Genome-wide identification of interferon-sensitive mutations enables influenza vaccine design

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In conventional attenuated viral vaccines, immunogenicity is often suboptimal. Here we present a systematic approach for vaccine development that eliminates interferon (IFN)—modulating functions genome-wide while maintaining virus replication fitness. We applied a quantitative high-throughput genomics system to influenza A virus that simultaneously measured the replication fitness and IFN sensitivity of mutations across the entire genome. By incorporating eight IFN-sensitive mutations, we generated a hyper–interferon-sensitive (HIS) virus as a vaccine candidate. HIS virus is highly attenuated in IFN-competent hosts but able to induce transient IFN responses, elicits robust humoral and cellular immune responses, and provides protection against homologous and heterologous viral challenges. Our approach, which attenuates the virus and promotes immune responses concurrently, is broadly applicable for vaccine development against other pathogens.

repression of hundreds of IFN-stimulated genes (ISGs), many of which have antiviral activities (7). The IFN response is also critical for dendritic cell maturation, development of B and T cells, and memory formation, bridging innate and adaptive immunity (8–12). Most viruses have evolved to efficiently suppress the production and function of IFN to allow replication in vivo. Thus, systematic elimination of IFN-modulating functions from the virus presents a potential approach for vaccine development (fig. S1) (13, 14). Removing the most well-characterized IFN modulator in influenza virus—namely, the NS1 protein—has shown promise in a vaccine candidate (delNS1) in phase 1/2 clinical trials (14, 15). Although studies have suggested that influenza proteins other than NS1 have IFN-modulating functions (16, 17), genome-wide identification and elimination of IFN-modulating functions without affecting viral replication fitness in vitro have remained challenging tasks.

To tackle this challenge, we developed a quantitative high-throughput genomics system, which combines saturation mutagenesis and next-generation sequencing, to comprehensively identify IFN-modulating functions in the entire viral genome (18). This system has enabled us to quantitatively measure the replication capacity of a large number of mutants in parallel under specific conditions (18, 19). We performed comparative profiling of the entire influenza genome with and without IFN selection, which led to the identification of IFN-modulating functions on multiple viral segments. By combining eight IFN-sensitive mutations across the viral genome, we generated a hyper–interferon-sensitive (HIS) virus that is replication-competent in vitro but highly attenuated in IFN-competent hosts in vivo. The HIS virus showed desired properties as a safe and effective live attenuated influenza vaccine with robust humoral and cellular responses, and it provided broad protection against homologous and heterologous viral challenges in mice and ferrets.

Systematic identification of IFN-sensitive mutations

The RF scores of most mutants are correlated in the presence and absence of exogenous IFN treatment; however, we observed a set of mutations that were nearly neutral in the absence of IFN but highly deleterious under IFN selection (Fig. 1B and fig. S6). These putative IFN-sensitive mutations were widespread on multiple viral segments. Among all influenza A viral proteins, NS1 has been extensively studied for its interaction with the IFN pathway (19, 26, 27), which is validated both in our fitness profiling and individually constructed NSI mutant viruses (fig. S7).
To further explore IFN-modulating functions across the genome, we focused on IFN-sensitive mutations outside NS1, especially the solvent-exposed and structurally clustered residues in the polymerase complex (PB2, PB1, PA, and NP), as well as the M1 and M2 proteins (Fig. 1C and fig. S6). Twenty-six mutations were constructed individually, most of which were nearly neutral for viral replication with nearly intact polymerase activity (fig. S8). These included the previously characterized mutations PB2-N9D, which is known to counteract the inhibition of MAVS (mitochondrial antiviral signaling protein)–induced IFN-β production (16), and M1-D30N, which has been shown to induce IFN-β production (17). Several mutations significantly increased IFN sensitivity compared with WT, and the top eight were chosen for further characterization (Fig. 1D). Six of them (PB2-N9D, PB2-Q75H, PB2-T76A, M1-N36Y, M1-R72Q, and M1-S225T) elevated the expression of IFN-β and ISG54 (Fig. 1E and fig. S9) and stimulated nuclear translocation of IRF3 (fig. S10). We also observed that the IFN induction was MAVS-dependent and STING (stimulator of interferon genes)–independent (fig. S11). Moreover, these six mutants were not sensitive to IFN treatment in Vero cells, which are deficient in IFN production. However, the other two mutations (PB1-L155H and PA-E181D) did not induce higher IFN production (Fig. 1E) and were still IFN-sensitive in Vero cells, suggesting that these mutants likely affect processes downstream of IFN production.

