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Universal influenza DNA vaccine encoding conserved CD4⁺ T cell epitopes protects against lethal viral challenge in HLA-DR transgenic mice

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Abstract

The goal of the present study was to design a vaccine that would provide universal protection against infection of humans with diverse influenza A viruses. Accordingly, protein sequences from influenza A virus strains currently in circulation (H1N1, H3N2), agents of past pandemics (H1N1, H2N2, H3N2) and zoonotic infections of man (H1N1, H5N1, H7N2, H7N3, H7N7, H9N2) were evaluated for the presence of amino acid sequences, motifs, that are predicted to mediate peptide epitope binding with high affinity to the most frequent HLA-DR allelic products. Peptides conserved among diverse influenza strains were then synthesized, evaluated for binding to purified HLA-DR molecules and for their capacity to induce influenza-specific immune recall responses using human donor peripheral blood mononuclear cells (PBMC). Accordingly, 20 epitopes were selected for further investigation based on their conservancy among diverse influenza strains, predicted population coverage in diverse ethnic groups and capacity to recall influenza-specific responses. A DNA plasmid encoding the epitopes was constructed using amino acid spacers between epitopes to promote optimum processing and presentation. Immunogenicity of the DNA vaccine was measured using HLA-DR4 transgenic mice and the TriGrid™ *in vivo* electroporation device. Vaccination resulted in peptide-specific immune responses, augmented HA-specific antibody responses and protection of HLA-DR4 transgenic mice from lethal PR8 influenza virus challenge. These studies demonstrate the utility of this vaccine format and the contribution of CD4⁺ T cell responses to protection against influenza infection.

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Keywords

Influenza; CD4⁺ T cell epitopes; HA-specific antibodies

1. Introduction

Influenza viruses are single-stranded RNA viruses of the family Orthomyxoviridae. Of the 3 types (A, B, C) infectious in humans, only influenza A and B viruses occur in highly pathogenic forms and only the A virus is known to cause pandemics. Seasonal epidemics affect up to 15% of the world's population resulting in 3–5 million cases of severe illness and approximately 500,000 deaths per year [1]. The very young, immunologically naïve, and very old, undergoing immune senescence, are most susceptible to infections. During epidemics, approximately 90% of all influenza-related deaths occur among people 65 years of age or older [2].

Pandemics of influenza A viruses continue to occur at sporadic intervals in human populations. Beveridge reported that at least 20 major influenza pandemics occurred between 1729 and 1968 at irregular intervals of between 3 and 28 years [3]. Three have occurred in the twentieth century alone in 1918, 1957 and 1968. These worldwide pandemics are noted for their high mortality and it is estimated that approximately 50 million people died in the 1918 pandemic and at least 1.5 million people in the 1957 and 1968 outbreaks combined [4]. The critical issue with development of a pandemic vaccine is the uncertainty of which subtype will cause the next event. Since 1996, several novel avian subtypes, H5N1, H7N1, H7N2, H7N3, H7N7 and H9N2 have crossed the species barrier from domestic poultry to humans and have caused a spectrum of mild to severe and even fatal human disease[5–7]. At present, a novel swine-associated H1N1 virus is being transmitted among humans. The severity of disease has been comparable to illness associated to seasonal influenza. However, it remains unknown whether this variant will further adapt to humans and become more virulent. Thus, in spite of the success of currently licensed influenza vaccines, development of new vaccines with increased effectiveness in specific age groups and development of pandemic influenza vaccines appear to be of significant interest.

Hemagglutinin (HA)-specific antibody response is the surrogate most commonly used to evaluate vaccine protection. Additional surrogate markers currently under study include matrix2-specific antibodies and CD4⁺ and CD8⁺ T cell responses [8,9]. Several lines of evidence support a role of cellular immune responses in protection against influenza. Epstein and colleagues demonstrated that either CD8⁺ or CD4⁺ T-lymphocytes promoted survival in mice immunized with an experimental DNA vaccine encoding internal viral proteins [10]. Crowe and colleagues have demonstrated that vaccination of a single HA-specific helper T-lymphocyte (HTL) epitope in mice increased epitope-specific T cell numbers and a significant reduction in viral titers following challenge [11].

Early human studies also indicated cellular immune responses play a role in controlling influenza infection. McMichael and colleagues inoculated 63 volunteers intranasally with live unattenuated influenza A/Munich/1/79 virus and found that all subjects with demonstrable T cell responses cleared virus effectively [12]. Sonoguchi and colleagues found that students previously infected with H3N2 virus were partially protected against subsequent infection with H1N1 subtype virus suggesting cross-subtype protection in humans during sequential epidemics [13]. The protective effect of cellular immune responses may be particularly relevant in the elderly where studies suggest that cellular immune responses may be an important predictor of protection [14]. Recent studies have

also demonstrated a correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children [15]. In part, the mechanisms employed by CD4⁺ T lymphocytes may be a result of ‘help’ for enhancing protective antibody responses and generation on optimal CD8⁺ T cell responses [16–20].

Taken together, these findings indicate cellular immunity contributes to the control and clearance of infection and reduces pathogenesis. Our working hypothesis is that vaccination with conserved CD4⁺ influenza-specific epitopes can establish a pool of memory T cells, resulting in more rapid and vigorous induction of immune responses upon vaccination with seasonal vaccines or infection with live virus. While vaccine induction of cellular immunity alone would not be expected to provide complete protection; upon infection, helper-T cells assisted, humoral and cytotoxic responses would mediate control of infection and prevent death. Because of T cell epitope conservation in different strains, such strategy can be tested with seasonal strains but success would be expected to extend to new pandemic strains. Accordingly, this report describes the design and evaluation of an HLA-DR epitope-based universal influenza plasmid DNA (pDNA) vaccine.

