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## Live Attenuated Influenza Vaccines by Computer-Aided Rational Design

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### Abstract

Influenza claims 250,000 - 500,000 lives annually worldwide. Despite existing vaccines and enormous efforts in biomedical research, these staggering numbers have not changed significantly over the last two decades<sup>1</sup>, motivating the search for new, more effective, vaccines that can be rapidly designed and easily produced. Using influenza virus strain A/PR/8/34, we describe a systematic, rational approach, termed Synthetic Attenuated Virus Engineering (SAVE), to develop new, efficacious live attenuated influenza virus vaccine candidates through genome-scale changes in codon pair bias. Attenuation is based on many hundreds of nucleotide changes across the viral genome, offering high genetic stability and a wide margin of safety. The method can be applied rapidly to any emerging influenza virus in its entirety, an advantage that is significant for dealing with seasonal epidemics and pandemic threats, such as H5N1- or 2009-H1N1 influenza.

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Influenza viruses are negative stranded, enveloped orthomyxoviruses with eight gene segments, each encoding one or two proteins<sup>2</sup>. The signature antigenicity of the A and B types of influenza viruses is determined by the glycoproteins hemagglutinin (HA) and neuraminidase (NA). Antigenicity undergoes yearly genetic drift by point mutations, which is the basis for seasonal epidemics<sup>1-3</sup>. Swapping of gene segments by reassortment between viruses of aquatic birds, swine and humans produces new type A influenza viruses (genetic shift) with novel antigenicity that may cause devastating pandemics<sup>1-3</sup>.

The capacity of influenza viruses for immune escape demands annual updating of vaccine strains to reflect changes in the HA and NA genes within the impending seasonal strains. Two types of vaccines are currently used: a chemically inactivated virus delivered by injection, and a live attenuated influenza vaccine (LAIV) of cold-adapted virus<sup>4</sup>, delivered as a nasal-spray (“FluMist”) (CDC; <http://www.cdc.gov/flu/protect/keyfacts.htm>). Either vaccine comes with limitations. While cell-mediated responses are being recognized as a major determinant of influenza immunity<sup>5-8</sup>, the traditional, killed vaccines act mainly by

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inducing neutralizing antibodies. Unfortunately, this vaccine appears less effective than hoped in the elderly population (>65 yr)<sup>9</sup>. LAIV induces both humoral and cellular immunity but its administration is as yet restricted to healthy children, adolescents and adults (non-pregnant females), age 2 – 49. LAIV works relatively better in immunologically naive young children than in adults<sup>10, 11</sup>.

Here we illustrate a new technology we call Synthetic Attenuated *Virus Engineering (SAVE)*<sup>12</sup> as a rational design approach for building live attenuated influenza vaccines, as well as vaccines to other diseases.

The central idea of *SAVE* is to recode and synthesize a viral genome<sup>13</sup> in a way that perfectly preserves the wild-type amino acid sequence, but that re-arranges existing synonymous codons to create a sub-optimal arrangement of pairs of codons<sup>12</sup>. For reasons that are not understood, some pairs of codons occur more frequently while others occur less frequently than expected (codon pair bias)<sup>14</sup>. Every examined species has a statistically significant codon pair bias<sup>15</sup>. This bias evolves slowly; yeast and humans have a radically different codon pair bias, but all mammals share essentially the same codon pair bias (unpublished results).

Codon pair bias is independent of codon bias. For example, consider the amino acid pair Arg-Glu. Since there are six codons for Arg and two for Glu, there are 12 encodings for this pair of amino acids. Taking into account the frequency of the two contributing codons (codon bias), the pair CGC-GAA is expected 2397 times in the annotated human ORFeome, but in fact is observed only 268 times (observed/expected = 0.11). This is an infrequently used codon pair. In contrast, the Arg-Glu encoding AGA-GAA is expected 2644 times, but is observed 4195 times (observed/expected = 1.59); this is a frequently used codon pair. By whole genome synthesis<sup>13, 16</sup> we previously recoded poliovirus so as to contain “poor” (i.e., infrequently used) codon pairs, and found that this dramatically attenuated the virus<sup>12</sup>. Although the mechanism of attenuation is unclear, preliminary evidence suggests that translation is affected<sup>12</sup>. Attenuation can be “titrated” by adjusting the extent of codon pair deoptimization<sup>12</sup>. Because codon pair deoptimization is due to miniscule effects at each of hundreds or thousands of nucleotide mutations (without changing amino acid sequences), reversion to virulence is extremely unlikely<sup>12</sup>. Aided by computer algorithms<sup>12</sup>, codon pair-deoptimized viral genomes can be rapidly designed and synthesized, and live virus can be generated by reverse genetics.

