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# Identification of immunodominant CD8 epitope in the stalk domain of influenza B viral hemagglutinin

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ABSTRACT

Human infections by type B influenza virus constitute about 25% of all influenza cases. The viral hemagglutinin is comprised of two subunits, HA1 and HA2. While HA1 is constantly evolving in an unpredictable fashion, the HA2 subunit is highly conserved, making it a potential candidate for a universal vaccine. However, immunodominant epitopes in the HA2 subunit remain largely unknown. To delineate MHC Class I epitopes, we first identified 9-mer H-2K$^{d}$-restricted CD8 T cell epitopes in the HA2 domain by *in silico* analyses, followed by evaluating the immunodominance of these peptides in mice challenged with the virus. Of three peptides selected through *in silico* analysis, the universally conserved peptide, YYSTAASSL (B/HA2-190), possessed the highest predicted binding affinity to MHC Class I and was most effective in inducing IL-2 and TNF-α in mouse splenocytes. Importantly, the peptide demonstrated best capability of stimulating peptide-specific *ex-vivo* cytotoxicity against target cells. Taken together, this finding would be of value for assessment of cell-mediated immune responses elicited by vaccines based on the highly conserved HA2 stalk domain.



## 1. Introduction

Influenza B virus (IBV) on average causes 20–30% of all influenza cases but it can be the dominant strain in a given flu season [1] [2] [3] [4] [5] [6] [7] [8]. Of particular concern is the increasing influenza B-related mortality rate among infants and children under the age of 10 [9]. During the 2010–2011 epidemic, 25% of all influenza cases were caused by IBV with 38% of all pediatric deaths [5] [10], [11] [12], [13]. Furthermore, in 2017-18 flu season 46.8% of all reported cases worldwide were positive for influenza A whereas 53.2% were positive for influenza B with more severe symptoms [14]. These data indicate that efforts should be strengthened to prevent and contain IBV infections.

The most effective means against influenza is annual vaccination of susceptible populations. The current seasonal influenza vaccines are produced using the strains recommended by the World Health Organization (WHO) 6–8 months ahead of the targeted season [15]. However, there are inherent disadvantages associated with the preparation of conventional influenza vaccines such as the uncertainty of the actual circulating strains, the need for annual updating of the manufacturing process. Furthermore, mismatches between the strains selected for vaccine preparation and the circulating viruses can cause a marked reduction in the efficacy of seasonal influenza A/B vaccines [4] [16] [17].

IBV is broadly classified into two genetic lineages, i.e. Victoria and Yamagata; these two antigenically different groups of virus are known to co-circulate within the human population [16] [6] [9] [18]. It is noted that vaccine effectiveness in the 2017-18 flu season in the United States was estimated to be only 42% against influenza B viruses [19]. All these problems concerning the influenza vaccines are largely due to the frequent mutations of the virus surface proteins, particularly the hemagglutinin (HA) [20]. The mutations

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often take place in the HA1 subunit of the HA protein in both IAV and IBV, resulting in highly variable antigenicity of the HA protein whereas the other subunit, HA2, is highly conserved among all strains analyzed [21].

Viral infection or vaccination mainly induce antibodies targeting the variable HA1 subunit (head) whereas the HA2 subunit (stalk) is not the primary target of neutralizing antibodies, given that the latter is largely shielded by the head [22] [23]. Moreover, while antibodies targeting the stalk of HA can be broadly protective since it is highly conserved [1] [24], the role of cell mediated immune responses targeting the stalk region is not well studied, particularly in response to IBV infection or vaccination. In this study, we used *in silico* analyses to predict candidate epitopes in the HA2 stalk region of IBV and subsequently validated their immunodominance using H-2K$^d$-restricted mice.

## 2. Materials and methods

The detailed methods have been presented as supplementary information.

### 2.1. Bioinformatics and statistical analyses

H-2K$^d$-restricted MHC class I binding predictions was performed using syfpeithi. de [25], NeTMHC3.4, and Immune Epitope Database (IEDB) Analysis Resource [26] [27] [28] [29] [30]. Class I immunogenicity was predicted using IEDB [31]. The selected peptides were synthesized with over 95% purity (New England Peptides, Cambridge MA). Influenza B hemagglutinin sequences from 3488 strains in the Victoria lineage and 4572 strains in the Yamagata lineage deposited in public domains (NCBI Influenza Virus Resource Database) were retrieved for multiple gene alignment. They were aligned using Geneious 7.0.6 software to determine the universality of the selected peptides. For statistical analyses, all comparisons were conducted using one-way ANOVA with Bonferroni post-test. They were performed using GraphPad Prism 5.

