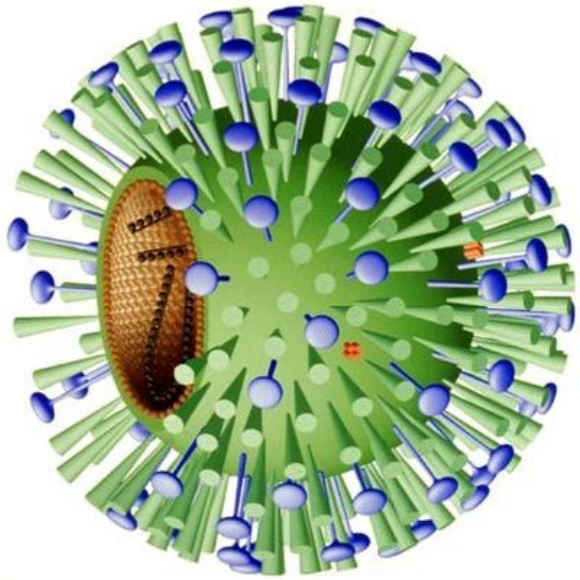
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A Rationally Designed Form of The TLR5 Agonist, Flagellin, Supports Superior Immunogenicity of Influenza B Globular Head Vaccines

Langzhou Song
VaxInnate Corporation
September 26, 2014

VaxInnate Influenza Vaccine Development

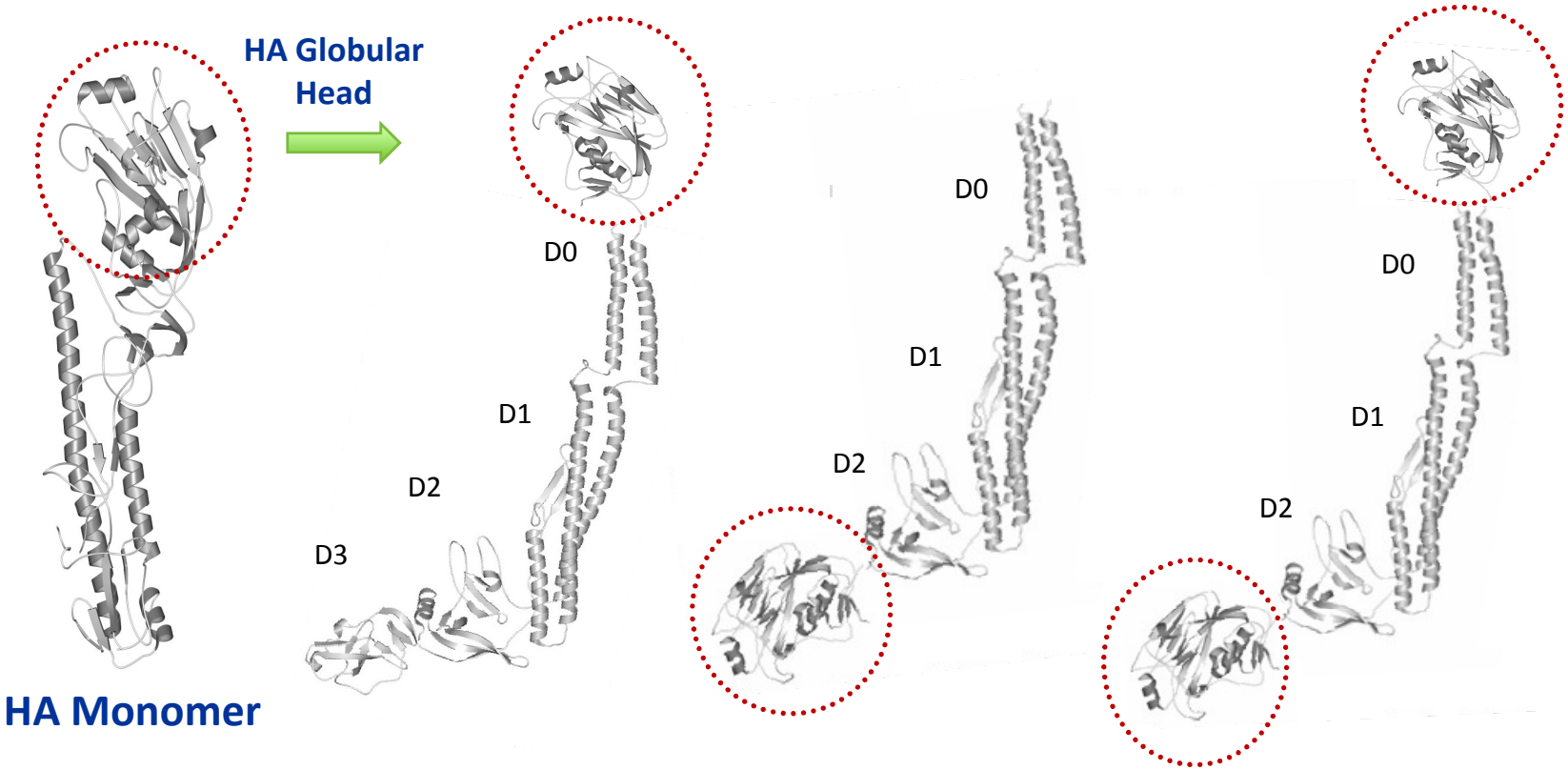


- Traditional flu vaccine contains three components: 1 H1, 1 H3 and 1 B
- More recently quadrivalent flu vaccine was introduced to the market which includes 1 H1, 1 H3 and 2 Bs from both lineages (Yamagata and Victoria).
- VaxInnate has successfully developed flu A vaccines that include H1, H3 and H5 subtypes which are immunogenic and safe in pre clinical animal models and in human clinical trials. However, influenza B vaccines based on the same vaccine formats are poor triggers of TLR5 and consequently poorly immunogenic.