To attenuate influenza virus, we redesigned large parts of the coding regions of the PB1, NP, and HA genes of influenza virus A/PR/8/34 (“PR8”), using our deoptimization computer program<sup>12</sup>. These genes play important roles in genome replication and assembly (although other genes may also have served). Without altering either amino acid sequence or codon bias, the program re-arranged existing synonymous codons to deoptimize codon pairs. This resulted in hundreds of silent mutations per genome segment without any amino acid changes. The characteristics of the synthetic genome segments and their changes in Codon Pair Bias (CPB) are summarized in Table 1 and in Figure S1.

The deoptimized segments were synthesized *de novo* and cloned into a standard ambisense, 8-plasmid system<sup>17,18</sup>. To generate influenza viruses carrying one or more deoptimized segments, the plasmids carrying the recoded, synthetic segments, together with the complement of the remaining PR8 wt plasmids, were transfected into susceptible cells (Materials and Methods). Each deoptimized segment PB1<sup>Min</sup>, NP<sup>Min</sup>, and HA<sup>Min</sup> in the background of the complementing 7 wt segments yielded a viable virus, as did any combination thereof, including the virus simultaneously containing all three deoptimized segments (PR8-PB1/NP/HA<sup>Min</sup>, abbreviated “PR8<sup>3F</sup>”) (Fig. 1; and data not shown).

Several of these new synthetic viruses were analyzed for their *in vitro* growth characteristics in MDCK cells. All mutant viruses formed plaques that were either indistinguishable from, or only slightly smaller than that of the wt virus (Fig. 1A). The mutant viruses grew less well than wt, but typically only to about ten-fold lower titers (Fig. 1B). The properties of viruses carrying combinations of synthetic segments other than depicted in Fig. 1 fall in between the curves or plaque phenotypes of PR8 and PR8<sup>3F</sup> (data not shown). The deoptimized virus PR8<sup>3F</sup> had about the same response to changes in temperature (to either 33°C or 39.5°C) as did the wild type virus (Fig. S3).

Previously, we found that codon or codon pair-deoptimized polioviruses had a reduced specific infectivity<sup>12,19</sup>. This was not the case for deoptimized influenza viruses, as their ratio of PFU to HA units was nearly identical to wt (data not shown).

Our working hypothesis postulates that deoptimization of viral ORFs results in reduced protein synthesis, which in turn brings forth a virus that is attenuated<sup>12</sup>. Therefore we tested protein synthesis driven from the deoptimized gene segments in PR8-NP<sup>Min</sup>, PR8-HA<sup>Min</sup>, and PR8-PB1<sup>Min</sup> by Western blotting of infected whole cell lysates (Fig. 1C). In all three viruses the deoptimized viral gene product was specifically reduced compared to other proteins from the same virus, or to the same protein in wild type-infected cells, or to the actin control in the same cells (Fig. 1C). Protein synthesis from the wild type segments in a deoptimized virus was apparently not affected. The exact molecular mechanisms responsible for the reduced protein production remain to be determined.

Despite their reasonably robust growth, codon pair-deoptimized influenza viruses proved to be remarkably attenuated in mice (Table 2). Each individual deoptimized segment had a demonstrable attenuating effect, leading to a reduction in LD<sub>50</sub> of about 10, 30, and 500 fold, for PR8-NP<sup>Min</sup>, PR8-HA<sup>Min</sup>, and PR8-PB1<sup>Min</sup>, respectively. Combinations of two deoptimized genes have not been tested, but combining all three attenuating genes into one virus (PR8<sup>3F</sup>) led to a cumulative attenuation of about 13,000 fold (Table 2).

To test the codon pair-deoptimized viruses in animals, BALB/c mice were infected intranasally with 10<sup>4</sup> PFU of PR8<sup>3F</sup> or PR8, and monitored for disease symptoms (ruffled fur, lethargy, weight loss, death). At this dose, mice infected with wild-type PR8 developed severe symptoms with rapid weight loss and did not survive beyond day 5 (Fig. 2A). Mice infected with PR8<sup>3F</sup>, on the other hand, experienced no observable symptoms or weight loss, save for a small, transient delay in weight gain as compared to mock-infected animals (Fig. 2A).