Combining mutations increases IFN sensitivity and IFN induction in vitro

To maximize IFN sensitivity and IFN induction, we combined three IFN-inducing mutations on PB2 (N9D, Q75H, and T76A), three on M1 (N36Y, R72Q, and S225T), and two previously reported ones on NS1 (R38A and K41A) to create the HIS virus. The growth of HIS virus in IFN-competent A549 cells showed significant attenuation compared to WT virus (Du et al., Science 359, 290–296 (2018)).
with that of WT virus (1.4-log decrease at 36 hours and 1.8-log decrease at 60 hours) but was fully restored in IFN-deficient Vero cells (Fig. 2, A and B). The IFN sensitivity of HIS virus was significantly higher than that of the NS1-R38A/K41A mutant, indicating an independent effect of mutations on PB2 and M1 (Fig. 2C). Gene expression data from lung epithelial and macrophage cell lines (A549 and THP1) showed that HIS virus induced higher IFN production and responses (Fig. 2D and fig. S12, A to C). Using RNA sequencing, we evaluated the global gene expression changes in A549 cells infected with WT, NS1-R38A/K41A, or HIS virus. At 6 hours post-infection, the expression of 120 genes was significantly up-regulated (fold change > 2 and \( P < 0.001 \)) in HIS-infected cells, of which 24% were IFN response genes (Fig. 2E, fig. S12D, and table S2). Gene Ontology (GO) enrichment analysis revealed that the pathways related to IFN production and response were the dominant ones activated by HIS virus, to a greater extent than by WT or mock infection (Fig. 2F). Furthermore, HIS virus induced negative regulators of apoptosis process, such as TNFAIP3, an important inhibitor of TNF-mediated apoptosis. Slower cell death was observed with HIS infection than with WT infection (fig. S12E).

We further defined the phenotypes of HIS virus with a panel of human lung cells, including immortalized small airway epithelial cells, bronchial epithelial cells, primary alveolar epithelial cells, and primary alveolar macrophages (Fig. 2G). HIS virus induced the strongest up-regulation of IFN-\( \beta \) expression (~50-fold relative to WT) in the primary alveolar macrophages, an important target for influenza infection (Fig. 2G), and greater up-regulation of ISGs than WT virus (Fig. 2F). HIS virus did not enhance the expression of other inflammatory cytokines [CXCL1, CXCL5, or interleukin-1\( \beta \) (IL-1\( \beta \))] in the infected macrophages, highlighting its specific effects on the IFN pathway (Fig. 2H). The phenotype of HIS virus is not limited to the WSN background: Introducing these eight mutations into another H1N1 strain of influenza, A/PR8/34 (PR8-HIS), led to a similar phenotype (fig. S12, F and G). The up-regulation of the IFN pathway requires active viral infection, given that formalin-inactivated HIS virus lost the ability to induce higher IFN-\( \beta \) expression (Fig. 2G).

**HIS virus is highly attenuated in IFN-competent mice and ferrets**

We next measured the replication and pathogenesis of HIS virus in mice and ferrets, the most commonly used animal models for influenza virus. BALB/c mice were intranasally inoculated with WT or HIS virus at different doses. Whereas the median lethal dose of WT virus was \( 5 \times 10^5 \) TCID50 (50% tissue culture infective dose), and \( 1 \times 10^3 \) TCID50 caused obvious body weight loss in all animals, neither weight loss nor indicative clinical symptoms were observed in HIS-infected mice given \( 1 \times 10^7 \) TCID50, the highest dose that we have tested (Fig. 3, A and B). To compare the HIS...
virus approach with the live attenuated vaccine strategy used in FluMist, we incorporated the five cold-adapted (CA) mutations from FluMist into the WSN background and generated a WSN-CA virus (28, 29). WSN-CA virus replicated well at 33°C but was highly attenuated at 39°C and induced IFN-β expression to a similar level as WT virus, which was significantly lower than that induced by HIS virus (fig. S13). By day 2 post-inoculation, replication of HIS virus in mouse lung tissues was examined by RNA sequencing (n = 2). (G) HE (hematoxylin and eosin) staining of lung tissues at day 9 post-infection. Thick arrows, bronchioles; thin arrows, vessels; red triangles, inflammatory cell infiltration.