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VaxInnate's HA Subunit Vaccines

Development of Initial Vaccine Formats



C Term
(Antigen Attached to C-terminus)

R3
(Antigen Replaces D3 Domain)

R3.2x
(2 Antigens Fused to Flagellin)

The vaccine format, or point of attachment of the vaccine antigen to flagellin, can dramatically affect the immunogenicity and safety profile of the vaccine.

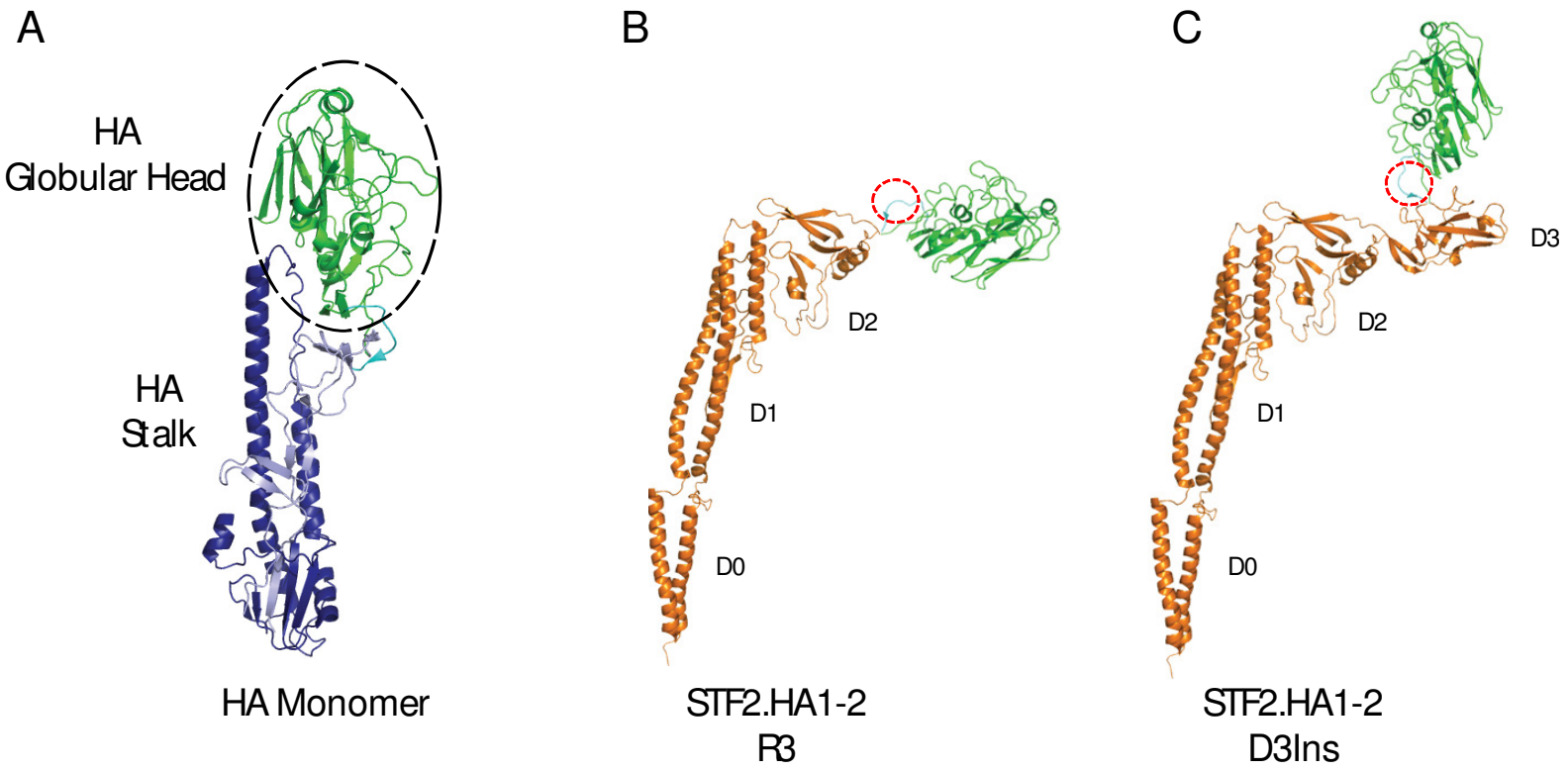
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Rationale for Flu B vaccine Design

- A major difference between the influenza A and B globular head domains is their iso-electric points (pIs), which for influenza B is among the highest (above 9.0 for the HA1 subunit) as compared to most influenza A HA1s. Flagellin, on the other hand, has pI around 5.
- We speculated that the positively charged influenza B HA heads might be interacting with the negatively charged flagellin at neutral pH, thereby interfering with TLR5 signaling and/or antigen presentation.
- To test this, we introduced two negatively charged residues, to mitigate potential charge interactions, in the region linking HA to flagellin in the R3 format.
- We also generated a second construct in which the globular head was inserted into D3 rather than fully replacing it, to form the D3Ins format. The latter design serves to move the globular head further away from the primary TLR5 binding sites in D1.