Live attenuated virus vaccines depend on a limited, yet safe, degree of replication within the host to stimulate the immune system. To assess the replicative potential of these viruses in an immune competent host, we monitored viral load in lungs of BALB/c mice infected intranasally with either  $10^3$  PFU of PR8<sup>3F</sup> or PR8 wt virus. Within 24 hours, wt-infected mice were marked by 3000 fold higher viral load in their lungs compared to PR8<sup>3F</sup>, leading to death in under 6 days (Fig. 2B). Conversely, in PR8<sup>3F</sup>-infected animals, amplification of the vaccine virus progressed slowly and peaked at a lower viral load than the wild type virus, resulting in a controlled infection with no overt disease symptoms, and virus clearance after nine days (Fig. 2B).

Infection by a sub-lethal dose of wild type virus can in principle provoke protective immunity, which is the usual course of natural human infections. The Chinese scholar Li Shizhen described the art of inoculating humans with live smallpox in his *Compendium of Materia Medica* (1593)<sup>18</sup>. This method of smallpox vaccination, practiced in China for centuries<sup>20</sup>, was very dangerous because the difference between the lethal dose (LD) and the immunizing protective dose (PD) of wt smallpox is very small. To address the issue of safety margin quantitatively with our influenza viruses, we determined the LD<sub>50</sub> and the protective dose 50 (PD<sub>50</sub>, the dose providing protective immunity to half the animals) for wild type PR8 and also for the attenuated strain, PR8<sup>3F</sup> (Fig. 3). PR8 had a very low PD<sub>50</sub> of 1 PFU (due to robust replication). (1 PFU of PR8, titred on MDCK cells, is about 40 virus particles<sup>21</sup>). The LD<sub>50</sub> of PR8 was 61 PFU, resulting in an LD<sub>50</sub>/PD<sub>50</sub> ratio of about 60. This ratio between the LD<sub>50</sub> dose and the PD<sub>50</sub> dose is the “safety margin” of a given virus if it were to be used as a vaccine. The safety margin of the wt (LD<sub>50</sub>/PD<sub>50</sub> = 60) is narrow--hence it is inadequate as a vaccine. In contrast, the attenuated virus PR8<sup>3F</sup> had a PD<sub>50</sub> of 13 PFU, higher than the PD<sub>50</sub> of the wild-type, but still very low. Strikingly, the attenuated PR8<sup>3F</sup> had an LD<sub>50</sub> of 790,000 PFU and, thus, an LD<sub>50</sub>/PD<sub>50</sub> ratio (safety margin) of 60,000, which is 1000-fold better than the wild-type virus (Fig. 3A versus Fig. 3B, shaded areas under the curve). Thus, it is easy to determine a dose of the attenuated virus PR8<sup>3F</sup> that is both safe to administer and effective in provoking immunity (see also Fig. S2).

To characterize the protective immunity in more detail, we immunized mice with a single intranasal vaccination of  $10^4$  PFU of PR8<sup>3F</sup>. After 28 days, these mice were challenged with  $1000 \times$  LD<sub>50</sub> of wt PR8. Three days after challenge, PR8 titres in lung homogenates were determined. In 80% of the mice, challenge virus was below the level of detection (suppressed at least  $10^6$ -fold) (Fig. 3C; open circles), whereas titers of  $\sim 10^7$  PFU were found in lungs of mock-vaccinated animals (Fig. 3C; open squares).

The mean anti-influenza serum IgG-antibody titer in mice immunized with  $0.01 \times$  LD<sub>50</sub> of the respective viruses was 312,500 for PR8<sup>3F</sup> and 27,540 for PR8 (Fig. 4C). At an even lower and, thus, safer vaccine dose of  $0.001 \times$  LD<sub>50</sub> the immune response toward PR8<sup>3F</sup> was nearly unchanged with an antibody titer of 237,500 (Fig. 4C). Thus, at identical doses relative to their respective LD<sub>50</sub>, PR8<sup>3F</sup> is a more potent inducer of influenza-specific antibodies than the wild type. These findings attest to the strong immunizing potential of a low-grade influenza virus infection in general, and to the safety profile of codon pair-optimized influenza viruses in particular.