### 2.2. Cell lines, virus and animal infection

Virus was grown in embryonated chicken eggs with titration determined with MDCK cells [1]. Twelve-to fourteen-week-old female DBA.2 mice (Jackson Laboratories) were intranasally infected with $1.5 \times 10^4$ PFU (10x $LD_{50}$) in 25 μl per mouse. Mice were monitored on a daily basis. Plaque assay was done as previously described [32].

### 2.3. Histology

Lungs from DBA.2 mice 3 days post infection were formalin-fixed for pathological analysis as described [33]. Cell infiltration refers to infiltrates of the predominant inflammatory cells in the perivascular stroma [34].

### 2.4. CD8$^+$ T cell intracellular cytokine staining

CD8$^+$ T cell IFN-γ, TNF-α, and IL-2 responses were evaluated 3 days post infection as previously described [32] in DBA.2 mice. In brief, splenocytes were cultured in RPMI 1640 medium with 10% FBS in the presence of 10 μg/ml synthetic H-2K$^d$ MHC class I–restricted peptides for *ex vivo* re-stimulation for 6 h. Stimulated cells ($2 \times 10^6$) were stained with a fixable viability dye (eBiosciences) for 30 min followed by a Fc block (eBiosciences) for 5 min and then a FITC–conjugated anti-mouse CD8a (clone 53–6.7; BD biosciences) for 30 min. Cells were then permeabilized with Cytofix/Cytoperm (BD biosciences) for 20 min and then stained with BV786–conjugated anti-mouse IFN-γ (clone XMG1.2; BD biosciences), PE-conjugated anti-mouse TNF-α (clone MP6-XT22; BD biosciences), and BV421–conjugated anti-mouse IL-2 (clone JES6-5H4; BD biosciences). Results for IFN-γ, TNF-α, and IL-2 were calculated as a percentage of CD8$^+$ T cells corrected using a control without peptide.

### 2.5. Cytotoxic T lymphocyte (CTL) assay

CTL assay was conducted using lactate dehydrogenase release assay as previously described [32]. In brief, $2 \times 10^7$ splenocytes from infected DBA.2 mice were collected 3 days post infection. They were re-stimulated *ex vivo* with 1 μg/ml peptide to generate effector CTLs. Five days later, cytotoxic activity was measured by lactate dehydrogenase release using peptide-pulsed P815 targets (H-2d). The percentage of cytotoxicity was calculated as (experimental release - effector spontaneous release - target spontaneous release)/(maximum release - target spontaneous release) x 100% and corrected with no peptide controls.

## 3. Results and discussion

### 3.1. H-2K$^d$-restricted epitope prediction in silico

The highly conserved HA2 subunit of Influenza B/Victoria/2/87 was subjected to *in silico* analyses using three different online MHC class I binding prediction programs, NeTMHC3.4, syfpeithi. de and IEDB Analysis Resource (Supp Table S1) [35]. A snapshot of the outputs from each program consisting of 9mer peptides is summarized in Supp Table S1 in descending order of MHC I binding affinity. All three programs predicted the same two peptides to have the highest binding affinity to H-2K$^d$-restricted MHC Class I, YYSTAASSL (B/HA2-190) and VYMVSRDNV (B/HA2-209). Some variation in the ordering based on binding affinity was observed between prediction tools from the third position (Supp Table S1).

We also used IEDB Analysis Resource to determine the immunogenicity of given peptides [31] [35]. The peptides predicted to have high affinity were analyzed. The strongest binding peptides were predicted not to have the highest immunogenicity (Supp Table S2). Specifically, the peptide with the highest binding affinity, B/HA2-190, had a low immunogenicity score. Therefore, for analysis of universality, two peptides predicted to have the highest binding affinity, B/HA2-190 and B/HA2-209, and 2 peptides predicted to have the highest immunogenicity, TFNAGEFSL (B/HA2-156) and STQEAINKI (B/HA2-40), were selected.

Three of the predicted epitopes are well conserved in majority of influenza B strains in both lineages.

To determine if the selected peptides were universally conserved, hemagglutinin sequence from 3488 influenza B strains from the Victoria lineage and 4572 strains from the Yamagata lineage were analyzed. We found that all amino acids in peptides B/HA2-190, B/HA2-40 and B/HA2-209 are conserved in over 98% of strains from both genetic lineages (Fig. 1). However, in B/HA2-156, all except one amino acid are conserved in above 99% of the strains. The amino acid in the third position, asparagine (N), is conserved in 99.4% of the Yamagata strains but only conserved in 2.1% of the Victoria strains (Fig. S1). Therefore, B/HA2-156 was not validated further in the *in vivo* model.

### 3.2. Confirming in vivo model for IBV infection

As the *in silico* algorithms can vary in their accuracy and the degree to which the predictions translate to biological systems, it is necessary to validate the predicted peptides for their immunological activities by *in vitro* or *in vivo* experiments [35]. To this end, a

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