2. Materials and methods

2.1 Identification of peptides predicted to be conserved CD4⁺ helper T cell epitopes

Algorithm-based T cell epitope scans of amino acid sequences of the influenza proteins for A/HK/156/97 (H5N1) and A/Singapore/1/57 (H2N2) were performed to identify those peptides predicted to bind with high affinity to HLA-DR molecules [21,22]. The analysis included sequences for nucleoprotein (NP), matrix 1 (M1), matrix 2 (M2), nonstructural protein 1 (NS1), nonstructural protein 2 (NS2), polymerase acid (PA), polymerase basic 1 (PB1) and polymerase basic 2 (PB2). Influenza hemagglutinin (HA) and neuraminidase (NA) were not analyzed due to their high variability in different influenza isolates. These predicted epitope peptides, 14 – 19 amino acids in length, were then evaluated for amino acid sequence conservation amongst 52 diverse influenza virus strains (NCBI Taxonomy Browser); including: (1) strains that are currently in circulation (H1N1, H3N2); (2) strains with potential for becoming components of an influenza virus pandemic including agents of past pandemics (H1N1, H2N2, H3N2); and (3) strains associated with zoonotic influenza infections of man (H1N1, H5N1, H7N2, H7N3, H7N7, H9N2) (Table 1). Of note, due to the recent outbreak of swine-associated H1N1 virus, the A/California/7/09 strain was included in the analysis. The conservancy of each epitope was defined as the number of the strains containing the epitope at a 100% identity level divided by the total number of strains used in the analysis. Generally, sequences that were conserved in greater than 30% of the strains were selected for peptide synthesis. Peptides were produced using standard F-moc chemistry, purified by reversed-phase HPLC and characterized using mass spectrometry or sequencing. Peptide binding to HLA-DR molecules representing 13 of the most common HLA-DR types was then measured using standardized processes [21,22].

2.2 Measurement of human memory CD4⁺ T cell responses

PBMC were obtained from HIV and HCV negative donors using apheresis (HemaCare Corporation, Van Nuys, CA), and purified using gradient separation prior to cryopreservation. The HLA-DR types of the donors were not known. Selected HLA-DR binding peptides were tested in two ways: (1) directly *ex vivo* where the PBMC were thawed, rested 5 days in media and responses measured by IFN- γ ELISPOT assay and (2) following a culture step with peptides to increase sensitivity. Specifically, the cryopreserved PBMC were thawed and rested overnight in media containing RPMI + 5% AB human serum/complete media followed by a 7 day expansion by culture with pools of 10 peptides, 1 μ g/ml final concentration of each peptide. On days 1 and 3, cultures were fed with fresh

media and IL2 (100U/ml). On day 7, the cultures were depleted of CD8⁺ lymphocytes using the MACS system (Miltenyi Biotec, Auburn, CA) prior to their use in an IFN- γ ELISPOT assay [22]. Spots were counted using an AID ELISpot Reader System (Straßberg, Germany). HLA-DR-restricted peptides from HIV, HCV and *Plasmodium falciparum* were used to determine background responses.

2.3 pDNA vaccine design and construction

A pDNA construct encoding 20 influenza-derived epitopes in addition to the universal Pan DR epitope (PADRE, seq; AKFVAAWTLKAAA) [23] was constructed. PADRE peptides were engineered by introducing anchor residues for different DR motifs within a poly-alanine background and introduction of bulky and charged residues at positions accessible to T cell recognition. PADRE elicits powerful responses *in vitro* from human PBMCs and in addition, cross-reacts on certain mouse Class II alleles [23]. CD4⁺ T cell epitopes were designed with GPGPG amino acid spacers to promote optimum epitope processing [24]. The gene was codon optimized for expression in mammalian cells (GeneArt, Regensburg, Germany) and was assembled using overlapping oligonucleotides in a PCR-based synthesis followed by subcloning into the pMB75.6 DNA plasmid vector.

The pDNA was produced by growth in *Escherichia coli* strain Stbl2 (Invitrogen, Carlsbad, CA) in terrific broth (TB, Becton Dickinson, Sparks, MD) with kanamycin (25 μ g/ml) and was purified using EndoFree Plasmid Mega kit columns according to the manufacturer's directions (Qiagen, Valencia, CA). The purified pDNA construct was dissolved in water and stored at -70°C .

2.4 Mouse immunogenicity and virus challenge studies

To study the efficacy of pDNA vaccine, HLA-DR4 transgenic mice (Taconic Farm, Hudson, NY) and the mouse adapted H1N1 PR8 virus (Charles River Laboratories, Wilmington, MA) were used. Studies were completed in accordance with the [Guide for the Care and Use of Laboratory Animals](#) with appropriate Institutional Animal Care and Use Committee review.

The pDNA vaccine was delivered intramuscularly (i.m.) using an *in vivo* electroporation device (TriGrid Delivery System®, Ichor Medical Systems, Inc., San Diego, CA) [25]. Briefly, the area proximal to Tibialis anterior muscles of anesthetized (Halocarbon, River Edge, NJ) mice weighing 25 to 30g were shaved and sterilized with isopropanol. The pDNA was delivered bilaterally, 25 μ g DNA/20 μ l in each muscle, 50 μ g total per mouse using 0.3 ml syringes with attached 1/2" 30G needle (BD Ultra-Fine, 328431) and the TriGrid electrode array, 2.5mm electrode length. Four seconds post pDNA injection, the electrical stimulation was applied at 62.5 Volts for a total duration of 40 mS over a 400 mS window. Mice in the positive control group were immunized once at the base of the tail with 15 μ g of inactivated PR8 virus (Charles River Laboratories) in 100 μ l of PBS. Cellular immune responses were measured using an IFN- γ ELISPOT assay following selection of mouse CD4⁺ MACS system as described [26].

HA antibody titers were measured using ELISA and hemagglutination inhibition (HAI). High-binding 96-well ELISA plates (Costar, Corning, NY) were coated with 10 μ g purified inactivated PR8 virus (Charles River Laboratories) and incubated overnight at 4 $^{\circ}\text{C}$. The use of inactivated PR8 virus as a coating antigen will not only detect HA-specific but also presumably other antigen-specific antibody responses such as NA. Plates were washed and blocked with PBS + 3% BSA prior to use. Serial dilutions of immune sera were incubated for 2 hours followed by plate washing. Antigen-specific serum antibodies were detected using HRP-coupled secondary antibody (Invitrogen Life Science, Carlsbad, CA). The plates

were developed with an HRP substrate reagent, stopped with acid, and absorbance measured at 450nm. End-point titers are expressed as the reciprocal of the highest dilution that gives a reading three standard deviations above the mean background.