Design of Flu B Vaccines

R3 and D3Ins Format

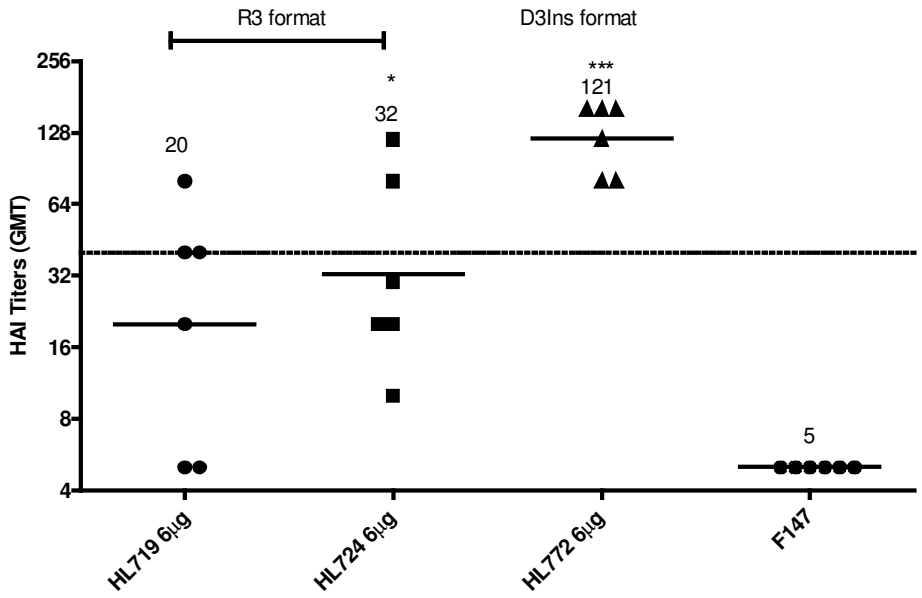


The vaccine format design aims to reduce charge-charge interactions between HA head and flagellin to facilitate antigen presentation and TLR5 signaling.

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Comparison of R3 and D3Ins formats in Mice

Neutralizing Antibody Responses (HAI)



- BALB/c mice (6 per group) were immunized s.c. twice (3 weeks apart) with 6 µg of Flu B vaccines.
- Sera were harvested two weeks post the booster and evaluated for HAI titers using ether extracted B/Wisconsin/01/2010 virus antigen. Titers of individual mice with GMTs are shown.

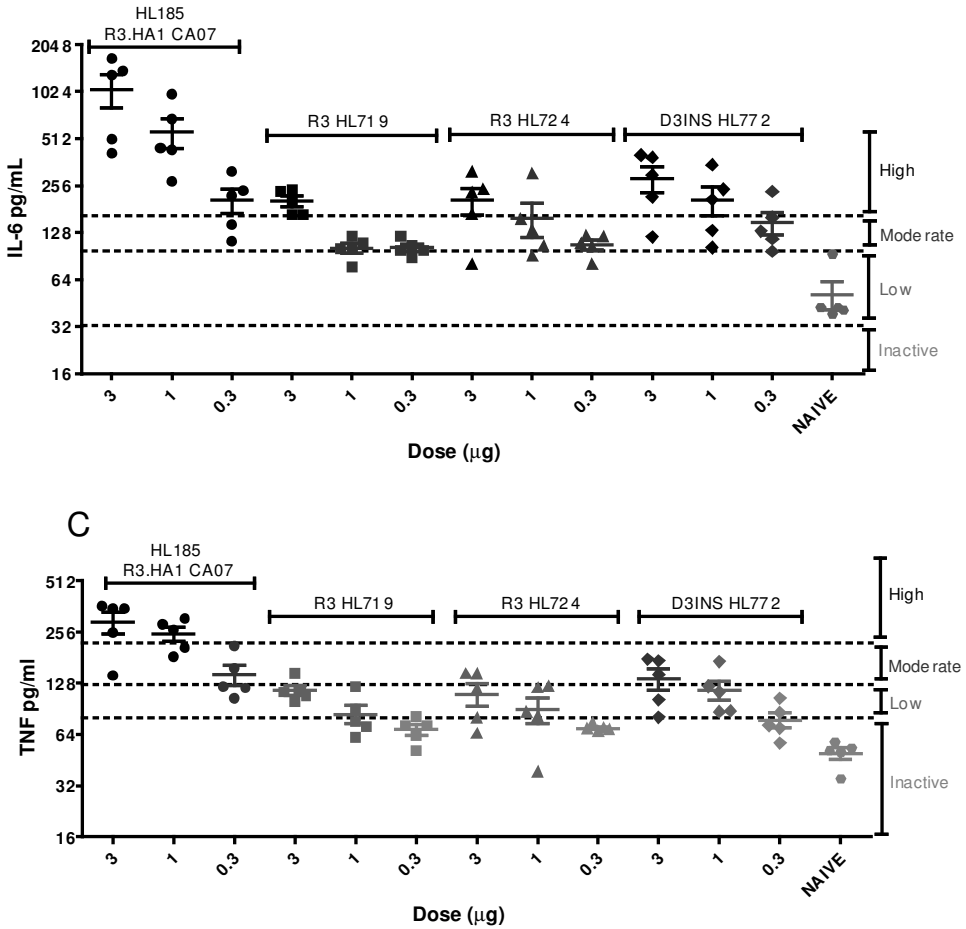
HL719 (R3 B/WI, an R3 format), **HL724** (R3 B/WI, an R3 with negative charges in the linker), **HL772** (D3Ins B/WI , a D3Ins format), F147 buffer

Introduction of the two negatively charged residues in the linking region of the R3 construct modestly improved titers. The use of the D3Ins format provided the most significant improvement in HAI titers.