Fig. 3. HIS virus is replication-deficient in vivo and induces a transient IFN response. (A and B) Survival rate and percentage of body weight loss after intranasal infection (n = 5). (C and D) Viral titers at day 2 post-infection (n = 4) (C) and replication kinetics (n = 3) (D) of WT and HIS viruses in mouse lung tissues. (E) Induction of indicated ISGs in mouse lung tissues at 6, 24, 48, and 120 hours (h) post-infection (n = 3), shown as fold of induction over mock infection. RNase H, ribonuclease H. (F) Gene expression of indicated inflammatory cytokines in mouse lung tissues was examined by RNA sequencing (n = 2). (G) HE (hematoxylin and eosin) staining of lung tissues at day 9 post-infection. Thick arrows, bronchioles; thin arrows, vessels; red triangles, inflammatory cell infiltration. (H) Percentage of neutrophils, monocytes, and lymphocytes in BAL cytospins at day 9 post-infection (n = 3). (I) Cytokines in BAL samples measured by Luminex multiplex assay (n = 4). (J) Replication of indicated viruses in lung tissues of IFNAR−/− mice (n = 4). (K) Viral titer of WT and HIS viruses in ferret nasal wash, trachea, and lung tissues (n = 3). Dashed lines represent detection limits. Error bars, SD. *P < 0.05, **P < 0.01, ***P < 0.001 [log-rank test for (A); ANOVA with Bonferroni multiple comparisons test for (C), (H), and (J); and two-tailed t test for (D), (E), (I), and (K)].
viral particles were detected in nasal washes of HIS-infected ferrets, in contrast to the robust viral shedding observed during WT infection.

**HIS virus induces strong and broad adaptive immune responses**

We then examined the ability of the HIS virus to induce humoral and cellular responses. Mouse sera and BAL samples were collected at day 28 after single-dose (1 × 10^4 TCID50) vaccination with WT, HIS, or WSN-CA virus. HIS virus induced robust antibody responses, as measured by ELISA (enzyme-linked immunosorbent assay) and hemagglutinin inhibition (HAI). The dashed line represents the detection limit. (G) Mutations not neutralized by mouse sera (red) were mapped onto the HA structure (PDB ID, 1RUZ; n = 5) (40). The other five colors represent five well-characterized neutralization epitopes. (H) Tetramer staining of antigen-specific CD8 T cells in mouse lung (left) and spleen (right) at day 10 post-vaccination (n = 10).

Fig. 4. HIS virus induces strong adaptive immune responses in mice and ferrets. (A to D) HA-binding IgG (n = 7), HA neutralizing antibody (n = 7), and NP- and NA-binding IgG (n = 4) in mouse sera at day 28 post-vaccination, HAI, hemagglutinin inhibition. (E) HA-binding IgA in BAL samples at day 28 post-vaccination (n = 4). The optical density (OD) in ELISA was 450 nm. (F) HA neutralizing antibody levels in ferret sera at day 22 post-vaccination (n = 3). The optical density (OD) in ELISA was 450 nm. (G) Mutations not neutralized by mouse sera (red) were mapped onto the HA structure (PDB ID, 1RUZ; n = 5) (40). The other five colors represent five well-characterized neutralization epitopes. (H) Tetramer staining of antigen-specific CD8 T cells in mouse lung (left) and spleen (right) at day 10 post-vaccination (n = 10). (J) Percentage of antigen-specific memory precursor effector cells in mouse lung and spleen (n = 3). (J) NP antigen-specific CD8 T cells during the secondary responses in lung tissues from mice vaccinated with indicated viruses (n = 4).