HAI was performed using chicken RBCs (Charles River Laboratories) incubated at 22–25°C with 4 HA units of the virus in the presence of serially diluted sera, previously treated to destroy heat stable non-specific inhibitor in a 96 well plate for 1 hour. Plates were then visually inspected for agglutination. The highest dilution of antibody that causes inhibition of hemagglutination is considered the HI titer.

Protection of mice against influenza virus challenge was assessed using the PR8 virus strain; completed using biosafety level-2 containment. Virus was administered for challenge 2 weeks post-immunization. HLA-DR4 transgenic mice were lightly anesthetized using Methoxyflurane (Springvale, Australia) and infected intranasally (i.n.) with 100 HAU (4MLD50) of virus in a 20 µl volume. This dose was used to assure that a majority of the unvaccinated animals would not survive the challenge. Additionally, we assumed that the protection afforded by cellular immunity would not be as potent as HA-specific humoral immunity and a high dose of 10–100 MLD50 would be too high to measure any protective effects induced by the HLA-DR-restricted epitopes. Each test group contained 14 mice with experiments organized as follows: (1) 2 mice/group were used to complete IFN-γ ELISPOT assays on day 14 post immunization; (2) 2 mice/group were used to complete IFN-γ ELISPOT assays 6 days post virus challenge and (3) the remaining 10 mice were evaluated for survival for a duration of 14 days post-challenge.

2.5 Statistical analyses

Triplicate well testing was used in the ELISA and ELISPOT and Student *t* test was used to document significant levels of immunogenicity obtained using immunized vs. naïve (non-immunized) mice. A *p* value of < 0.05 indicated a positive response. Statistically significant differences in survival times were established using the Fisher's exact test analysis. Differences were considered statistically significant if the *p* value was < 0.05.

In the assays using human PBMC we knew the donors were not previously exposed to HIV, HCV and assume lack of infection with *P. falciparum*. Therefore responses to peptides derived from these pathogens were used to determine assay background. A mean influenza-specific response was considered significant if the net-SFC number was greater than the mean response of the background plus 2 standard deviations. In addition, a minimum positive response greater than 100 IFN-γ SFC/10⁶ cells in the CD8⁺ cell depleted PBMC was considered the minimum for a positive response.

3. Results

3.1 Identification of influenza-derived CD4⁺ T cell epitopes

The HLA-DR peptide binding motif search of the selected influenza virus gene products resulted in the identification of 943 peptides. A total of 45 peptides were identified which bound with moderate to high affinity to at least 6 of the 13 most common HLA-DR molecules used in our study (Table 2). The 45 peptides were also highly conserved among the 52 diverse subtype strains with an indicated average conservation level of 74% (Table 2).

The 45 peptides were next evaluated for CD4⁺ T cell recognition using PBMC from 19 human volunteers. Recognition of multiple peptides was observed despite the unknown history of recent influenza virus infections; representative responses for 3 donors (753, 6018, 709) are presented in Fig. 1. Note that for donor 753, a total of 16 epitopes (M1.100,

NP.36, NP.186, NP.255, NS1.114, NS2.37, NS2.76, PA.175, PA.457, PB1.216, PB1.408, PB1.446, PB2.411, PB2.435 and PB2.551) were recognized using the *ex vivo*, version of the assay indicating the presence of circulating activated CD4⁺ T cells (Fig. 1A). The magnitude of CD4⁺ T cell responses specific for these epitopes ranged from 160 to 2,040 IFN- γ SFC/10⁶ cells. Following the 7 day peptide expansion step, response to an additional peptide, M1.57, was also observed (Fig. 1B). Responses with somewhat reduced breadth are shown for two other PBMC donors, 6018 and 709, Fig. 2 C, D, E and F.

We believe the observed reactivity is most likely mediated by CD4⁺ T cells since the PBMC preparations were depleted of CD8⁺ cells prior to the IFN- γ ELISPOT analysis. Also, the reactivity was detected in donors expressing different HLA Class I alleles. For example, the M1.57 helper epitope has an embedded HLA-A2-restricted CD8⁺ epitope, indicated by italics and underlined, *K*GILGFVFTLVPSE, but the CD4⁺ epitope was recognized using PBMC from 6 different donors, of which two donors were HLA-A1 and HLA-A3 but not HLA-A2.

Together these data demonstrate the utility for the motif scanning and HLA-DR peptide binding assays for identifying CD4⁺ T cell epitopes. In total, 37 of the 45 selected peptides were documented to be epitopes based on recognition by T cells from at least 2 donors (Table 2).

3.2 Testing of the experimental pDNA vaccine in HLA-DR4 transgenic mice

Based on positive recall responses using human PBMC, conservancy among diverse influenza strains and representation of multiple influenza proteins, 20 of the potential 37 epitopes were selected for use in the experimental 'prototype' vaccine (Table 2 and Fig 2). HLA-DR4 transgenic mice did not respond to highly significant levels of epitope-specific immunity following immunization with the 20 HTL pDNA vaccine (Fig.3A). However, vaccine immunization followed by live PR8 challenge induced detectable CD4⁺ T cell responses to multiple epitopes (M1.57, M1.100, M1.202, NP.36, NP.186, NP.403 and NS1.153), ranging from 54 to 559 IFN- γ SFC/10⁶ cells whereas empty vector DNA immunization followed by live PR8 challenge did not induce detectable responses (Fig.3A).

Vaccine induced protection was assessed using PR8 lethal challenge in the HLA-DR4 transgenic mice. In the 20 HTL pDNA vaccinated group, 7 of 10 immunized animals survived the 15 day duration of infection (Fig. 3B) whereas only a single animal in the empty vector DNA control animal survived. Immunization with inactivated PR8 virus induced a higher level of protection as the entire positive control group survived. These results demonstrate that influenza-derived HLA-DR-restricted CD4⁺ T cell epitopes can induce immune responses that partially protect against influenza infection.

Influenza-derived CD4⁺ T cell epitopes may afford protection by providing 'help' to HA-specific B cells and thus increase the efficiency or rate of antibody production. To evaluate this potential mechanism, we immunized mice with the 20 HTL pDNA vaccine followed by immunization with the inactivated PR8 influenza virus. Seven days following the administration of the virus, significant levels of antibodies were measured in the animals primed with the 20 HTL pDNA vaccine; antibody titers varied from 7,300 to 36,900, mean = 14,000 (Fig. 3C). In contrast, unprimed animals generated significantly lower responses ($P=0.0033$) with mean antibody titers of 3,000. HAI antibody titers were also significantly higher ($P=0.0015$) in the 20 HTL pDNA immunized mice; antibody titers in the 80 to 320 range in contrast to a titer of 80 for control animals. Significant differences in antibody responses were also noted day 14 post-inactivated PR8 immunization (Fig. 3C).