Vaccines created in this way encode all viral proteins with the exact wild-type amino acid sequences. Thus the viruses express the entire wild-type repertoire of antigenic sites and would have the maximum chance of inducing both cellular and humoral immunity against all epitopes. Since attenuation results from hundreds or thousands of nucleotide changes the probability of reversion to virulence is extremely low, an advantage over other approaches to making live vaccines. Co-infection of the vaccine recipient with a naturally circulating wt virus could lead to gene reassortment, but this is unlikely to produce variants more virulent than the co-infecting virus. The deoptimized segments of the vaccine strain would “poison” any such reassortant.

In spite of striking genetic dissimilarities, codon pair deoptimization has given similar results with both influenza virus and poliovirus<sup>12</sup>. However, poliovirus is a plus-stranded RNA virus whose genome encodes a single polyprotein. Codon pair-deoptimization in the 5'-region of the poliovirus genome disturbs the synthesis of all viral proteins with drastic effects on replication<sup>12</sup>. In contrast, influenza virus, a minus-stranded RNA virus, has a genome divided into eight segments and its proteins are expressed independently from individual mRNAs. The fact that two such extremely different viruses each are highly sensitive to codon pair deoptimization suggests that the strategy may operate quite generally.

As with any new technology, there are issues to resolve before applying this approach for human use. These include our incomplete knowledge of the molecular mechanism(s) involved in attenuation, an in-depth assessment of the genetic stability of the attenuation phenotype, and a need to find the right balance between attenuation (for provoking an immune response) and robust replication (for purposes of production). For seasonal epidemics, SAVE may allow a different strategy than the existing live vaccines, such as FluMist™. For FluMist™, the attenuating mutations (obtained by lengthy selection procedures) map to six viral “backbone” genes, which therefore must be kept constant every year, with only HA and NA being updated annually (“6:2 recombinants”). Individuals immunized yearly could develop immunity against “backbone” proteins, limiting the replication and thus the efficacy of the vaccine (possibly helping to explain the relatively poor efficacy of FluMist in previously-immunized army personnel<sup>22</sup>). In contrast, using SAVE one could attenuate the entire impending seasonal or pandemic strain's genome, providing a perfect antigenic match between the vaccine and target virus. However, this approach would require an appropriate regulatory environment that might, for instance, approve a method of attenuating each new strain to a certain standardized degree with a standardized change in codon pair bias (just as the current inactivated virus vaccine uses a standardized method of inactivation). To attenuate in a standardized and predictable way will require many more experiments to exhaustively explore the variables.

The recoded influenza viruses described here present a novel paradigm for vaccines, since attenuation is the result of hundreds or thousands of nucleotide changes without change of a single amino acid. The attenuated phenotype results from large-scale rearrangements of existing synonymous codons, producing under-represented pairs of codons<sup>12</sup>. Combined with (i) the expected high genetic stability of the attenuating genetic changes (“attenuation by thousand cuts”<sup>23</sup>), (ii) the possibility of systematically designing such viruses, the favorable growth kinetics in tissue culture ( $10^8$  PFU/ml), (iii) the small protective dose of

“deoptimized” influenza viruses, (iv) the efficacy of PR8<sup>3F</sup> as revealed in challenge experiments, and, particularly, (v) the wide safety margin, the *SAVE* technology sets the stage for making efficient live attenuated influenza vaccines. While it is difficult to extrapolate to human use, in our system 10 milliliter of culture supernatant would appear to contain enough virus to vaccinate and protect approximately 1 million mice with a single vaccination of 100 PD<sub>50</sub> doses of PR8<sup>3F</sup> (Fig. 3A, Fig. S2).

Vaccines based on changes in codon pair bias could be generated within weeks for any emerging influenza virus once its genome sequence is known, although of course a further period of testing would be required before the vaccine could be used. The margin of safety appears to be unusually high. This new strategy will be applicable to the rapid development of human vaccines against seasonal flu epidemics and pandemics.

## Materials and Methods

### Cell lines

293T and Madin Darby Canine Kidney cells (MDCK) cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin (Invitrogen).

### Reverse Genetics and Generation of Synthetic Influenza Viruses

All synthetic influenza viruses used here are based on the strain A/PR/8/34 (Mt. Sinai variant, short PR8). The reference sequences of the 8 gene segments for this strain are available under genbank accession numbers AF389115, AF389116, AF389117, AF389118, AF389119, AF389120, AF389121, AF389122). An 8-plasmid ambisense system for this strain cloned in the vector pDZ<sup>24</sup> was kindly made available by Peter Palese and Adolfo García-Sastre (Mt. Sinai School of Medicine).