**HIS virus induces strong and broad adaptive immune responses in ferrets.**

We then examined the ability of the HIS virus to induce humoral and cellular responses. Mouse sera and BAL samples were collected at day 28 after single-dose (1 × 10^4 TCID50) vaccination with WT, HIS, or WSN-CA virus. HIS virus induced robust antibody responses, as measured by ELISA (enzyme-linked immunosorbent assay) and hemagglutinin inhibition (HAI) inhibition and neutralization antibody assays (Fig. 4, A to E, and fig. S15). The level of HA antibody responses elicited by HIS virus was lower than for WT virus, yet significantly higher than for the WSN-CA, inactivated WT, and inactivated HIS viruses (Fig. 4, A and B, and fig. S15, C and D). Immunoglobulin G (IgG) antibodies against NP, NA, and M1 proteins, which have been shown to play an important role in limiting viral replication (30,31), were also detected in the sera of HIS-vaccinated mice at a level comparable to that in WT-infected mice (Fig. 4, C and D, and fig. S15C). Furthermore, mucosal immune responses, indicated by secretory IgA antibodies against HA and NP proteins, were elicited by HIS vaccination (Fig. 4E and fig. S15F). Robust HA antibody responses were also observed in ferrets vaccinated with HIS virus (Fig. 4F and fig. S15G), which were sustained for at least 50 days post-vaccination. To examine the epitope coverage of the neutralizing antibodies generated by HIS virus, we profiled the HA mutants in the presence or absence of mouse serum antibodies by using the high-throughput genomic approach (32). Mutations not neutralized by sera were observed in both head (Ca2 and Sa sites) and stem regions, with no significant difference in the number or the distribution of mutations between the WT and HIS viruses (Fig. 4G, fig. S16, and table S3). This suggests that the breadth and diversity of neutralizing antibodies induced by the HIS virus are comparable to those induced by the WT virus.

In addition to humoral responses, HIS virus elicited NP and PB1 antigen-specific CD8 T cell responses, similarly to WT virus and much more...
strongly than the WSN-CA, inactivated WT, and inactivated HIS viruses (Fig. 4H and fig. S17, A to D). The CD8 T cells induced by the WT and HIS viruses had a similar capacity for IFN-γ production upon stimulation by viral epitope peptides (fig. S17E). We further examined the phenotypes of virus-specific T cells by quantifying the expression of KLRG1, CD127, CD44, CD62L, and CCR7. By day 21 post-infection, the NP and P1B antigen-specific CD8 T cells induced by the WT and HIS viruses displayed similar levels of memory precursor effector cells with a CD62L<sup>−</sup>CD127<sup>−</sup>KLGR1<sup>−</sup> phenotype and short-lived effector cells with a CD62L<sup>−</sup>CD127<sup>−</sup>KLGR1<sup>−</sup>phenotype (Fig. 4G and fig. S17F). These virus-specific CD8 T cells also displayed a similar effector/memory phenotype, as measured by CD62L, CD127, and CCR7 expression (fig. S17, G and H). Consistently, after challenge infection at 1 month post-vaccination, HIS virus induced the secondary CD8 T cell responses similarly to WT but more strongly than WSN-CA virus (Fig. 4J and fig. S17). Moreover, similar frequencies of influenza-specific CD4 T cells were elicited by the WT and HIS viruses (Fig. 4K). To examine the diversity of the primary and secondary T cell responses, we analyzed the T cell receptor repertoire by sequencing the β T cell receptor (TCR) locus of NP-specific CD8 T cells in mice vaccinated with WT or HIS virus. The Vβ usage and clonality for both primary and secondary T cell responses were comparable between the WT and HIS viruses, documenting the diversity of T cell lineages induced by HIS vaccination (Fig. 4, L and M, and fig. S18).

We analyzed the potential impact of immune responses on the viral genome at the population level. Our whole-genome fitness profiling provides a data set for examining the genetic flexibility of viral sequences. We calculated the fitness cost of mutations in the previously identified B and T cell epitopes. Mutations on several T cell epitopes, but not on antibody epitopes, were generally correlated with lower fitness scores (Fig. 4N and table S4). Our results suggest that an escape from T cell selection will impose a higher fitness cost for the virus, and thus T cell responses will be effective against vaccine escape.

