sera from mice immunized with PBS were collected as negative controls, while sera from mice immunized (priming and boosting at the same time of the vaccinated group) with M2e peptide in Al adjuvant were used as positive controls in the antibody detection experiments using enzyme-linked immunosorbent assay (ELISA). For interferon-γ (IFN-γ) enzyme-linked immune-spot (ELISPOT) assays, mice were euthanized 8 days after the booster and spleens were collected.

2.7 | Antibody detection by ELISA

NPCTL- and M2e-specific antibodies were measured by ELISA. Briefly, Nunc-Immuno plates (Nunc 442404) were coated with 50 μL PBS-diluted M2e peptide (for M2e-immunized positive control) or NPCTL peptide (for all NPCTL-immunized samples), 5 μg/ml, at 4°C overnight. Unspecific binding was blocked with 200 μl of 0.25% gelatin in PBS-T solution (0.5% Tween-20 in PBS). Fifty microliters of the serially diluted sera was added to each well and incubated at 37°C for one and half hours. After extensive washing with PBS-T solution, binding antibodies were detected by horseradish peroxidase-labeled goat anti-mouse IgG antibody (Agilent Dako; P044701-2) and o-phenylenediamine dihydrochloride substrate (Sigma), following the manufacturer's instructions.

2.8 | IFN-γ detection by ELISPOT assay

ELISPOT assay kit (U-Cytech) was used to determine antigen-specific IFN-γ production, following the manufacturer's instructions. Briefly, an anti-mouse IFN-γ monoclonal antibody was coated on the 96-well ELISPOT plates at 4°C for 8 h. The coated plates were then blocked with 200 μl of 1% bovine serum albumin in PBS at 37°C for 4 h. Lymphocytes in the spleens were enriched by density separation on mouse lymphocyte cell separation buffer (LTS1092; TBD) and resuspended in complete RPMI-1640 medium (Hyclone) with 10% fetal bovine serum. A total of 1×10^5 cells were added into each well of the ELISPOT plates, followed by 1 μg/ml of the NPCTL peptide antigen. HIV-1 GP41 2F5 epitopes were used as a nonspecific antigen target and as a negative control. Cells with peptide stimulation were then incubated at 37°C for 40 hours with 5% CO_2. Subsequently, the plates were then extensively washed, followed by a biotinylated detection antibody and φ-labeled anti-biotin antibody. After incubation with freshly prepared activator I/II for 15 min, the visible spots of IFN-γ-secreting cells were photographed with a microscope system and enumerated by TotalLab software (UK).

2.9 | Influenza virus challenge

To determine the efficacy of cross-protection by the CTL epitope vaccines, eight mice per group were used for each vaccine formula being tested, following a priming–boosting vaccination regimen (Figure 3A). Seven days after the booster dose, mice with the indicated immunizations following the procedure described above were challenged with 5 mouse lethal dose 50 (MLD50) of the PR8 virus (equal to 7.5×10^3 $TCID_{50}$ as determined in MDCK cells). Briefly, mice were anesthetized by isoflurane (Fluriso; VETone) and the viruses were delivered to the mice intranasally in 50 μl of sterile PBS. Mouse body weight and survival rates were monitored daily for 2 weeks post infection. Mice that lost ≥30% of their original weight were euthanized and recorded as a death event.

2.10 | Statistical analysis

Data between groups were compared by one- or two-way analysis of variance, or *t* test. The survival rate was compared by a log-rank test. All the statistical analysis was performed by GraphPad Prism version 8.00 (GraphPad), with $p \leq 0.05$ considered statistically significant. "NS" refers to "no significance."

3 | RESULTS

3.1 | Determination of NP residuals that are historically positively selected and conserved amongst heterosubtypes