4. Discussion

Currently, influenza vaccines are not capable of conferring protection against emerging variants of even a single influenza virus subtype. Herein, we explored the feasibility of an influenza DNA vaccine encoding HLA-DR-restricted CD4⁺ T cell epitopes derived from highly conserved regions of influenza virus proteins from past, current and potential emerging viral strains. Our vaccine differs from that of standard commercial influenza vaccines in that the vaccine is specifically designed to induce T helper cell activity and its utility would remain effective against both circulating or newly emerging influenza virus because conserved epitopes are targeted.

Influenza A-derived CD4⁺ T cell epitopes were identified using a well established process allowing us to identify 45 conserved CD4⁺ T cell epitopes, 20 which were selected for inclusion in a prototype DNA vaccine. Interestingly, 10 of these epitopes were also identified in independent studies (see Table 2 legend) but 27 are described herein for the first time.

We focused our analyses on the internal proteins of influenza A, specifically; M1, M2, NP, PB1, PB2, PA, NS1 and NS2. This bias was based on data from studies using animal models indicating that protective cellular responses were induced by internal proteins. Numerous studies using the nucleoprotein (NP) and the matrix (M) proteins as vaccine immunogens have been published [10,27,28]. Our results expanded the antigen sources, with a test package consisting of 20 epitopes, 10 of which derived from antigens other than M and NP.

Data from multiple studies in mice have demonstrated the contribution of cellular immune responses in vaccine-induced protection against disease and death; using various vaccine formats [10,27,29–32] and adoptive transfer of CD4⁺ T cells [16]. In our study, a DNA construct encoding the CD4⁺ T cell helper epitopes was produced because this format provides an effective means to deliver multiple epitopes [26]. It is interesting to note that immunization of HLA-DR4 transgenic mice, using the DNA vaccine and the *in vivo* electroporation device, induced only minimal primary responses (Fig. 3). However, this treatment effectively primed CD4⁺ T cell responses; which was demonstrated by the rapid and high magnitude responses observed after infection with the PR8 virus. The vaccine induced priming specific for these peptides in HLA-DR4 transgenic mice which is consistent with their measured HLA-DRB1*0401 (DR4) binding affinities. Five of the 7 peptides exhibited high binding affinity to DR4 of 53nM or less, while the others were characterized with somewhat reduced levels of binding. In contrast, similar responses were not boosted using inactivated PR8 virus in place of the infection; perhaps because the amount of antigen entering the Class II pathway was higher for live viral replication.

To test benefit of these CD4⁺ T cells, mice were first immunized with the DNA vaccine, or control empty vector, and the challenged with a lethal dose of the PR8 virus. A majority of the immunized animals (7 of 10) survived whereas only 1 of the 10 control animals survived. Thus the benefit of primed helper T cell epitope responses was established although the level of protection was not absolute and less than achieved using inactivated PR8 virus. We propose that the vaccine would be ‘universal’ and thus protective against various strains of influenza representing relevant epidemic and potential pandemic strains. However, it should be noted that this assumption is based solely on the fact that the epitopes encoded within the vaccine are conserved among multiple influenza strains and not that multiple strains of influenza were used as challenge virus in this study.

The mechanism of protection mediated by CD4⁺ T cells appeared most likely to be indirect since the survival rate was not as high as that achieved using the inactivated PR8 virus. More specifically, the effect could be related to the action of antibodies and the CD4⁺ T

cells provided support for antibody production. To test this hypothesis, mice were primed with the DNA vaccine and boosted with inactivated PR8 and significantly higher PR8- and HA-specific antibody responses were observed; relative to mice immunized only with the empty vector DNA control. Thus the benefit of the DNA vaccine was likely the positive effect on antibody production. The effect of the vaccine on augmenting cytotoxic T-cell activity was not evaluated but should be considered as a possible explanation for the protective effects of the vaccine.

Our belief is that primed CD4⁺ T cells specific to the internal proteins were able to support B cell differentiation and maturation resulting in enhanced influenza-specific antibody response kinetics. Several investigators have generated data that support this hypothesis using active vaccination experiments [18,19] and the passive transfer of CD4⁺ effector T-cells [16]. As such, our finding does not appear to be unusual.

Several studies, including ours, demonstrated the presence of effector and memory CD4⁺ T cells in humans specific for highly conserved epitopes; epitopes that occur in both human season influenza virus strains and the avian H5N1 virus [33,34]. It is tempting to speculate that expanding these reactive CD4⁺ T cells in the human population by deliberate vaccination might, at least to some degree, afford increased protection against influenza infection in vulnerable subpopulations, as well as provide protection against new seasonal variants and possibly even new pandemic strains and this could be the basis for a new approach to augmenting the efficacy of current influenza virus vaccines.

The HLA system is highly polymorphic and the use of individual epitopes to design a vaccine could result in a product that fails to provide the desired efficacy in diverse ethnicities. However, we reasoned that the use of multiple epitopes, each binding multiple HLA molecules, would enable the development of a vaccine that would be sufficiently immunogenic in most major ethnic populations. The potential population coverage was calculated using peptide-HLA-DR binding affinities and reported gene frequencies for HLA-DR types to estimate the fraction of individuals with the genetic potential to respond to the vaccine encoded epitopes [35]. Based on the 20 epitopes used in this study and the 13 HLA-DR allelic products used in the binding analyses (Table 2) we calculated a theoretical minimal population coverage of 89%. Thus, this approach appears well suited for use in the design new influenza virus vaccine product.