Codon pair-deoptimized genome segments were designed using a computer algorithm previously described<sup>12</sup>. Coding regions of the segments PB1, HA, and NP were targeted to be recoded. A minimum of 120 nucleotides at either segment terminus were left unaltered since these sequences contain packaging signals<sup>2</sup>. All of the codon changes were synonymous; i.e., none of them introduced any amino acid changes. The precise extent of recoded sequence for each target segment and the resulting changes in codon pair bias are summarized in Table 1.

The recoded gene segments were synthesized *de novo* (Mr. Gene GmbH, Regensburg, Germany) and introduced into wt plasmids to replace the respective wt counterpart sequence. Codon pair-deoptimized viruses were derived by DNA transfection of either one, or combinations of two, or three deoptimized segments together with the remaining wt plasmids. For this purpose a total of 2µg plasmid DNA (250ng of each of 8 plasmids) was transfected into co-cultures of 293T and MDCK cells in 35mm dishes using Lipofectamine 2000 (Invitrogen) according to manufacturers recommendations. After 6 hours of incubation at 37 °C, the serum free Opti-MEM containing the transfection mix was replaced with DMEM containing 0.2% Bovine Serum Albumin (BSA). After a further 24 hours of

incubation, 1 µg/ml TPCK-Trypsin was added to the dishes. Two days thereafter virus containing cell supernatants were collected and amplified on MDCK cells.

### **In vitro Growth characteristics and titration of synthetic Influenza viruses**

The growth characteristics of codon pair-deoptimized synthetic viruses were analyzed by infecting confluent monolayers of MDCK cells in 100 mm dishes with 0.001 multiplicities of infection (MOI). Infected cells were incubated at 37°C in DMEM, containing 0.2% Bovine Serum Albumin (BSA) and 2µg/ml TPCK-Trypsin (Pierce, Rockford, IL). At the given time points 200 µl of supernatant was removed and stored at -80°C until titration. Viral titers and plaque phenotypes were determined by plaque assay on confluent monolayers of MDCK cells in 35mm six well plates using a semisolid overlay of 0.6% tragacanth gum (Sigma-Aldrich) in minimal Eagle medium (MEM) containing 0.2% Bovine Serum Albumin (BSA) and 4µg/ml TPCK-Trypsin. After 72 hours of incubation at 37°C plaques were visualized by staining the wells with crystal violet.

### **Mouse pathogenicity, in vivo virus replication, and vaccination**

A minimum of 5 BALB/c mice (5-6 weeks old) per group were infected once by intranasal inoculation with doses ranging from  $10^0$  to  $10^6$  PFU of PR8<sup>3F</sup> or of wt PR8. Inoculum virus was diluted in 25µl PBS and administered evenly into both nostrils. A control group of 5 mice was inoculated with PBS only (mock). Venous blood from the tail vein was collected from all animals prior to initial infection for subsequent determination of pre-vaccination antibody titers.

Morbidity and mortality (weight loss, reduced activity, death) was monitored. The Lethal Dose 50, LD<sub>50</sub>, of the wt virus and the vaccine candidates was calculated by the method of Reed and Muench<sup>25</sup>. Mice experiencing severe disease symptoms (rapid, excessive weight loss over 25%) were euthanized and scored as a lethal outcome.

For vaccination experiments mice were infected as above. 28 days following the initial infection (vaccination), venous blood from the tail vein was drawn for subsequent determination of post-vaccination antibody titers.

The mice were then challenged with  $10^5$  PFU of the wt virus PR8 corresponding to more than 1000 times the LD<sub>50</sub>. Mortality and morbidity (weight loss, reduced activity, death) was monitored. The Protective Dose 50 (PD<sub>50</sub>) of codon pair-deoptimized PR8-3F versus that of the PR8 was determined as the dose required to protect 50% of mice from a challenge with  $1000 \times$  LD<sub>50</sub> of the wt virus, 28 days after a single inoculation with the vaccine virus.

To assess virus replication in the lungs of infected animals, BALB/c mice were infected intranasally with  $10^3$  PFU of either PR8 or PR8<sup>3F</sup>. At 1, 3, 5, 7, and 9 days post infection the lungs of three mice each were collected (wt infected mice did not survive beyond day 6). Lungs were homogenized in 1 ml of PBS and the virus load per organ was determined by plaque assay on MDCK cells, as described above. Similarly, replication of PR8 challenge virus in lungs of PR8<sup>3F</sup>-vaccinated mice were determined. 28 days following a single intranasal inoculation with  $10^4$  PFU of PR8<sup>3F</sup> or saline, 5 animals each were challenged

with  $1000 \times LD_{50}$  of PR8. 3 days after challenge (the usual peak of viral replication), lungs were processed and viral load per organ determined as described above.