IAV NP is highly conserved in subtypes, regardless of the host specificity, and phylogenetic clustering based on NP sequences is more suitable than that based on HA/NA sequences to reveal the lineage evolution of IAVs.[27,28] It has been shown that the ratios between nonsynonymous and synonymous (*dN*/*dS*) mutations in the NP CTL epitope regions are higher than that in the rest of the NP AA sequences.[29] To illustrate the relationship of the NP regions that are conserved across heterosubtypes and those subjected to CTL selections, we combined the analyses of NP conservation among IAV subtypes and positive selection in H3N2 under constant pressure. To reveal the conserved domains across IAV heterosubtypes, we selected the reference NP sequences of four well-studied isolates, A/Puerto Rico/8/34 (H1N1), A/New York/392/2004 (H3N2), A/Goose/Guangdong/1/96 (H5N1), and A/Hong Kong/1073/99 (H9N2), and then computed and plotted the nonconserved AA sites (Table 1 and Figure 1, red dots). Gaps between nonconserved sites represent regions that are conserved amongst the NPs in IAV heterosubtypes, including H1N1, H3N2, H5N1, and H9N2. To determine the historically positively selected regions of influenza A NP, we selected viral isolates obtained from New York during 1993–2007, because multiple H3N2 isolates from the same population in this area were sequenced, and during the selected period, there was no dramatic change in circulating viral strains or pandemic that could disrupt the model of stable immune pressure-mediated historical positive selection. We randomly selected four isolates from each year, with an accumulative number of 60 NP sequences of IAV H3N2 subtypes. These NP sequences were analyzed in ClustalX and a phylogenetic tree was generated using TreeView, followed by analyses in PAML[22] to determine IAV H3N2 NP AAs that are positively selected ($dN/dS > 1$). We identified 24 positively selected sites (Table 2), six of which were within the conserved regions (Figure 1, blue dots).

3.2 | Immunogenicity of a CTL epitope within the longest conserved region that contains a positively selected site

Within the longest conserved region that contained a positively selected site, our computation identified $NP_{147–155}$ (TYQRTRALV), a functional CTL epitope validated by HLA binding and cytotoxicity by IFN-γ ELISPOT assay in human cells.[30,31] Major histocompatibility complex class I (MHC-I) recognizes and presents peptide antigens by binding to the essential sequence of the epitope and allows conformational plasticity in the group of peptides being presented on the

TABLE 1 Nonconserved amino acid residuals in H1N1, H3N2, H9N2, and H5N1 nucleoprotein

Site	H1N1	H3N2	H9N2	H5N1	Score
16	D	D	G	G	29
18	E	D	E	E	69
31	K	K	R	R	66
33	I	I	V	V	65
34	G	D	G	G	39
41	I	I	V	I	72
52	Y	H	Q	Y	13
61	L	L	I	I	69
65	R	K	R	R	73
77	K	K	R	K	73
100	V	V	R	R	16
101	N	D	D	D	62
105	M	M	V	V	56
109	I	V	I	I	72
127	D	E	E	E	69
136	M	I	M	M	70
183	V	V	I	V	72
186	V	I	V	V	72
194	V	I	I	I	72
197	I	V	I	I	72
210	E	E	D	E	69
214	K	K	R	R	66
217	I	S	I	I	24
236	K	R	R	R	73
239	M	V	M	M	65
247	D	N	N	N	62
253	F	I	I	I	33
257	T	I	I	I	30
280	V	A	V	V	39
283	P	P	L	L	10
286	A	S	A	A	73
293	R	K	R	R	73
305	R	K	R	R	73
312	V	I	V	V	72
313	Y	Y	F	F	44
343	V	L	V	V	60
344	L	L	S	S	14
348	K	R	R	R	73

(Continues)

TABLE 1 (Continued)

Site	H1N1	H3N2	H9N2	H5N1	Score
351	K	K	R	R	66
353	V	S	I	A	20
357	K	K	Q	Q	69
371	M	M	V	M	65
372	E	D	E	E	69
373	T	N	A	T	46
375	E	G	D	D	35
384	R	G	R	R	31
406	I	T	I	I	30
408	I	V	V	V	72
421	D	E	E	E	69
422	R	K	R	R	73
423	T	S	P	A	36
425	V	I	I	I	72
430	T	T	K	T	53
442	T	A	T	T	72
450	S	G	S	S	39
451	A	A	A	S	73
452	R	K	R	R	73
455	D	E	D	D	69
459	Q	R	Q	Q	65
472	A	T	T	T	72
473	S	N	N	N	60