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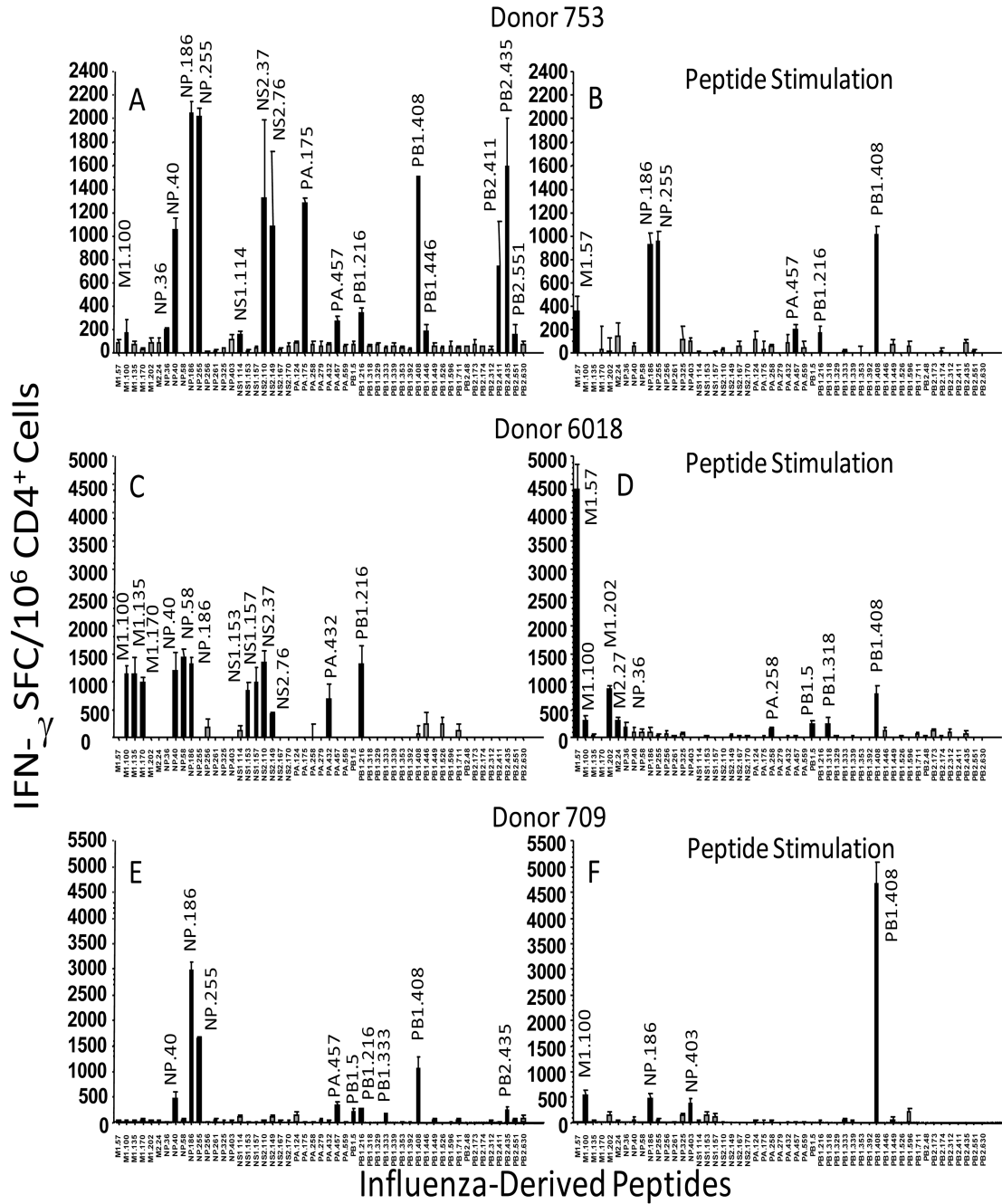


Fig. 1. HLA-DR-restricted influenza recall responses from human donors. Human donor 753 panels A and B; donor 6018 panels C and D and donor 709 panels E and F. Panels A, C and E depict recall responses measured in the absence of *in vitro* peptide expansion while panels B, D and F depict recall responses measured using an *in vitro* peptide expansion step. CD4⁺ cells from normal human donors were enriched for assay use. The experimental values are expressed as mean IFN- γ net spots/10⁶ CD4⁺ lymphocytes \pm SEM for each peptide; tests were performed using triplicate wells. Significant responses from the indicated epitopes were defined as responses > than mean of background responses + (2.0 \times Std. Dev.). Background responses were determined using supertype HLA-DR binding peptides from

pathogens to which donors were not exposed, i.e., HIV, HBV, HCV and *Plasmodium falciparum*.

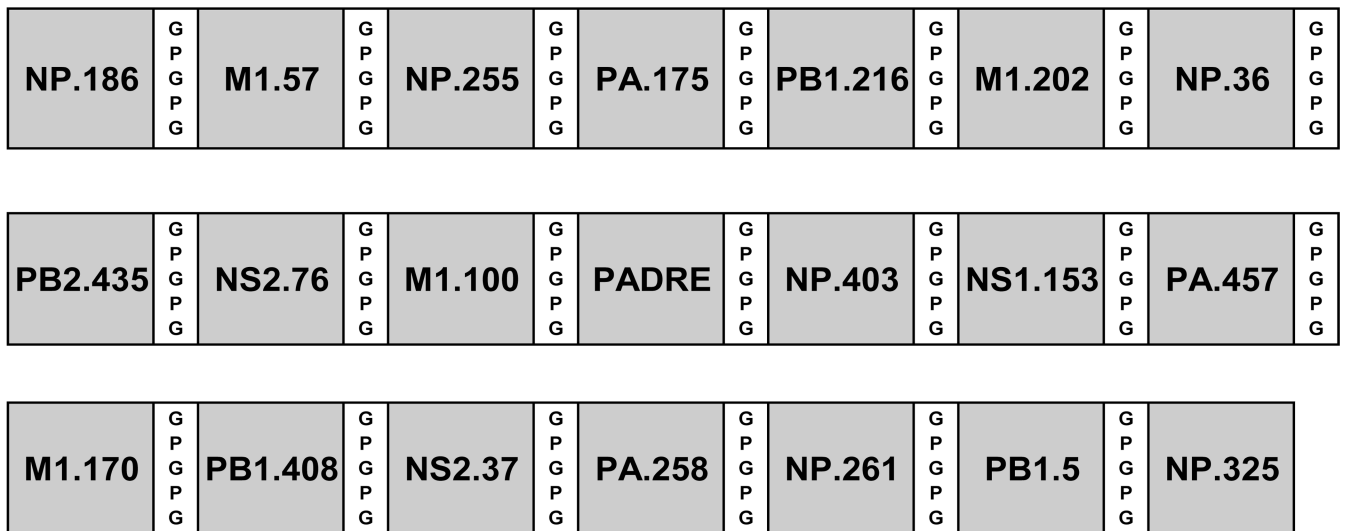


Fig. 2. Schematic diagram of the CD4⁺ 20 epitope DNA insert. The epitope order and amino acid spacer usage are shown. Influenza-derived epitopes were used in addition to the universal helper epitope, PADRE [23].