### Western Blot analysis of Influenza virus proteins in infected cells

MDCK cells were infected with virus at a multiplicity of infection of 5 and incubated for 4 hours at 37°C. Subsequently, the cells were harvested in Laemmli buffer. The proteins were resolved by SDS-PAGE, analyzed by Western blotting with  $\alpha$ -HA,  $\alpha$ -PB1 and  $\alpha$ -actin antibodies (Santa Cruz Biotechnology), and  $\alpha$ -NP antibody (generous gift from Dr. Peter Palese), and HRP-conjugated secondary antibodies, and detected by autoradiography.

### ELISA Determination of Influenza-specific serum IgG-antibodies after vaccination

Nunc Maxisorp ELISA 96 well plates were coated over night with 100 ng purified Influenza PR8 virus in 100 $\mu$ l PBS followed by blocking with 100  $\mu$ l 1% BSA in PBS. Serial 5-fold dilutions in PBS/1% BSA of mouse sera obtained prior to and 28 days after a single intranasal vaccination were incubated for 2 hours at room temperature. Mice were previously vaccinated with approximately 0.01 or 0.001  $\times LD_{50}$  of PR8<sup>3F</sup> ( $10^3$  PFU or  $10^4$  PFU, respectively), 0.01  $\times LD_{50}$  of PR8 wt ( $10^0$  PFU) or mock vaccinated. After 4 washes with PBS the wells were incubated with 1:500 of anti mouse-alkaline phosphatase conjugated secondary anti-mouse IgG antibody (Santa Cruz) for another 2 hours at room temperature. Following 4 washes with PBS and brief rinsing with distilled water 100  $\mu$ l of a chromogenic substrate solution containing 9mg/ml p-nitrophenylphosphate in 200 mM diethanolamine, 1 mM MgCl<sub>2</sub>, pH 9.8 was added. The color reaction was stopped by addition of an equal volume of 500 mM NaOH. Absorbance at 405 nm was read using a Molecular Devices ELISA reader. The endpoint antibody titer was defined as the highest dilution of serum that gave a signal greater than 5 standard deviations above background. Background level was determined from wells processed identically to experimental samples, in the absence of any mouse serum.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

