A rationally designed form of the TLR5 agonist, flagellin, supports superior immunogenicity of Influenza B globular head vaccines

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ABSTRACT

Previously, we demonstrated that for H1N1 and H5N1 influenza strains, the globular head of the hemagglutinin (HA) antigen fused to flagellin of Salmonella typhimurium fliB (STF2) is highly immunogenic in preclinical models and man (Song et al. (2008) [13]; Song et al. (2009) [14]; Taylor et al. (2012) [12]). Further we showed that the vaccine format, or point of attachment of the vaccine antigen to flagellin, can dramatically affect the immunogenicity and safety profile of the vaccine. However, Influenza B vaccines based on these formats are poor triggers of TLR5 and consequently are poorly immunogenic. Through rational design, here we show that we have identified a fusion position within domain 3 of flagellin that improves TLR5 signaling and consequentially, immunogenicity of multiple influenza B vaccines. Our results demonstrate that, similar to influenza A strains, the protective subunit of the influenza B HA can be fused to flagellin and produced in a standard prokaryotic expression system thereby allowing for cost and time efficient production of multivalent seasonal influenza vaccines.

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1. Introduction

Pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), recognize conserved components (known as pathogen associated patterns or PAMPs) of bacteria, viruses, fungi, and protozoa [1–4]. Recognition initiates a conserved signaling cascade that orchestrates adaptive immunity [5]. These basic aspects of TLR biology have provided a strong rationale for the use of their agonists as vaccine adjuvants.

Flagellin, the primary protein component of bacterial flagella, acts as a TLR5 agonist [6–8]. Structural analyses of flagellin have revealed a boomerang shaped protein with four major domains [9]. The primary TLR5 binding sites are located in Domain 1 (D1) [9–11]. Our vaccine platform is based on the genetic fusion of vaccine antigens to flagellin. A number of different vaccine formats which differ in the nature of the attachment (linkage) of the antigen to flagellin and/or the number of flagellin domains that are retained have been developed. Interestingly, the vaccine format not only affects the immunogenicity of the vaccine antigen it can also affect the safety window of the vaccine in preclinical models and in man – presumably through altering the agonist properties of flagellin [12].

Our influenza vaccines fuse the globular head domain of the major protective antigen, hemagglutinin (HA), to flagellin. Early vaccine formats fused the HA globular head to the C-terminus of flagellin (C-term format) [13], replaced domain 3 of flagellin (R3 format) [14] or placed one copy of the HA head at the C terminus while a second copy replaced domain 3 (R3.2x format) [15]. In preclinical and clinical studies comparing these formats, the R3 and R3.2x formats were superior to the C-term in their immunogenicity and safety profiles. However, influenza B vaccines based on these formats are poor triggers of TLR5 and consequently poorly immunogenic. Here we present the rational design of a vaccine format that supports optimal TLR5 signaling through the flagellin moiety and immunogenicity of the influenza B globular head.

2. Materials and methods

2.1. Cells, eggs and viruses

Madin-Darby canine kidney (MDCK) cells were obtained from ATCC (Manassas, Virginia) and maintained in minimal essential medium (MEM). Specific Pathogen Free research eggs were purchase from Charles River Laboratories (Storrs, CT). Influenza B viruses were obtained from Influenza Division/CDC (Atlanta, Georgia). Mouse-adapted B/Sichuan/379/1999 was kindly provided by Dr. Don Smee (Utah State University). Virus stocks were obtained...
by infection of chicken embryonated eggs for 3 days at 33 °C. Aliquots of harvested virus (allantoic fluid) were stored at −80 °C until use. Virus stock was titrated by standard hemagglutination or plaque assays.

2.2. Cloning and purification

2.2.1. Cloning of recombinant HA genes

An HA1-2 [13] globular head (aa60–aa308, Yamagata lineage and aa60–aa309, Victoria Lineage) was used as the influenza B antigen. The codon optimized synthetic genes of influenza B viruses were used to replace domain 3 (D3, aa191–aa292) of the full-length flagellin sequence to form R3 format vaccines. The same length globular head was inserted into the D3 or D2 domain (Fig. 1A and B) to form D3Ins/D2Ins format vaccines. Constructs were cloned into the pET24a vector to generate STF2R3.HA1-2 and STF2D3Ins.HA1-2/STF2D2Ins.HA1-2 plasmids. BLR (DE3) cells were transformed with plasmids (Novagen, San Diego, CA; Cat #69053) to generate working cell banks.