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Flagellin function of the fusion vaccines

TLR5 Activity by *In Vivo* Cytokine Assay



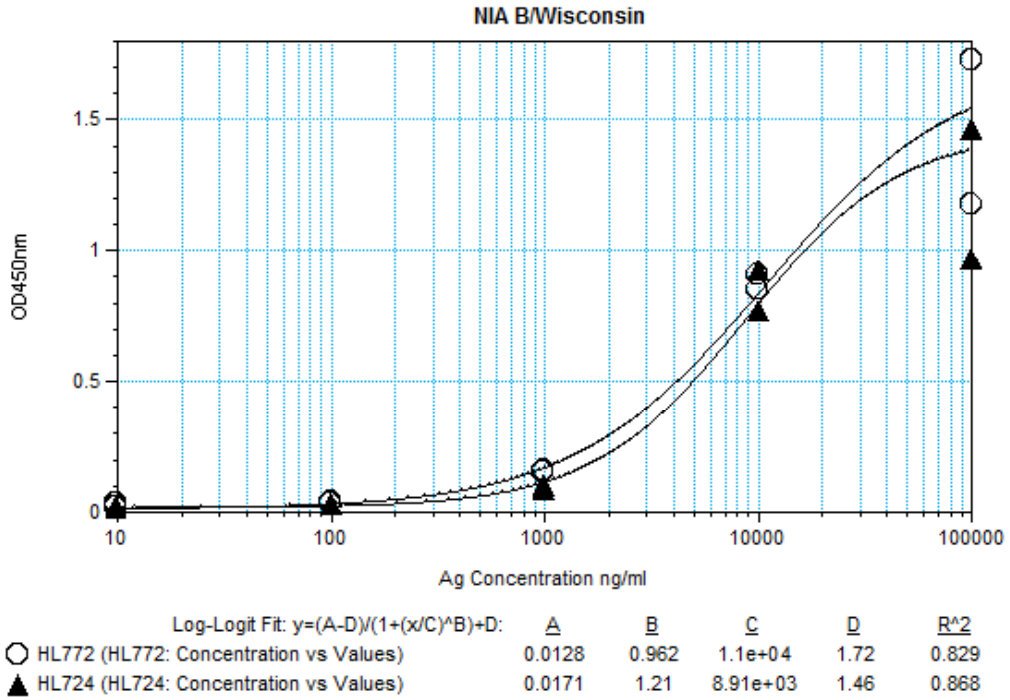
- BALB/c mice (n = 5) were immunized once (i.m.), sera harvested 3 h later and evaluated for IL-6 and TNF levels using a mouse cytometric bead array (CBA) from BD Biosciences.
- HL185 (R3 A/CA07): positive control; HL719, (R3 format, B/WI), HL724 (R3 B/WI with negative charges in the linker) and HL772 (D3Ins format, B/WI).

The results show that introduction of the two negatively charged residues provides an incremental improvement while movement of the globular head to the D3Ins position provides a distinct improvement in the TLR5 activity, particularly as measured by IL-6 production

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Antigenicity of HA in Fusion Vaccines

Neutralizing Inhibition Assay (NIA)



Log-Logit Fit: y=(A-D)/(1+(x/C)^B)+D:	A	B	C	D	R^2
○ HL772 (HL772: Concentration vs Values)	0.0128	0.962	1.1e+04	1.72	0.829
▲ HL724 (HL724: Concentration vs Values)	0.0171	1.21	8.91e+03	1.46	0.868

HL724 (R3 B/WI, an R3 with negative charges in the linker),
HL772 (D3Ins B/WI , a D3Ins format)

- NIA measures the ability of a vaccine candidate to bind and deplete neutralizing antibodies presented in the immune serum, thereby assessing the integrity of neutralizing epitopes on the flagellin-HA fusion immunogen.
- The higher potency of the vaccine candidate, the greater the depletion of the neutralizing antibodies in the serum that allows the test virus to infect MDCK cells.

The R3 format and the D3Ins format compete equally well for binding to neutralizing antibodies in the hyper-immune serum indicating that the antigenicity of the HA head is unaltered across the different constructs. Thus the poor immunogenicity of the R3 format of the B/Wisconsin vaccine likely relates primarily to impaired TLR5 signaling activity

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Insertion Points of Flu B Vaccine Variants