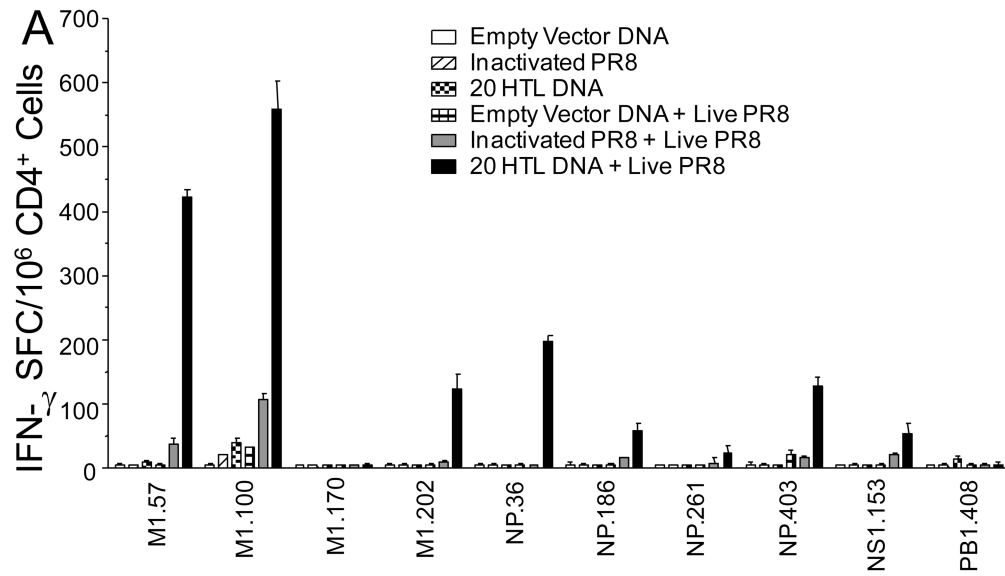


Fig. 3A

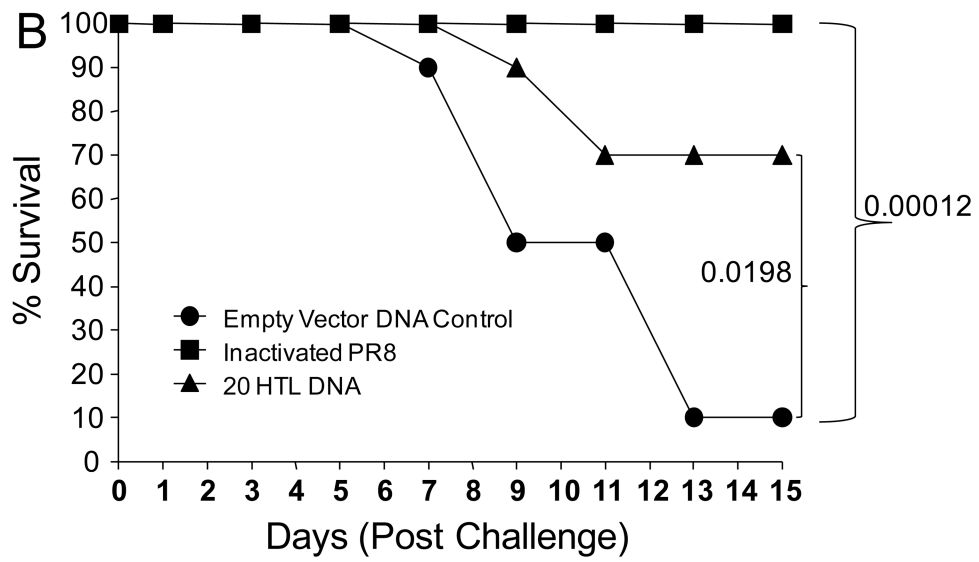


Fig. 3B

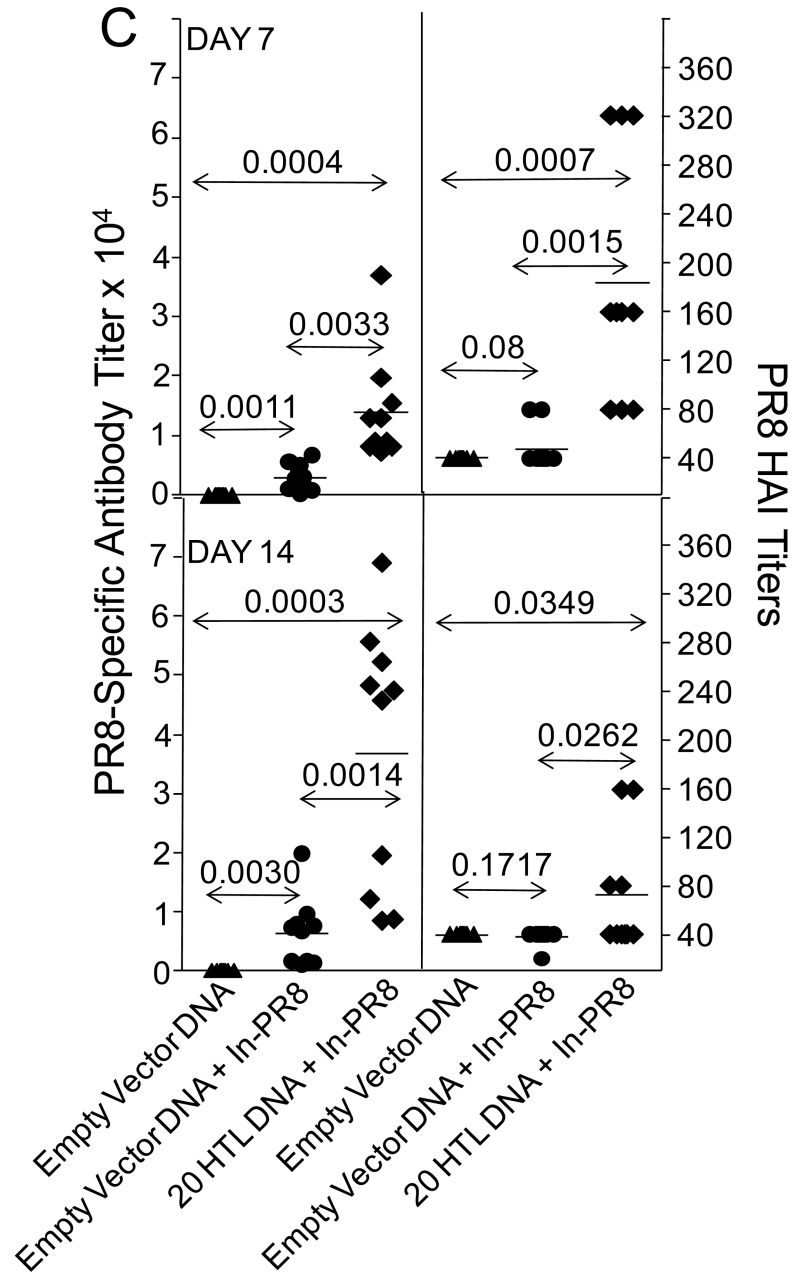


Fig. 3C

Fig. 3. Epitope-specific immune responses, animal survival and augmentation of PR8-specific antibody responses. HLA-DR4 transgenic H-2^b mice, (n=14), were immunized with CD4⁺20 epitope DNA construct or empty vector DNA control using Ichor's *in vivo* electroporation device with 50 µg per mouse. Additionally, a group of mice was also immunized with inactivated PR8. Two weeks post-immunization, two mice per group were sacrificed and splenocytes prepared for an epitope-specific IFN-γ ELISPOT (A). All 20 CD4⁺ epitope-specific responses were evaluated. Only positive responses plus three negative responses as comparators are shown. At this same time, the remaining 12 mice were infected with 100 HAU PR8 (4MLD50). Six days following the PR8 infection, two

additional mice were sacrificed from each group for a second epitope-specific ELISPOT evaluation (A). The remaining 10 mice were monitored for survival of the animals (B). HLA-DR4 transgenic mice, (n=10) were immunized with CD4⁺20 epitope DNA vaccine or empty vector DNA control. Two weeks later, mice were immunized with 15 µg inactivated PR8. Seven and 14 days following inactivated PR8 immunization, mice were bled for determination of HA-specific antibody responses (C).