2.2.2. Expression and purification of HA globular head-flagellin fusion proteins

Flagellin fusion proteins were manufactured utilizing a fed-batch fermentation process in Escherichia coli as described previously [13,14]. Protein purity and concentration was determined by RP HPLC and UV280. Residual endotoxin was assayed by standard Chromogenic Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD) as directed by the manufacturer.

2.3. Characterization of flagellin-HA globular head fusion proteins

2.3.1. In vivo TLR5 assay

BALB/c mice (5–6 per group) were injected intramuscularly (i.m.) with control or test vaccines at 0.3–3 μg each. Three hours later serum was prepared and analyzed for inflammatory cytokines using a mouse cytometric bead array (CBA) from BD Biosciences (BD FACSCalibur). Cytokine values (pg/mL) were calculated against a standard curve fit with a 4-parameter logistic equation.
2.3.2. Hemagglutination inhibition (HAI) test
HAI antibody titer against influenza B virus was measured as previously described [14,16]. Sheep serum generated against the relevant virus (CBER) or ferret post infection serum (CDC) was used as reference serum. Data was reported as results of individual mice with geometric mean titers (GMT).

2.3.3. Neutralization inhibition assay (NIA)
The NIA measures the ability of a vaccine molecule to compete with virus for binding to neutralizing antibodies in the reference serum. Sheep hyperimmune serum (CBER/FDA) raised against a specific influenza B HA was incubated with a serially diluted vaccine candidate for 75 min. Influenza virus B/Wisconsin/01/2010 (50 TCID50) was added and allowed to incubate for 1 hr at 37°C prior to addition of 1.5 × 105 per well MDCK cells in Opti Pro SFM (Invitrogen) supplemented with 100 IU/ml Penicillin and 100 mg/ml Streptomycin. Following a 20-h incubation at 37°C, the cells were washed, fixed with cold acetone, air-dried and incubated with a monoclonal anti-influenza B nucleoprotein antibody (primary) and goat anti-mouse Fcγ specific IgG:HRP (secondary). Signals were detected by OD450. NIA curves are generated with Log-logit fit model using SoftMax pro 5.2 (Molecular Devices).

2.4. Animal studies

2.4.1. Animals
All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Princeton University according to NIH guidelines and IACUC-approved protocols. Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, Massachusetts) and maintained at the AAALAC-accredited animal facility of Princeton University.

2.4.2. Vaccination
Female BALB/c mice (6–8 weeks old) were vaccinated subcutaneously (s.c.) or i.m. with two doses of vaccine (days 0 and 21) in 100 or 50 μl of formulation buffer F147 (10 mM histidine, 10 mM Tris, 150 mM NaCl, 5% trehalose, 0.02% P80, 0.1 mM EDTA, 0.5% ethanol, pH 7.0). The animals were bled via retro orbital puncture on day 35 following anesthesia with isoflurane inhalation. Serum samples were prepared and stored at −20°C until use.

2.4.3. Virus challenge
Mice were infected intranasally (i.n.) with 5 LD50 (105 pfu) of influenza B/Sichuan/379/1999 virus in 50 μl of PBS on day 42 following intraperitoneal injection of a mixture of Ketamine (100 mg/kg) and Xylazine (5 mg/kg). Mortality was monitored daily for 3 weeks and weights were recorded for 14 consecutive days. Animals that lost 25% of their initial weight were euthanized.

2.5. Statistical analyses
For HAI analysis, HAI titers were log-transformed and ANOVA/Tukey’s multiple comparison tests were performed to determine a vaccine needs to have low to moderate levels of activity in the 0.3–1 μg dose range in order to be immunogenic. A complete panel of cytokines and chemokines were measured (IL-12 p70, TNF, γ-IFN, MCP-1, IL-10 and IL-6). Only IL-6 (B) and TNF (C) levels for individual mice are shown, along with bars representing the mean and standard error. The rest are none-discriminative in this assay. B Wisconsin formats compared were: R3 (HL719 without negative charges in the linker), R3 (HL724 with negative charges in the linker) and D3Ins format (HL772). A/H1 CA07 R3 (HL185) was included as a positive control. (D) NIA activity: B/Wisconsin/01/10 vaccines in the format of R3 (HL724) or D3Ins (HL772) were subjected to NIA. After color development with TMB substrate, the virus is quantified by measuring OD450. NIA curves were generated by Log-Logit fit model.
Table 1
Relative in vivo TLR5 activity of different insertion vaccine variants.