D3Ins Format Optimization

```
        10         20         30         40         50         60
MAQVINTNSL SLLTQNNLNK SQSALGTAIE RLSSGLRINS AKDDAGQAI ANRFTANIKG

        70         80         90        100        110        120
LTQASRNAND GISIAQTTEG ALNEINNNLQ RVRELAVQSA NSTNSQSDLD SIQAEITQRL
                                           ↑D1I-o1

       130        140        150        160        170        180
NEIDRVSGQT QFNGVKVLAQ DNTLTIQVGA NDGETIDIDL KQINSQTLGL DSLNVQAYD
                                                      ↑D2I-o2

       190        200        210        220        230        240
VKDTAVTTKA YANNGTTLDV SGLDDAAIKA ATGGTNGTAS VTGGAVKFDA DNNKYFVTI
        ↑D2I-o2                                        ↑D3I-o2

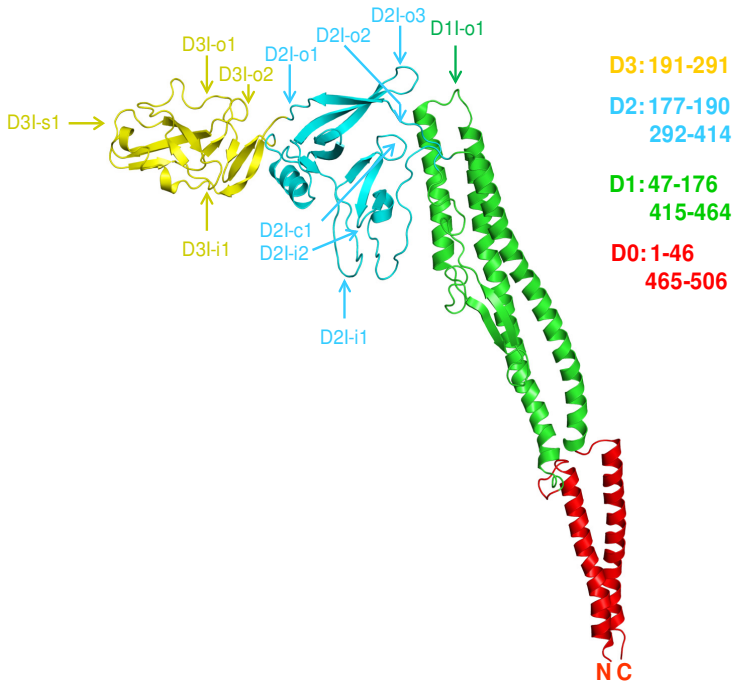
       250        260        270        280        290        300
GFTGADAAKN GDYEVNVATD GTVTLAAGAT KTTMPAGATT KTEVQELKDTPAVVSADAKN
   ↑D3I-s1          ↑D3I-i1              ↑D3I-o1

       310        320        330        340        350        360
ALIAGGVDAT DANGAELVKM SYTDKNGKTI EGGYALKAGD KYYAADYDEA TGAIKAKTTS
                              ↑D2I-o3

       370        380        390        400        410        420
YTAADGTTKT AANQLGGVDG KTEVVTIDGK TYNASKAAGH DFKAQPELAE AATTENPL
     ↑D2I-i1        ↑D2I-c1   ↑D2I-i2

       430        440        450        460        470        480
QKIDAALAQV DALRSDLGAV QNRFNSAITN LGNTVNNLSE AKSIEDSDY ATEVSNMSRA

       490        500
QILQQAGTSV LAQANQVPQN VLSLR
```



Various insertion points in D3 and D2 were tested. The D3Ins vaccines were found to outperform the D2Ins vaccine candidates.

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Insertion Points of Flu B Vaccine Variants

D3Ins Format Optimization

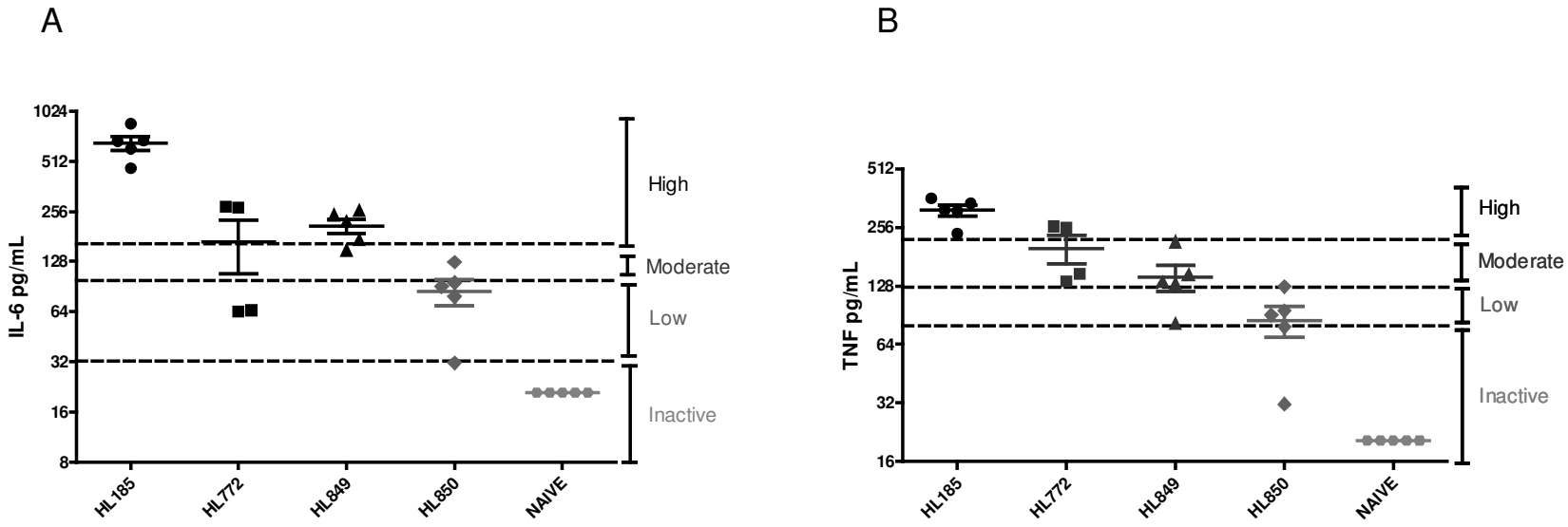
Construct	*In Vivo* TLR5 Activity	Construct	*In Vivo* TLR5 Activity
HL772, D3I-o1	High (IL-6), Moderate (TNF-α)	HL826, D2I-o2	Low (IL-6), Inactive (TNF-α)
HL849, D3I-i1	High (IL-6), Moderate (TNF-α)	HL827, D2I-o3	Low (IL-6), Inactive (TNF-α)
HL848, D3I-s1	Moderate (IL-6), Inactive (TNF-α),	HL850, D2I-i1	Low (IL-6), Inactive (TNF-α)
HL888, D3I-o2	Low (IL-6), Inactive (TNF-α)	HL892, D2I-i2	Low (IL-6), Inactive (TNF-α)
HL825, D2I-o1	Moderate (IL-6), Low (TNF-α)	HL828, D1I-o1	Inactive (IL-6), Inactive (TNF-α)

Various insertion points in D3 and D2 were tested. The D3Ins vaccines were found to outperform the D2Ins vaccine candidates.