**HIS virus protects against homologous and heterologous viral challenge**

We examined whether HIS vaccination could offer protection against homologous and heterologous viral challenges. Immunized mice were challenged 28 days post-vaccination with 1 × 10<sup>6</sup> TCID<sub>50</sub> of WT virus. Vaccination by HIS virus reduced viral replication by ~3 log, with no sign of weight loss (Fig. 5 and fig. S19). Complete protection without detectable viral titers in the lung was achieved with one vaccination at a high dose (1 × 10<sup>8</sup>TCID<sub>50</sub>) or two vaccinations at a low dose (1 × 10<sup>4</sup>TCID<sub>50</sub>) (Fig. 5B and fig. S19B). Similar protective effects were observed in ferrets, which were challenged with 1 × 10<sup>7</sup>TCID<sub>50</sub> of WT virus at day 35 post-vaccination. Nasal washes were collected at days 1, 3, 4, 7, and 9 post-challenge, and no infectious viral particles were detected in nasal washes from HIS-vaccinated ferrets throughout this time period (Fig. 5C).

To test whether HIS vaccination provides protection against heterologous strains, we first challenged immunized mice with PR8 virus and examined viral titer at day 2 post-challenge. HIS vaccination reduced viral titer by ~3 log compared with mock vaccination and significantly more than WSN-CA vaccination (fig. S19C). We further challenged vaccinated mice with a lethal dose of three different influenza strains: H1N1 subtypes A/PR8/34 and A/Cal/04/09 and H3N2 subtype A/X-31. Protection by HIS vaccination was observed in all measures, including survival rate, percentage of body weight loss, and clinical scores (Fig. 5, D and E, and fig. S19D). Strong secondary antigen-specific T cell responses were observed in the challenged mice for all strains (fig. S20). HIS vaccination also protected ferrets from heterologous A/Cal/07/09 challenge, as shown by viral titer in nasal washes and percentage of body weight loss (Fig. 5F and fig. S19E).

**Discussion**

Conventional approaches to develop vaccines render the virus avirulent but also reduce immunogenicity. We developed a quantitative high-throughput genomics approach to systematically identify and eliminate immune-modulating functions in the virus genome while maintaining replication fitness in vitro. This is a systems-based strategy to enhance viral immunogenicity while attenuating replication and pathogenesis. In this proof-of-principle study, we generated a HIS virus with a combination of eight IFN-sensitive mutations. These mutations also induced higher IFN production and response. We demonstrated that HIS virus is highly attenuated in vivo but is able to induce transient IFN responses, elicit robust and diverse humoral and cellular immunity, and provide protection against homologous and heterologous viral challenges in mice and ferrets.

Recent studies have suggested several strategies to design live attenuated vaccines (14, 15, 33-37). Our method is distinctive in the following aspects: (i) We systematically investigated the whole viral genome, and we eliminated immune-evasion functions at multiple loci to obtain a safe strain that has no detectable replication in vivo; (ii) we selected mutants that induce a higher IFN response, because a transient IFN response has been shown to be essential for adaptive immunity, including the strong and diverse T cell responses; (iii) HIS virus selectively induced a transient IFN response but no other tested inflammatory responses, which reduced potential pathogenesis or side effects for future
clinical usage. We have also applied this approach to a DNA virus and generated an effective vaccine candidate.

In general, this unbiased and quantitative high-throughput genomics system can be widely applied to other pathogens to define the impact of genome-wide mutations under certain selection conditions. Similar profiling of a viral genome genome-wide mutations under certain selection conditions. Similar profiling of a viral genome can be performed with other immune components, such as cytokines, natural killer cells, or T cells, in vitro and in vivo. Inactivating additional immune evasion functions in the virus will further increase the safety and immunogenicity of its derivatives for prevention or therapy.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S20
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Avoiding interferon avoidance

Interferon (IFN) expression is a mammal's first response to viral infection. Many viruses have thus evolved mechanisms to evade IFN. Du et al. developed a method to systematically ablate IFN evasion genes from live, attenuated influenza virus (see the Perspective by Teijaro and Burton). A combination of mutants was assembled to construct a virus that triggered transient IFN responses in mice but that was unable to replicate effectively. The transient IFN responses led to robust antibody and memory responses that protected against subsequent challenge with different influenza viruses. This approach could be adapted to improve other RNA virus vaccines.

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