same MHC-I molecule.[32,33] $NP_{147-155}$ (TYQRTRALV) is an immunodominant CTL epitope in mice,[34] and to minimize the epitope size in the interest of avoiding induction of humoral responses, we chose $NP_{147-155}$ (TYQRTRALV) to further define the potential of inducing cross-protective CTL responses using CTL epitopes that are within conserved regions that contain positively selected sites. The protective effects of CTL epitopes as vaccine antigens in murine models variesd depending on the route and formula of vaccination.[35–38] To determine the immunogenicity, including the ability of inducing humoral and cellular immunities, of the $NP_{147-155}$ immune epitope, we tested the antigenicity of $NP_{147-155}$ in combination with different adjuvant formulas, including incomplete Freund's, aluminum, oil-in-water, CpG DNA, and combined adjuvants. Mice were immunized with a priming and boosting dose, and sera and splenocytes were analyzed 7–8 days post the booster for antibody and cytotoxicity assays, respectively.

As $NP_{147-155}$ (TYQRTRALV) was predicted to be a CTL epitope within minimal humoral antigenicity, we included M2e peptide immunization (as we previously reported[23]) as a positive control of antibody induction (Figure 2A). As predicted, we observed minimal humoral responses in mice immunized with the $NP_{147-155}$ epitope (below the limit of detection of the ELISA assay kit). To further determine the cellular antigenicity, we restimulated the splenocytes with the antigen peptide, using an irrelevant peptide (HIV 2F5 epitope) as a negative control. In contrast to antibody production, the BALB/c mice that received two immunizations developed a robust CTL response (determined by IFN-γ ELISPOT) when CpG adjuvant was present, alone, or in combination with incomplete Freund's, Alum, or MF59 adjuvant (Figure 2B). Of note, the combination of Alum and CpG adjuvants for $NP_{147-155}$ epitope peptide immunization allowed the induction of the most robust epitope-specific IFN-γ producing cells.

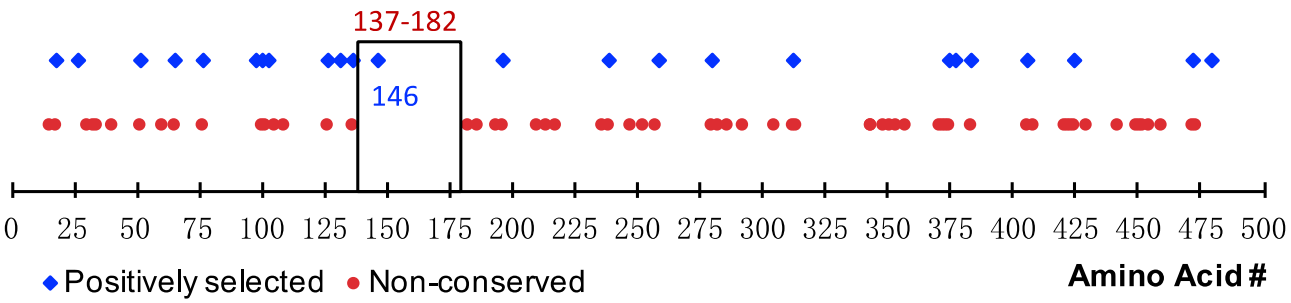

FIGURE 1 Distributions of heterosubtypically nonconserved and historically positively selected amino acids in influenza A virus nucleoprotein (IAV NP). A/Puerto Rico/8/34 (H1N1), A/New York/392/2004 (H3N2), A/Goose/Guangdong/1/96 (H5N1), and A/Hong Kong/1073/99 (H9N2) NP reference sequences were downloaded from the NCBI database and analyzed using ClustalX to reveal the heterosubtypically nonconserved amino acids; NP sequences of H3N2 isolates isolated from New York, 1993–2007, 4 isolates per year × 15 years = 60 isolates were obtained from NCBI database and analyzed using a combination of ClustalX, TreeView, and phylogenetic analysis by maximum likelihood to reveal the historically positively selected amino acid locations. The longest conserved region 137–182 contains a positively selected site 146

TABLE 2 Historically positively selected amino acids in IAV H3N2 NP

Residual site	18	27[a]	52	65	77	98	100	103[a]
Positive AA	D	A	H	K	K	R	V	K
Probability	0.938	0.938	0.938	0.894	0.938	0.938	0.918	0.938
Residual site	127	131[a]	136	146[a]	197	239	259[a]	280
Positively AA	E	S	I	A	V	V	L	A
Probability	0.938	0.932	0.938	0.938	0.938	0.938	0.937	0.938
Residual site	312	375	377	384	406	425	472	480[a]
Positively AA	I	G	S	G	T	I	T	D
Probability	0.938	0.916	0.938	0.928	0.938	0.938	0.93	0.938

Note: NP sequences of H3N2 isolates isolated from New York, 1993–2007, 4 isolates per year × 15 years = 60 isolates were analyzed using PAML to reveal the historically positively selected AA sites/locations. "Positive AA" annotates AAs that are determined as positively selected in the indicated positions.