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Insertion Points of Flu B Vaccine Variants

In Vivo TLR5 Assay for the Representative Vaccine Variants



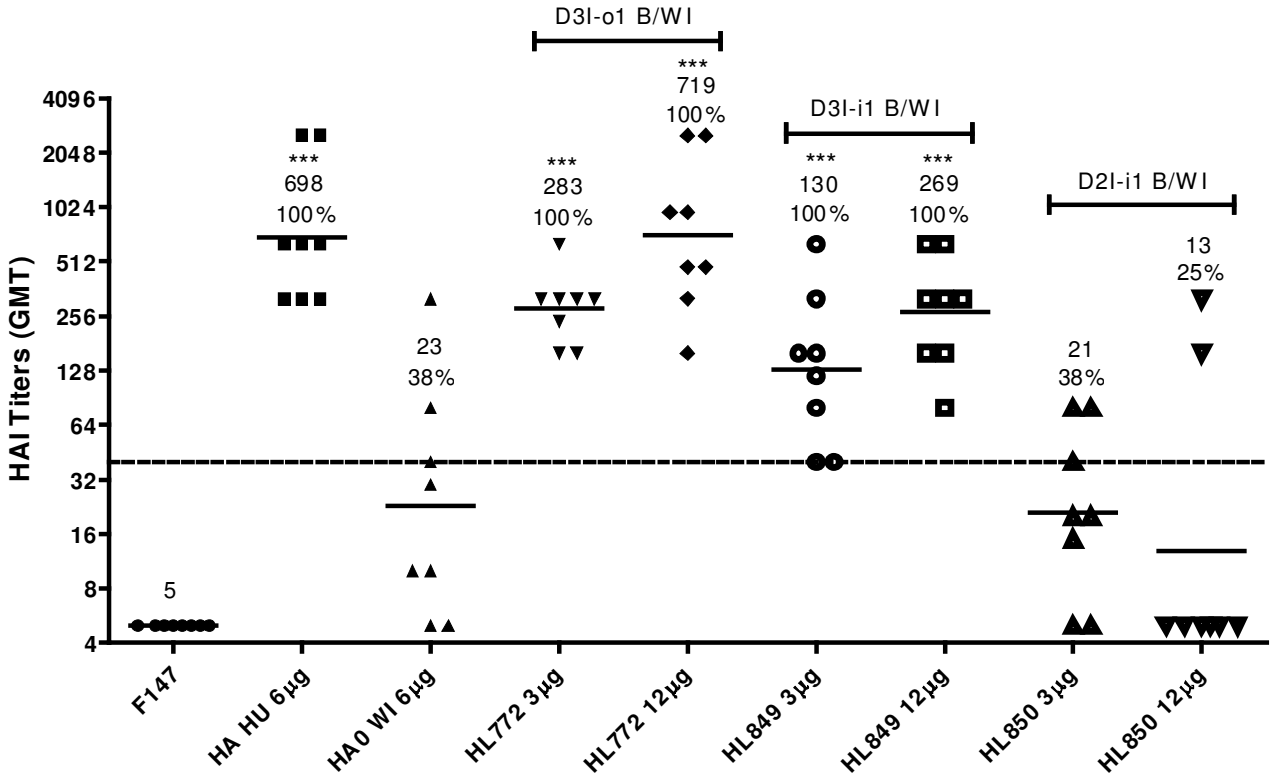
D3Ins: HL772 (D3I-o1, B/WI) and HL849 (D3I-i1, B/WI)
D2Ins: HL850 (D2I-i1, B/WI)
Positive Control: HL185 (R3 A/CA07)

Various insertion points in D3 and D2 were tested. The D3Ins vaccines were found to outperform the D2Ins vaccine candidates.

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Insertion Points of Insertion Variants

Mouse Immunogenicity by HAI



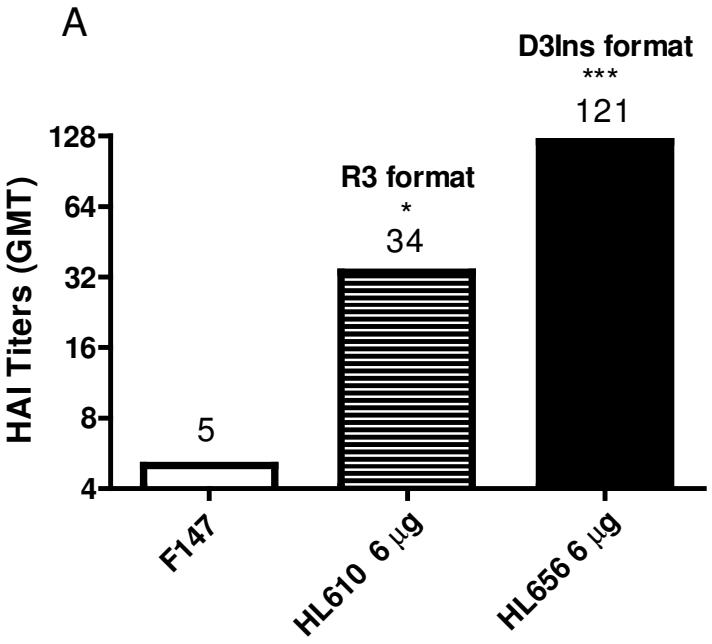
Various insertion points in D3 and D2 were tested. The D3Ins vaccines were found to outperform the D2Ins vaccine candidates.