<table>
<thead>
<tr>
<th>Construct</th>
<th>In vivo TLR5 activity</th>
<th>Construct</th>
<th>In vivo TLR5 activity</th>
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<tbody>
<tr>
<td>HL772, D3I-o1</td>
<td>High (IL-6), moderate (TNF-α)</td>
<td>HL826, D2I-o2</td>
<td>Low (IL-6), inactive (TNF-α)</td>
</tr>
<tr>
<td>HL849, D3I-i1</td>
<td>High (IL-6), moderate (TNF-α)</td>
<td>HL827, D2I-o3</td>
<td>Low (IL-6), inactive (TNF-α)</td>
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<td>HL848, D3I-s1</td>
<td>Moderate (IL-6), inactive (TNF-α)</td>
<td>HL850, D2I-i1</td>
<td>Low (IL-6), inactive (TNF-α)</td>
</tr>
<tr>
<td>HL888, D3I-o2</td>
<td>Low (IL-6), inactive (TNF-α)</td>
<td>HL892, D2I-i2</td>
<td>Low (IL-6), inactive (TNF-α)</td>
</tr>
<tr>
<td>HL825, D2I-o1</td>
<td>Moderate (IL-6), low (TNF-α)</td>
<td>HL828, D1I-o1</td>
<td>Inactive (IL-6), inactive (TNF-α)</td>
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3. Results

While influenza A vaccines using an R3 or R3.2x format are highly immunogenic and well tolerated in preclinical models and man [1–4,15], R3 formats of influenza B vaccine were poorly immunogenic in mice (data not shown). A major difference between the influenza A and B globular head domains is their isoelectric points (pI), which for influenza B is among the highest (above 9.0 for the HA1 subunit) as compared to most influenza A HA1s. Given that flagellin has several negatively charged patches, with an overall pI of ~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 (Fig. 1A). The latter design serves to move the globular head further away from the primary TLR5 binding sites in D1. We assessed the effects on immunogenicity and TLR5 activity of the vaccine.

In an immunogenicity study, mice were immunized twice, at a 3 week interval with 6 μg of each construct. Three weeks later sera were harvested and evaluated for HAI titers. The results in Fig. 2A show that introduction of the two negatively charged residues in the linking region of the R3 construct modestly improved titers but use of the D3Ins format provided the most significant improvement in HAI titers.

TLR5 activity was assessed using an in vivo assay. Mice were immunized once, sera harvested 3 h later and evaluated for IL-6 and TNF levels. By comparing levels of cytokines elicited by poorly immunogenic versus highly immunogenic vaccines we have been able to establish ‘activity thresholds’ that provide a guide as to the minimum level of activity required for the vaccine to be immunogenic. The results (Fig. 2B and C) 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 (Fig. 2B). Importantly, the activity of the D3Ins construct approaches that of the positive control, which is an R3 format vaccine (HL185) based on the A/California/07/2009 (H1N1) strain. This vaccine as well as other H1 and H5 R3 format vaccines are known to be immunogenic in the clinic [12,15,17].

To rule out a difference in refolding of the HA head between constructs we evaluated the R3 format (HL724) and the D3Ins format (HL772) constructs in a neutralization inhibition assay (or NIA). Poor refolding of the head domain manifests itself in this assay as ineffective depletion of the neutralizing antibodies in the sheep hyper-immune serum which in turn allows the test virus to infect MDCK cells. The results (Fig. 2D) show that 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 and this can be improved with changes that improve TLR5 signaling – presumably by alleviating interactions between the HA head and flagellin.

To ensure that we have selected the most optimal insertion point in the hinge region of flagellin, additional constructs with different insertion sites (in the D3 domain and also in the D2 domain) were assessed. All insertion sites were located in the loop regions and therefore could theoretically accommodate the insertion without disturbing the overall flagellin structure as illustrated in Fig. 1B. The primary sequence with the insertion sites marked is also listed in Fig. 1B.

For these studies we again based the constructs on the B/Wisconsin/01/2010 strain (see Table 1 for the list of constructs). Constructs were initially evaluated in the in vivo TLR5 assay. The results are summarized in Table 1 and representative results for the two highest and one of the lowest in this assay are shown in Fig. 3A and B.