Abbreviations: AA, amino acid; IAV, influenza A virus; NP, nucleoprotein; PAML, phylogenetic analysis by maximum likelihood.

[a]AA positions that are conserved.

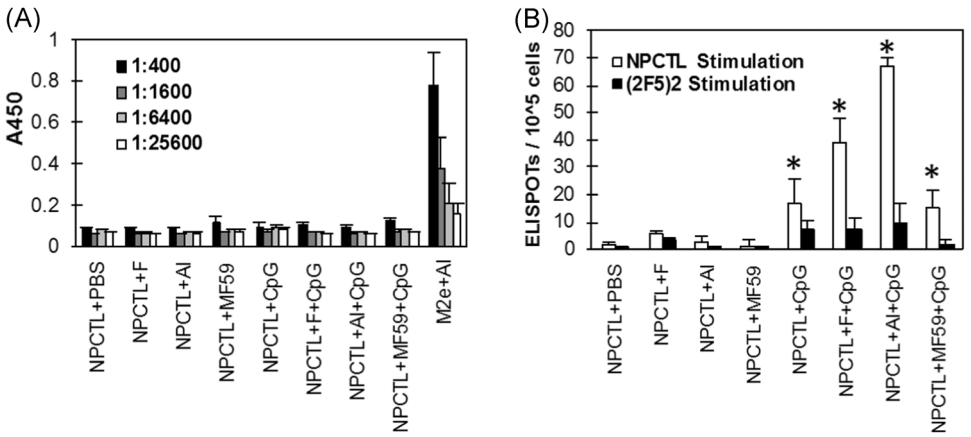

FIGURE 2 NPCTL immunization elicits cytotoxic T lymphocytes (CTLs) but not humoral responses in BALB/c mice. Female BALB/c mice were intraperitoneally immunized with NPCTL peptide (10 μg per mouse at a final volume of 200 μl) in incomplete Freund's (F), aluminum (Al), oil-in-water (MF59), CpG DNA, or combined adjuvants as indicated. A booster immunization was given with the same vaccine formula 2 weeks later. Sera and splenocytes were collected 7–8 days after the second immunization. Phosphate-buffered saline (PBS) was used as adjuvant control, M2e peptide in Al was used as a positive control for antibody induction, and HIV-1 gp41 2F5 epitope peptide was used as a negative control of nucleoprotein (NP)-specific CTL stimulus. (A) Peptide antigen-specific antibody titer determined by the average value of absorbance at 450 nm (*A*450) using enzyme-linked immunosorbent assay. (B) NP-specific IFN-γ production determined by ELISPOT (# of spots generated with 1e5 splenocytes were presented). Data were presented in mean ± SD. *$p < 0.05$ by Student's *t* test, comparing to NPCTL + PBS group restimulated with NPCTL epitope peptide. $N = 3$

3.3 | Immunization with H3N2 $NP_{147–155}$ epitope provides protective immunity against lethal H1N1 infection

Although seasonal flu vaccines rely on the odds of successful prediction of the upcoming viral strains, they trigger mainly humoral immunity that allows neutralizing antibodies to effectively bind to and neutralize flu viruses that are the same or similar to the predicted strains, before viral entry into the host cells to prevent the establishment of vibrant virus replication. CTL immune effects may function more predominantly during the later stage of infection, that is, after the host cells were infected and process the viral antigens for MHC presentation and T-cell activation. It is possible that CTL immune response is not sufficient to provide protective immunity against lethal IAV infection, even if the immune epitope is highly conserved and immunogenic. To further determine whether the predicted H3N2 NPCTL epitope immunization can provide sufficient immune protection against a heterosubtype IAV infection, we immunized female BALB/c mice with NPCTL peptide in the presence of Alum, CpG, or CpG + Alum adjuvants, and infected the immunized