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Application of D3Ins Format to Both Flu B Lineages

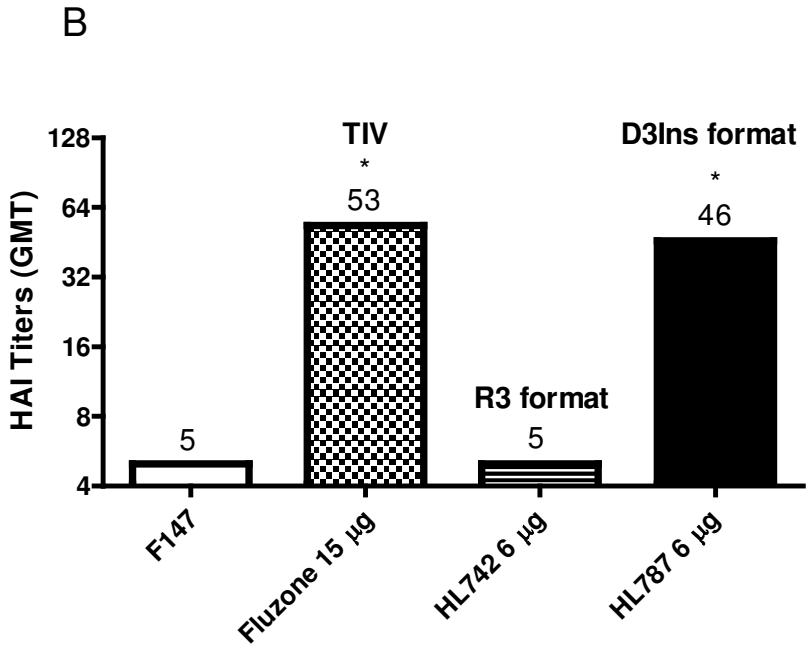
HAI Titers of Flu B Vaccines of Two Lineages

Yamagata Lineage: B/Florida/4/2006



F147: Formulation buffer
HL610 (R3 B/FL, an R3 with negative charges in the linker),
HL772 (D3Ins B/FL, a D3Ins format)

Victoria Lineage: B/Bangladesh/5945/09



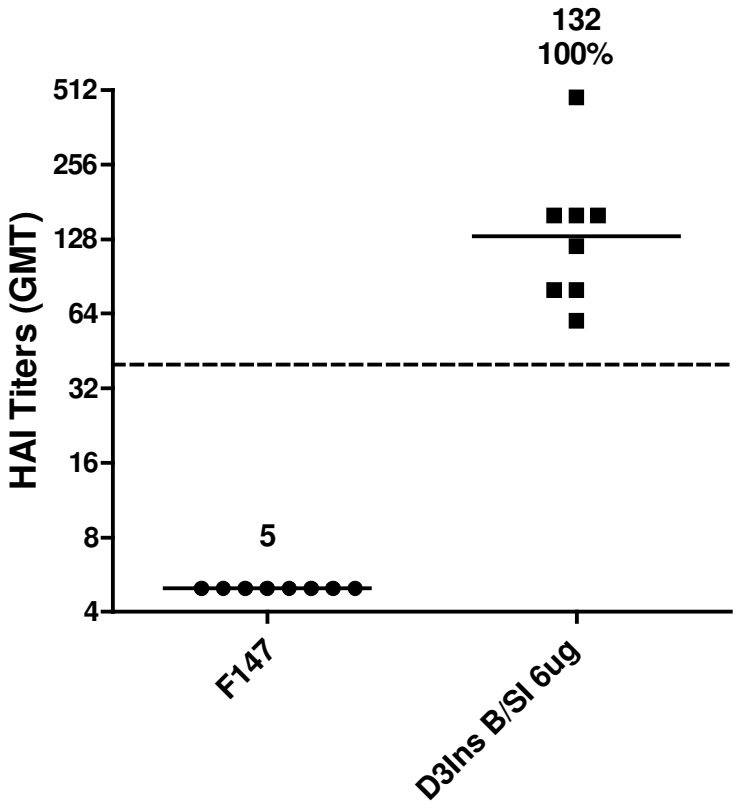
F147: Formulation buffer; **TIV**, Fluzone positive control
HL742 (R3 B/BD, an R3 with negative charges in the linker),
HL787 (D3Ins B/BD, a D3Ins format)

D3Ins format is applicable to both B lineages.

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Immunogenicity and Efficacy of Flu B Vaccines

Flu B Challenge Model (B/Sichuan/379/1999)



- Flu B challenge mouse model using B/Sichuan/379/1999 (D3Ins B/SI, Yamagata lineage).
- Groups of 10 BALB/c mice were immunized twice with 6 µg of a D3Ins format vaccine against the B/SI.
- Sera were harvested two weeks post the booster dose and evaluated for HAI titers.

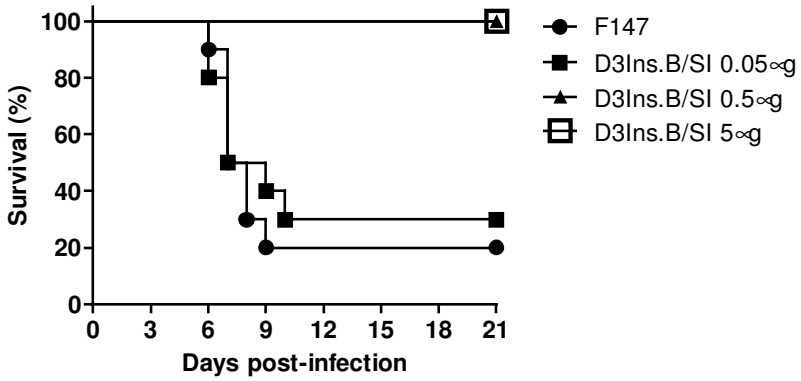
A robust immune response was shown in all mice in the challenge model.