Table 1

Fifty-two diverse influenza strains used for epitope conservancy analysis

H1N1	H2N2	H3N2
A/Brevig Mission/1/1918	A/Leningrad/134/57	A/Hong Kong/1/68
A/PR/8/34	A/Singapore/1/57	A/Albany/1/76
A/Fort Monmouth/1/47	A/Ann Arbor/6/60	A/Moscow/10/99
A/USSR/90/1977	A/Berlin/3/64	A/Hong Kong/1774/99
A/New Caledonia/20/1999	A/Tokyo/3/67	A/Wisconsin/67/2005
A/California/UR06-0125/2007		A/Brisbane/10/2007
A/California/07/09		
H5N1	H7N1 & H7N2 & H7N3 & H7N7	H9N2
A/goose/Guangdong/1/1996	A/chicken/Italy/2335/2000	A/chicken/Beijing/1/94
A/Hong Kong/483/97	A/turkey/Italy/3675/99	A/Hong Kong/1073/1999
A/Hong Kong/156/97	A/turkey/Virginia/55/02	A/chicken/Hong Kong/G9/1997
A/Hong Kong/213/03	A/chicken/New York/21211-2/05	A/quail/Hong Kong/G1/1997
A/Vietnam/1203/04	A/New York/107/03	A/Korea/KBNP-0028/00
A/Indonesia/5/2005	A/chicken/Brit. Col./GSC human B/04	A/ck/HK/CSW161/2003
A/turkey/Turkey/1/2005	A/Canada/rv504/04	A/chicken/Shantou/6781/2005
A/Egypt/902782/2006	A/equine/San Paulo/4/76	A/chicken/Jiangsu/L1/2004
A/China/GD01/2006	A/seal/Mass/1/1980	
A/Bar-headed Goose/Qinghai/12/05	A/chicken/Victoria/1/1985	
	A/Netherlands/219/203	
H4N6	H6N1 & H6N2	
A/swine/Ontario/01911-1/99	A/teal/Hong Kong/W312/97	
	A/chicken/Taiwan/0204/05	
	A/chicken/California/139/01	
	A/chicken/Shantou/20741/05	