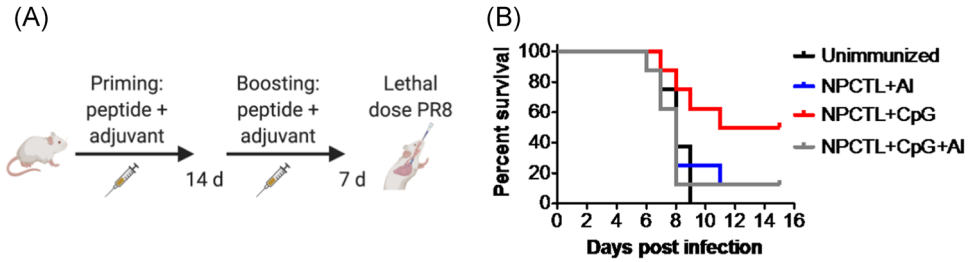

FIGURE 3 H3N2 NPCTL immunization with CpG provides partial cross-protection against lethal infection by H1N1 PR8. (A) Female BALB/C mice were intraperitoneally immunized twice (14 days apart) with NPCTL peptide in aluminum (Al), CpG DNA, or combined CpG + Al adjuvant. Seven days post the second immunization, mice were infected intranasally with 5 mouse lethal dose 50 (MLD50) of the H1N1 PR8 virus. (B) Survival of mice that were immunized and infected with a lethal dose of PR8. Survival curves are presented, and $p < 0.05$ between NPCTL + CpG group versus all others by log-rank test. $N = 8$

mice (7 days after the priming and boosting vaccinations) with 5 MLD50 of H1N1 (PR8) viruses. Interestingly, while we observed the most robust NPCTL-specific CTL responses in mice immunized with $NP_{147-155}$ in the presence of CpG + Alum (Figure 2B), these mice succumbed to the lethal dose infection of PR8 (Figure 3). In contrast, in mice that were immunized with the $NP_{147-155}$ in the presence of CpG, we observed a modest $NP_{147-155}$ specific CTL response (Figure 2B), but these mice were better protected against the lethal infection by PR8 (Figure 3). These data suggest that H3N2 NPCTL epitope immunization can elicit protective immunity against lethal dose infection of H1N1 and that an overreactive CTL response may result in adverse effects during acute lethal infection, likely through off-target cytotoxicity that can lead to host immunopathology.

4 | DISCUSSION

IAVs are among the viruses with the highest mutation rates,[39] along with active re-assortment of vRNA segments,[40] allowing IAVs a diverse repertoire of genetic materials for evolutionary selection. The world's population is repeatedly vaccinated against and infected with influenza; therefore, a robust immune memory is expected to place constant selecting pressure on the circulating IAV strains. Viral mutants that managed to substitute the immune epitopes targeted by immune memory and remained vibrant are thought to have the advantageous niche in evolution and evolve by positive selection.[41] Positive selection has been proposed to explain preferential segment combinations during IAV reassortment[42] and accumulative mutations in the IAV surface glycoproteins HA and NA that escape neutralizing antibodies,[8,43] as well as mutations on IAV internal proteins M1 and NP that escape CTLs.[10,16] CTL epitopes are bound and presented by MHC-I to the T-cell receptor (TCR) on the CTLs to elicit the production of cytotoxic cytokines including IFN-γ and granzymes to eliminate infected cells.[44] IAV NP mutations on CTL epitopes that disrupted the epitope binding to MHC-I molecules or peptide-loaded MHC-I interaction with TCR have been demonstrated with the ability to escape CTLs.[9,45,46] The loss of immunodominant CTL epitopes led to compromised IAV-specific IFN-γ production and cytotoxic activities of the CTLs,[47] which may explain the result of positive selection of the observed mutant epitopes, as these epitopes managed to escape the CTL immune surveillance.