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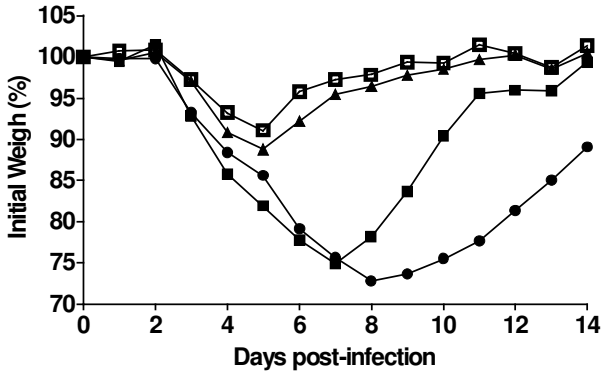
Immunogenicity and Efficacy of Flu B Vaccines

Flu B Challenge Model (B/Sichuan/379/1999)

A



B



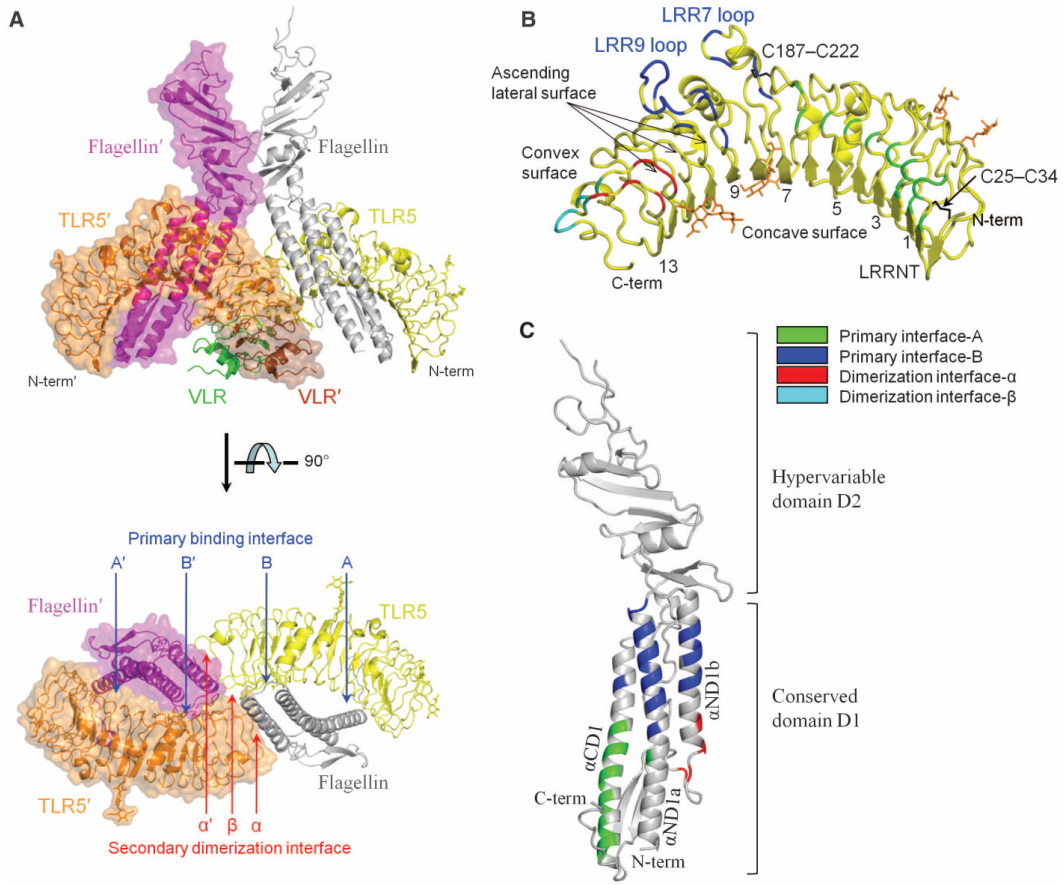
Groups of 10 BALB/c mice were immunized s.c. with the indicated doses of D3Ins B/SI or F147 formulation buffer on days 0 and 21. On day 42, mice were challenged i.n. with 5 LD_{50} of B/SI, and monitored daily for mortality for 21 days and weight change for 14 days. Survival rates (A) and weights (mean percentage of initial weight) (B) are plotted.

D3Ins vaccine of B/Sichuan/379/1999 is efficacious at a submicrogram dose. The efficacy results of D3Ins B/SI thus support the general suitability of D3Ins format for our influenza B vaccines.

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Flagellin and TLR5 (Yoon, et al., Science 2012)

X-Ray Crystal Structure of Flagellin and TLR5 Complex



The Flagellin/TLR5 structure confirmed that all known agonist-activated TLR structures form a similar dimer organization, which brings their C-terminal regions into juxtaposition so that their intracellular TIR domains can initiate a signaling cascade.

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Summary

- We have developed an alternative vaccine format for influenza B vaccines for which the HA head is inserted into domain 3 of flagellin and we have confirmed the optimal insertion point within domain 3.
- We have shown that the redesigned format maximizes TLR5 signaling and immunogenicity for multiple influenza B strains.
- Similar to the A strain vaccines, the Flu B vaccines are economically and efficiently produced in our standard prokaryotic system which therefore allows for time and cost efficient production of a multivalent seasonal product comprising A and B strains.

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