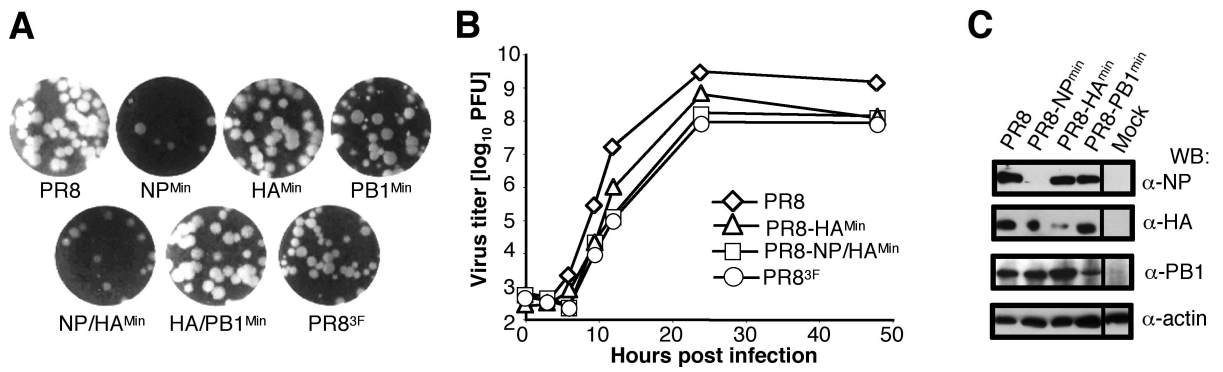
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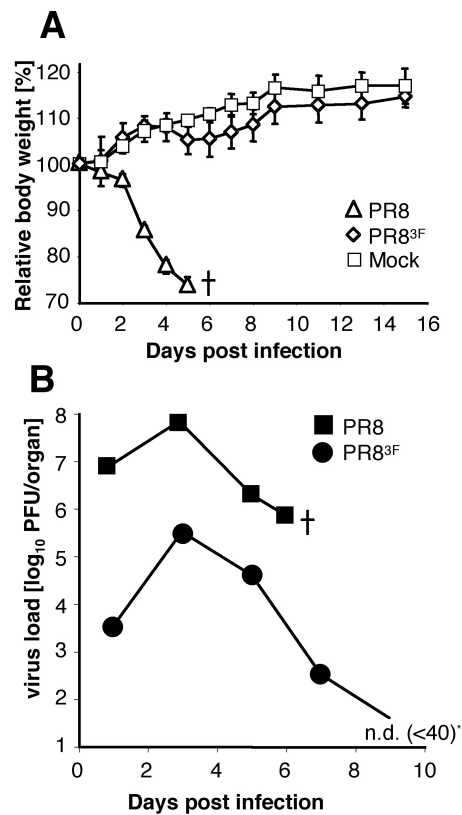
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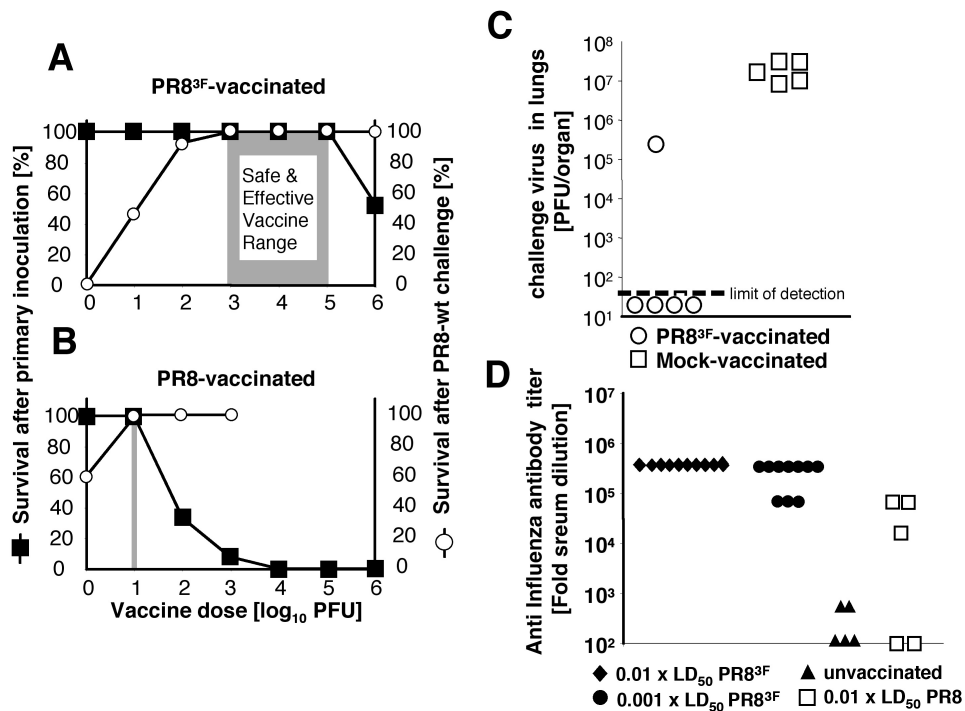
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**Fig. 1. Characterization in tissue culture of synthetic codon pair-deoptimized Influenza viruses** (A) Plaque phenotypes on MDCK cells of PR8 wild type virus and synthetic PR8 derivatives, carrying one (NP<sup>Min</sup>, HA<sup>Min</sup>, PB1<sup>Min</sup>), two (NP/HA<sup>Min</sup>, HA/PB1<sup>Min</sup>) or three (PR8<sup>3F</sup>) deoptimized gene segments. (B) Growth kinetics of PR8 wild type virus and three synthetic PR8 derivatives in MDCK cells after infection with 0.001 MOI of the indicated viruses. All combinations grew within log 8-9 although only PR8-HA<sup>Min</sup>, PR8-NP/HA<sup>Min</sup> and PR8<sup>3F</sup> are shown as compared to wild type. (C) Analysis of Influenza virus protein expression in infected cells. MDCK cells were infected with PR8 wild type virus or synthetic PR8 derivatives, carrying one deoptimized gene segment each (NP<sup>Min</sup>, HA<sup>Min</sup>, PB1<sup>Min</sup>), as indicated. Western blot (WB) analysis of proteins extracted from whole cell lysates was carried out with PB1, NP, HA, or actin antibodies. Actin was used to indicate equal protein loading.