From the results, we conclude that D3 insertion constructs outperformed D2 insertion constructs with respect to TLR5 activity. Among the D3Ins variants, D3I-o1 (HL772) and D3I-i1 (HL849) were more active than D3I-s1 (HL848) in the TLR5 assay. It is noteworthy that both the D3I-o1 (HL772) and D3I-i1 (HL849) variants place the insertion on the side of the D3 domain while the less active construct, D3I-s1 (HL848) has the insertion site at the tip of the D3 domain (Fig. 2B). This may reflect differences among the constructs in the known conformational change of the D3 domain that takes place in TLR5 binding and signaling [11]. Consistent with this, both HL772 and HL849 elicited robust HAI titers in mice whereas the D2 insertion variant, HL850 which had low TLR5 activity was poorly immunogenic (Fig. 3C). Taken together the results indicate that the D3I-o1 and D3I-i1 insertion points are the most optimal for insertion of the Flu B globular head. To confirm the general applicability of the D3Ins format for B vaccines, we tested candidate vaccines for two additional strains. In the first study, we generated R3 and D3Ins format vaccine against the B/Florida/04/2006 strain. Mice received two doses of 6 μg. The results shown in Fig. 4A demonstrate that the D3Ins vaccine elicited significant titers with a GMT of 121, again outperforming R3 format which had GMT of 34.

In the second experiment we generated R3 and D3Ins constructs for the currently circulating B Victoria lineage strain, B/Bangladesh/5945/2009 (B/Brisbane/60/2008-like) and evaluated immunogenicity in mice. Mice were immunized twice with 6 μg of R3 and D3Ins format vaccines. The HAI results show that the D3Ins format elicited significantly higher titers than the R3 format of B/Bangladesh/5945/2009 (Fig. 4B), suggesting D3Ins format is applicable to both B lineages.

Finally, we performed immunogenicity and efficacy studies of the D3Ins format for the historical B virus, B/Sichuan/379/1999 (D3Ins B/SI, Yamagata lineage). For the immunogenicity study,
**Fig. 3.** In vivo TLR5 activity and relative immunogenicity of D2 and D3 insertion variants. (A and B) In vivo TLR5 activity: BALB/c mice (n = 5) were immunized i.m. with 1 µg of the indicated vaccine candidate or left naïve. At 3 h, sera were harvested and cytokine levels were quantified using a mouse cytometric bead array (BD). Dotted lines indicate thresholds of cytokines levels correlated with immunogenicity. IL-6 (A) and TNF (B) levels of individual mice are shown, along with bars representing the mean and standard error. Two D3 insertion variants (HL772, D3-I-o1 and HL849, D3-I-1) are more active in inducing both IL-6 and TNF compared to D2 insertion variant (HL850, D2-I-1). (C) HAI Titers: Female BALB/c mice (n = 8) were immunized s.c. with 3 µg and 12 µg of D3 insertion vaccine variants including D3-I-o1 (B/WI, HL772), D3-I-1 (B/WI, HL849) and D2-I-1 (B/WI, HL850). The formulation buffer, F147, 6 µg of rHA WI (Protein Sciences) and inactivated B/Hubei-Wujiapang/158/2009 antigen (CBER HU) were included as controls. Post boost sera were tested with HAI assays using ether extracted B/Wisconsin/01/2010 virus antigen. Data are shown as titers of individual mice with GMTs. Statistical differences were determined in 1-way ANOVA/Tukey’s posttests. **p < 0.001 vs F147 group.

**Fig. 4.** HAI titers of mouse sera following immunizations with R3 and D3Ins vaccines of B Florida (A) and B Bangladesh (B) strains. (A) Yamagata Lineage: Groups of 5 BALB/c were immunized s.c. on days 0 and 21 with 6 µg of a R3 and D3Ins vaccines against the B/Florida/4/2006 strain or F147 buffer. Neutralizing antibody titers of the serum samples were measured by HAI test and expressed as GMTs. *p < 0.05; **p < 0.01 vs F147 group in 1-way ANOVA/Tukey’s posttests. (B) B Victoria Lineage: Groups of 6 BALB/c were immunized s.c. on days 0 and 21 with 6 µg R3 or D3Ins vaccines of B/Bangladesh/5945/09, 15 µg Fluzone or F147 buffer. Neutralizing antibody titers of the serum samples were measured by HAI test and expressed as GMTs. Vaccine constructs used were HL742 (R3.HAB, B/B) and HL787 (D3Ins.HAB, B/B). *p < 0.05 vs F147 group in 1-way ANOVA/Tukey’s posttests.
groups of 10 BALB/c mice were immunized twice with 6 μg of a D3Ins format vaccine against the B Sichuan strain. Sera were harvested two weeks post the booster dose and evaluated for HAI titers. The results showed robust immunogenicity with all mice developing an immune response with a GMT of 132.