Table 2

CD4⁺ epitope summary

Sequence	Source	Cons. (% ^a / ^b)	Human Recall															No. of HLA-DR alleles IC ₅₀ 1000nM
			Positive Donors (19 Total)		IC ₅₀ nM to purified HLA ^(c)													
			DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101			
KGILGFVFTLTPSE ^(dn)	M1.57	94	15	2610	5	1	6	11	33	33	37	10	127	356	2206	11		
YRKLKREITHGAKE ^(e)	M1.100	60	20	4633	13	20	951	976	47	432	28	-- ^(c)	327	58	459	11		
MGTVTTEVALGLYCA	M1.135	21	58	348	321	355	--	924	--	4234	--	769	142	1589	7609	7		
NPLRPHENRMVLA ^(f)	M1.170	98	17	40	779	31	1588	298	108	95	32	6	3	690	3691	11		
AMEVNSQARQMVQAM ^(g)	M1.202	73	641	833	4252	1561	263	424	1142	1437	1406	185	310	759	786	8		
DPLVA ^(h) AASIHGILHL	M2.24	46	78	--	92	23	630	1304	3591	709	1804	1859	2196	252	--	6		
IGRFQMCTELKLS ⁽ⁱ⁾ DYEG	NP.36	65	31	291	53	116	29	443	486	193	236	2704	23	210	28	12		
QNSITHE ^(j) RMVLSAFD	NP.58	69	890	298	430	188	4137	806	534	5615	4231	1421	891	39	--	8		
VGTV ^(k) MELIRMIKRG ^(b)	NP.186	71	22	16	1092	13	1997	495	47	8886	32	256	738	10	25	10		
DLIF ^(l) ARSALILRGS ^(j)	NP.255	92	7	857	315	9	348	13	15	365	321	6	8	2243	35	12		
RSAL ^(m) LRGSAHKSC ^(j)	NP.261	100	13	1482	269	17	599	14	16	63	265	268	119	480	61	12		
KSQ ⁽ⁿ⁾ LS ^(m) WMA ^(h) CHSAAFE ^(k)	NP.325	71	35	1118	194	34	127	172	324	201	472	--	259	149	462	11		
AGQ ^(o) EV ^(p) PTFSVQRN ^(l)	NP.403	62	12	1632	41	11	335	290	--	589	3744	55	9	34	163	10		
EGAI ^(q) CGEISPLPSP ^(r) PGHTD	NS1.153	27	64	8334	14	6	98	--	227	6365	88	1773	7	131	1142	8		
SLKLYRDSLGEAVMR	NS2.37	44	11	46	903	996	5993	790	--	707	--	800	19	--	--	8		
IRW ^(s) LIEV ^(r) RHLRIT	NS2.76	8	137	73	1045	3841	3511	877	1672	6597	703	932	189	2944	39	7		
FEQITFMQALQLLLE	NS2.94	60	5	4551	345	160	335	38	2696	47	765	1083	10	387	314	10		
ITEMQALQLLLEVEQ	NS2.97	60	13	2511	161	124	224	26	2426	20	1375	242	104	149	46	10		
RREVHIYYLEKANKI	PA.124	77	4	--	63	181	1490	23	95	851	96	150	4	5	25	11		
LFTIRQEMASRGLWD	PA.175	69	25	523	53	4	768	3317	88	2428	890	304	237	15	43	11		
EPFLKTT ^(t) PRPLRPD	PA.258	35	17	373	961	2021	--	51	117	109	131	412	570	7660	62	10		
RSKFL ^(u) MDALKLSIED	PA.279	90	2	1	125	53	14	35	25	96	15	49	1	639	16	13		

Sequence	Source	Cons. (% ^b)	Human Recall																No. of HLA-DR alleles IC ₅₀ 1000nM
			IC ₅₀ nM to purified HLA ^{a)}																
			Positive Donors (19 Total)	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0404	DRB1 *0405	DRB1 *0701	DRB1 *0802	DRB1 *0901	DRB1 *1101	DRB1 *1302	DRB1 *1501	DRB4 *0101	DRB5 *0101			
VAPIEHASMRNYF	PA.432	75	4	375	114	503	41	7658	543	643	7437	70	221	147	529	4	11		
EYIMKGVYINTALLN	PA.457	98	4	22	3758	1064	67	425	1041	133	1200	2732	7	70	1337	585	7		
RPMFLYVRTNGTSKI	PA.559	90	3	5	--	7	7	400	15	12	82	8	1	83	694	6	12		
PTLLFLKVPQAQNAIST	PB1.5	69	2	8	--	17	9	152	34	15	69	49	1380	285	64	50	11		
SYLRALTLNTMTKD	PB1.216	87	2	7	4900	42	14	429	76	56	121	280	87	7	3	386	12		
FLAMTYITRNQPEW	PB1.318	77	3	8	--	126	64	200	590	65	769	59	174	74	1140	60	11		
QPEWTRNNVLSIAPIME	PB1.329	75	2	37	8991	100	236	374	12	228	197	288	98	723	104	656	12		
FRNVA-SIAPIMFSNKM	PB1.333	75	1	10	1450	25	8	409	10	71	88	68	77	62	43	33	12		
IAPIMF-SNKMARLGK	PB1.339	83	1	58	51	404	180	--	315	27	1836	140	77	25	3733	100	10		
KGYVDFESKSMKLRITQI	PB1.352	75	2	7	547	173	63	806	6	60	80	33	9	2	34	8	13		
MMGFENMLSTVLGVS ^m	PB1.408	100	10	3	9191	6	1	72	85	15	20	13	40	48	37	42	12		
DFALVYNAPNHEGIQ	PB1.446	85	3	47	--	37	10	52	8663	295	724	153	9	26	46	208	11		
YGFVAFNFSMELPSFG	PB1.499	90	3	5	5376	40	61	617	8	1177	13	700	1	17	2632	1320	9		
GVTWTKNNMINNDLGP	PB1.526	92	2	37	--	956	180	457	153	239	1880	--	8	8	3762	--	8		
PNLYSIRNLHIPEVC	PB1.596	81	2	68	--	1159	9	259	10	71	914	1535	148	9	409	2309	9		
ISSMVLAMVSRARID	PB1.711	79	1	112	4284	93	10	--	2942	310	1076	294	1108	902	108	13	8		
KWVMAMKYPITADKR	PB2.48	81	1	415	5884	--	182	8335	114	13	446	19	6773	198	1257	101	8		
GARVFSESQLTITK	PB2.173	81	2	46	967	103	65	5479	376	--	300	4800	19	19	1181	7735	8		
KAAAGLRISSFSFG	PB2.312	77	3	37	501	47	4	452	4	466	58	398	3	1	79	45	13		
IKAVRGDLNFVNRAN	PB2.411	90	4	729	123	75	17	510	--	419	--	1914	264	517	515	--	9		
LRHFQKDAKVLQNW	PB2.435	88	1	153	51	1431	1348	9917	578	536	5727	318	240	207	979	1412	8		
QWTRNWETVKIQWS	PB2.551	75	3	47	4422	49	16	124	24	1573	243	729	9	2	49	156	11		
RMQFSSLTVNVRGSG	PB2.630	96	2	9	9323	33	71	988	310	833	306	437	149	102	3725	8	11		

^{a)}IC₅₀ nM to purified HLA (In general, IC₅₀ nM < 1,000 is immunogenic)

^{b)} amino acid conservancy among H1N1, H2N2, H3N2, H5N1, H7N7, H9N2, etc. (52 strains total)

^{c)}--indicates IC₅₀ nM > 10,000

^{d)}Refs (d-f, Yang 2006 (ref. 36); g-l, Roti 2008 (ref. 34); m, Assarsson 2008 (ref. 37))

^{n/20} epitopes selected for inclusion in 'prototype' DNA construct are indicated by shading

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