Studies in both humans and mice have demonstrated a protective role of pre-existing influenza virus-specific $CD8^+$ T cells in reducing the severity of disease and enhancing viral clearance,[6,48–53] including cases reported during the 2009 H1N1 pandemic.[54] Influenza NP is the most abundantly expressed protein during the course of infection and has been reported to be the major target of immunodominant $CD8^+$ T-cell responses.[11,15] Although vaccine-elicited antibodies provide protection against seasonal flu when they are well matched to the viral strains,[55,56] virus-specific CTLs directed towards the internal and conserved viral components such as the NP may offer a universal protection.[48,57,58] These CTLs are protective against different heterosubtypes as well as viruses transmitted across species. For instance, H3N2 NP-specific CTLs are cross-reactive to H1N1 NP and their presence is correlated with a decrease of symptomatic infection over multiyear in a large human cohort (2006–2010, >1400 participants) during the period covering both epidemics and the 2009 pandemic.[48] These human H3N2 NP-specific CTLs also have the ability to recognize the avian H5N1 NP and H5N1-infected human peripheral blood monocytes (PBMCs).[59] In avian H7N9-derived flu in human patients diagnosed in Shanghai China during 2013, $CD8^+$ T-cell responses were correlated with a protective effect against severe influenza transmitted from birds to humans.[53] Despite the known contribution of CTLs against IAVs, our knowledge of the evolution of the specific epitopes, the kinetics and types of T-cell responses, the ability of NP epitopes in eliciting protective CTLs as a vaccine antigen, and the extent of cross-reactivity with diverse influenza viruses is limited.

Our preliminary studies exploited the relationship between the evolutionarily conserved and historically positively selected regions on the IAV NPs and identified a region that contains the longest conserved AA sequences along with a positively selected residue (Figure 1). This region contains an epitope, $NP_{147-155}$, referred to as NPCTL epitope in this study. Our studies also validated the immunogenicity of $NP_{147-155}$ in eliciting NP-specific CTL responses that can partially protect mice from lethal dose IAV heterosubtype

infection (Figures 2 and 3). Our proof-of-concept research supports the hypothesis that the evolutionary conservation and positive selection of influenza NP can be exploited to identify functional CTL epitope to elicit cross-protection against different influenza heterosubtypes.

We observed more robust CTL responses in mice immunized with NPCTL epitope in the presence of Alum, incomplete Freund's, and CpG combined adjuvant formulas than in mice immunized with peptide + CpG alone (Figure 2), suggesting that combined adjuvants are more potent in stimulating antigen-presenting function and therefore stronger CTL responses to short peptide antigens. However, although Alum and CpG combined adjuvant elicited a stronger CTL response than CpG alone as determined by IFN-γ ELISPOT (Figure 2B), CpG alone with NP peptide is more capable of eliciting protective immunity against lethal dose heterosubtypic influenza A infection (Figure 3). Our data suggested that extremely strong CTL responses may not favor host survival, because CTLs mainly function to eliminate infected host cells and clear viruses, which may also cause host immunopathology. Indeed, while $CD8^+$ T cells produce inflammatory cytokines such as IFN-γ and granzymes, they can also produce the immunomodulatory cytokine interleukin-10[60–62], which is critical for limiting the immunopathology caused by the CTL responses.[63,64]

Our current research focused on the proof of concept of the evolutionary conservation and historically positive selection of NP epitopes and their potential application in inducing cross-protective CTL immunity. Our predicted results suggested that CTL immune response alone can provide only partial protection against lethal dose flu infection. Future designs of universal flu vaccines shall incorporate conserved immune epitopes that are more accessible by neutralizing antibodies. We have demonstrated that M2e peptide immunization can induce cross-protective neutralizing antibodies and CTL responses and protect mice against lethal dose PR8 infection.[23,25] It is possible that, by combining the highly conserved NPCTL epitopes with the M2e immune epitope in vaccination, a more robust and broadly protective vaccine-induced immunity may be achieved by inducing M2-specific antibodies, M2, and NP-specific CTLs.

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CONFLICTS OF INTEREST

Weishan Huang receives research support from MegaRobo Technologies Corporation, which was not used in this study. Michael C. McGee declares that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Michael C. McGee and Weishan Huang performed experiments and analyzed the data. Weishan Huang designed experiments, wrote the manuscript, secured funding, and supervised the research.

DATA AVAILABILITY STATEMENT

Influenza nucleocapsid protein sequences were obtained from the NCBI gene database as described in the "Materials and methods" section, and data generated from the wet lab experiments are available from the corresponding author upon reasonable request.

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