An established mouse lethal challenge model was used to evaluate the efficacy of D3Ins vaccine of B/Sichuan/379/1999. Immunizations with doses of 5 μg and 0.5 μg D3Ins B/SI resulted in a 100% survival rate and <12% weight loss in mice challenged with a lethal dose of B/Sichuan/379/1999 virus (Fig. 5A and B). In contrast, mice in the placebo group showed a 20% survival rate and >75% weight loss. These results indicate that the 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.

4. Discussion

Although R3 format vaccines of influenza A (H1N1 & H5N1) are well tolerated and immunogenic [12,15,17] in preclinical models and man, the R3 format of a prototypic Flu B vaccine was found to be poorly immunogenic in mice. In our investigation of the underlying mechanism of the poor immunogenicity, we determined that the HA1-2 globular head of Flu B has a much higher pI, generally over 9.0, as compared to H1, which is usually near neutral pH. We hypothesized that the positively charged HA head may interact with the negatively charged flagellin at neutral pH and that the poor immunogenicity may largely be due to compromised TLR5 signaling and/or antigenic presentation of the HA head.

Consistent with this, we find that modifications either to the linking region, such as the incorporation of negatively charged residues, or the redesign of the vaccine format to move the head domain away from domain 1 of flagellin improved TLR5 signaling and vaccine immunogenicity without altering the antigenicity of the vaccine. Further, we find that the most optimal insertion points are those located on the side of domain 3, as these work to preserve TLR5 signaling. Our interpretation of the results is that construct changes which alleviate the charge-charge interactions between a high pI antigen and domain 1 of flagellin allow flagellin to bind to TLR5 more efficiently and thus better to orchestrate the downstream adaptive immune responses.

Recently, Yoon et al. [11] published the crystal structure of the ectodomain of TLR5 in complex with flagellin at 2.47 Å. The 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. In the crystal structure, flagellin binds to TLR5 predominantly through its D1 domain and forms a 1:1 heterodimer with TLR5 and further assembles into a 2:2 tail-to-tail signaling complex which is a key event to initiate signal transduction. In this process, the D2 domain of flagellin undergoes significant conformational changes to adapt into a dimer configuration. The D3 domain then repositions itself relative to the D1 as a result of D2’s conformational change, even though D3 could not be clearly defined in the electron density map. Since a D2 conformational change is necessary for dimer formation and TLR5 signaling, it becomes clear that the relative orientation, the overall surface chemistry and the position of an inserted antigen in the hinge region of flagellin are critical. More specifically, for the R3 format, the size and the position of the antigen could greatly impact dimer formation and consequently, TLR5 signaling. Preliminary EM images of an R3 format of our influenza B vaccine suggest the molecules are folded over rather than extended as compared to influenza A vaccines of the same format (Andrew Ward, Scripps, personal communication). Such a conformation could create a barrier to 2:2 heterodimer formation and subsequent signaling.

While the addition of negatively charged residues between the HA head and flagellin appeared to reduce such interactions, TLR5 signaling and immunogenicity remained relatively low. Since the conformational change during dimerization centers on the D2 domain of flagellin, moving the globular head further away from the D2 domain by inserting the antigen into the D3 domain may provide more room and flexibility for the antigen moiety to adapt to the dimer conformation. Interestingly, the insertion of the antigen on the sides of D3 rather than at the tip of D3 provided the largest rescue of TLR5 activity and immunogenicity. These results suggest that it is possible that the tip insertion is too extended and that the side insertions create a more compact molecule that better facilitates the conformational change, resulting in more active TLR5 signaling.

5. Conclusion

In 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. Further, we have shown that the redesigned format maximizes TLR5 signaling and immunogenicity for multiple influenza B strains. Finally, similar to the A strain vaccines, the 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.

Conflict of interests

All the authors are employees of VaxImmune Corporation.
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References