**Fig. 2. Attenuation of codon pair-deoptimized Influenza virus PR8<sup>3F</sup> in BALB/c mice**  
**(A)** Body weight curve following intranasal infection with 10<sup>4</sup> PFU of PR8 wild type (triangles), 10<sup>4</sup> PFU of deoptimized PR8<sup>3F</sup> (diamonds), or mock-infected (saline; squares). The average of 5 mice per time point and standard deviations are indicated. Wild type-infected mice did not survive beyond day 5 (indicated by a cross). **(B)** Virus titer in whole lung homogenate after infection with either 10<sup>3</sup> PFU PR8 wild type (squares), or deoptimized PR8<sup>3F</sup> (circles). Average of three mice per time point. \* On day 9 post infection, PR8<sup>3F</sup> was no longer detectable (below 40 PFU/lung).



**Fig. 3. Immune responses and protection**

(A, B) Vaccine Margin of Safety for PR8 wild type and deoptimized PR8<sup>3F</sup> viruses. The left ordinate indicates the percentage of animals surviving the primary inoculation (black squares) with (A) PR8<sup>3F</sup> or (B) wt PR8, at doses ranging between 10<sup>0</sup> to 10<sup>6</sup> PFU. After 28 days, the surviving, vaccinated animals were challenged with a single 1000 × lethal dose 50 of PR8 wild type virus. Disease and survival were monitored (right ordinate; open circles) for (A) PR8<sup>3F</sup>- and (B) PR8-vaccinated mice. (C) Virus load in mouse lungs following wild type challenge of PR8<sup>3F</sup>-vaccinated animals. 28 days following a single intranasal vaccination with 10<sup>4</sup> PFU PR8<sup>3F</sup> mice were challenged with 1000 × LD<sub>50</sub> of PR8 wt virus. Three days thereafter the level of challenge virus in lung homogenates was determined. (D) ELSIA determination of influenza-specific serum antibodies. 28 days after a primary infection, serum was collected, and anti-influenza IgG serum antibody titers were determined from animals that had received a primary inoculation of 0.01 × LD<sub>50</sub> (black diamonds) or 0.001 × LD<sub>50</sub> of PR8<sup>3F</sup> (black circles), 0.01 × LD<sub>50</sub> of PR8 (white squares), or saline (black triangles). ELISA antibody titer against PR8 virus antigen is expressed as the lowest reciprocal serum dilution that resulted in a positive ELISA signal (5 standard deviation above background).

**Table 1**

Characteristics of deoptimized Influenza Genome Segments

Gene Segment	Deoptimized Coding Region <sup>a</sup>	CPB of wt Segment <sup>b</sup>	CPB of deoptimized Segment <sup>c</sup>	Number of silent Mutations
NP <sup>Min</sup>	125-1426	0.012	-0.421	314
PB1 <sup>Min</sup>	519-1494	0.007	-0.386	236
HA <sup>Min</sup>	157-1654	0.019	-0.420	353

<sup>a</sup> nucleotide position within the genome segment that underwent the codon pair-deoptimization algorithm

<sup>b</sup> original codon pair bias (CPB) of the corresponding wt sequence

<sup>c</sup> codon pair bias (CPB) of the synthetic, codon pair-deoptimized gene segment

**Table 2**

Lethal Dose 50 and Protective Dose 50 of deoptimized Influenza Viruses

Virus	LD <sub>50</sub> (PFU) <sup>a</sup>	PD <sub>50</sub> (PFU) <sup>b</sup>
PR8 (wt)	$6.1 \times 10^1$	$\sim 1.0 \times 10^{0c}$
PR8-NP <sup>Min</sup>	$5.0 \times 10^2$	n.d. <sup>d</sup>
PR8-PB1 <sup>Min</sup>	$3.2 \times 10^4$	n.d.
PR8-HA <sup>Min</sup>	$1.7 \times 10^3$	n.d.
PR8-NP/HA/PB1 <sup>Min</sup> (PR8 <sup>3F</sup> )	$7.9 \times 10^5$	$1.3 \times 10^1$

<sup>a</sup> The dose required to result in lethal disease in 50% of inoculated mice, calculated by the method of Reed and Muench <sup>25</sup>.

<sup>b</sup> The dose of vaccine required to protect 50% of mice with a single vaccination from a challenge infection with 1000 LD<sub>50</sub> of the PR8 wt virus on day 28 post vaccination.

<sup>c</sup> At the lowest of inoculum ( $1.0 \times 10^0$  PFU) 60% of mice were protected

<sup>d</sup> not determined