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H5N1 Strain-Specific Hemagglutinin CD4+ T cell Epitopes Restricted by HLA DR4

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Abstract

CD4+ T cells play a pivotal role in the viral immunity, and as such identification of unique strain specific HLA class II restricted epitopes is essential for monitoring cellular strain specific viral immunity. Using Tetramer-Guided Epitope Mapping technique, we identified HLA-DR0401 restricted HA epitopes that are strain-specific to H5N1 virion. Two immunodominant epitopes H5HA₄₄₁₋₄₆₀ and H5HA₅₇₋₇₆ were identified from in vitro stimulated human PBMC. Both epitopes elicit strong cellular immune responses when HLA-DR0401 transgenic mice are immunized with H5N1 subvirion indicating in vivo naturally processed immunodominant epitopes. The H5HA₅₇₋₇₆ epitope is unique for the H5N1 strain but conserved among all H5N1 clades recommended for vaccine development by World Health Organization. The unique H5HA₅₇₋₇₆ response was uncommon in unexposed individuals and only observed in the naïve T cell subset. Thus, H5N1 strain-specific H5HA₅₇₋₇₆ immunogenic epitope represents a unique marker for monitoring the efficacy of vaccination or as a candidate vaccine peptide.

Keywords

Avian Influenza; CD4+ T cell; T cell epitope

1.0 Introduction

In recent years, highly pathogenic influenza A H5N1 viruses have caused disease outbreaks in domestic poultry and wild birds, with sporadic human cases emerging in more than a dozen nations with approximately 60% mortality (243/385 death till June of 2008) [1–7]. Although to date no H5 strains have circulated in the human population, continued evolution of H5N1 viruses and the clustering of human infections have raised serious concerns that more virulent strains of H5N1 could emerge with efficacious human-to-human transmission potentially resulting in a catastrophic human pandemic[8–10].

Influenza A is an enveloped virus composed of Hemagglutinin (HA) and Neuraminidase (NA) surface glycoproteins. HA is involved in the viral attachment to and entry into host cells [11]. HA is synthesized as an HA₀ precursor and cleaved into HA₁ and HA₂ subunits by host proteases [12–14]. The HA₁ subunit mediates viral receptor binding to 2,3-linked sialic acid

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sugars on the surface of avian epithelial cells and 2,6-linked sialic acid on human cells. The other cleaved subunit (HA₂) mediates membrane fusion and entry into the host cell cytoplasm for the initiation of the virus life cycle [12–14]. Because of HA's importance in viral infection and initiation of the viral life cycle, humoral immunity to HA, especially the HA₁ subunit correlates with protection against influenza A [15,16]. Due to host immunological pressures, HA (and hence the HA₁ subunit) is a sequence diversified viral antigen [13,15–18]. Based on distinct serological antigenicity, the HA antigen has been classified into 16 types [12,15,19]. The homology of H5HA from human cases to H1, H2, and H3 HA expressed in current circulating strains of human influenza A, ranges from 40% to 70% identities in the order of H3HA, H1HA and H2HA [20,21]. Because of these HA sequence variations and the importance of HA in viral entry into the host cell, humoral and cellular immunity against one strain does not always afford a strong crossover protection to other strains.

CD4⁺ T cells play a central role in mediating adaptive anti-viral immunity [12,15,22,23]. They promote B cell differentiation into plasma cells to produce neutralizing antibodies, assist memory B cells for a swift recall response to re-infection [16,18,24], promote the optimum expansion of cytotoxic CD8⁺ T cells to clear the intracellular infection and the maintenance CD8⁺ T cell memory [16,18,25–28], communicate with innate immune cells such as macrophages and dendritic cells to regulate the type of adaptive immune responses by secretion of cytokines [12]. CD4⁺ T cells can themselves act as antiviral effector cells either by killing infected cells directly or by secretion of antiviral cytokines, such as IFN- γ and TNF- α [29, 30], and can become memory T helper cells [16,18,22]. The importance of CD4⁺ T cells in viral immunity is further underscored in that mice lacking CD4 T cells have an impaired or delayed ability to clear influenza infection [18,23,31]. However, few influenza A specific studies have been carried out to delineate the role CD4⁺ T cells play in influenza specific immunity, presumably because of a lack of knowledge of strain-specific CD4⁺ T cell epitopes and proper tools such as HLA class II tetramers for tracking the antigen-specific T cells [23].

This study is aimed to define antigenic CD4⁺ T cell epitopes contained in the H5HA protein restricted on HLA DR0401 allele prior to the epidemic. The DR0401 allele is a prevalent allele in European and North American populations (allele frequencies of 0.1 and 0.089 respectively) but is also observed as a minor allele in North-East Asia, South-West Asia, Sub-Saharan Africa, Australia, and North Africa [32]. Identifying these CD4⁺ T cell epitopes, especially H5N1 virion specific T cell epitopes, should provide important assistance to study mechanisms of pathogenesis, generation of protective immunity, tracking antigen exposure history, and instruction of vaccine development and evaluation of efficacy of vaccination.

2.0 Materials and Methods

2.1 Fluorescent Reagents

The following fluorescent reagents were used: anti-human CD3-FITC, CD3-Allophycocyanin (APC), CD25-APC, CD45RA-FITC (eBioscience, San Diego, CA), CD4-PerCP, CD4-PerCP-Cy5.5 (BD Biosciences, San Jose, CA), and streptavidin-R-PE (Biosource International, Camarillo, CA).

2.2 H5HA protein and Peptides

Recombinant H5HA protein and H5N1 subvirion from Influenza A/Vietnam/1203/04 (H5N1) was provided by NIH Biodefense and Emerging Infections Research Resource Repository (BEI Resources, Manassas, VA). A panel of 70 consecutive overlapping peptides covering the 568 aa of H5N1 HA protein (accession number AAW80717) were chemically synthesized (Mimotope, Clayton, Victoria, Australia) (Table 1). The peptides were 20 aa in length sharing 12 aa overlapping with adjacent peptides. Individual peptides were dissolved in Dimethyl

sulfoxide at 10 mg/ml. Five consecutive peptides were mixed at 2 mg/ml of each as peptide pools for T cell stimulation and tetramer loading. A total of 14 peptide pools encompassed the whole protein. Additional peptides, H1HA₅₈₋₇₇, LCLKGIAPLQLGNCSVAGW, H2HA₅₆₋₇₅, LCKLNGIPPELGDCSIAGW, H3HA₅₁₋₆₉, LCDSPHQILDGENCTLIDA, and H1HA₄₃₈₋₄₅₇, NAELLVLENERLTDFHDSN, were also generated.

2.3 CD4 T cells preparation and stimulation

Fresh blood samples were obtained from 10 local healthy donors carrying single HLA-DRA1*0101/DRB1*0401 (DR0401) allele with written consent. These donors had no history of wild bird exposure. Peripheral blood mononuclear cells (PBMC) were isolated from 150 ml of heparinized peripheral blood. CD4⁺CD25[−] T cells were enriched by auto-MACS using a “no touch” CD4⁺ cell isolation kit plus CD25 positive selection kit (Miltenyi Biotec, Auburn, CA). Enriched T cells were over 70% CD4⁺ and contained about 2.5% CD14⁺ monocytes. Cells were suspended in human T cell medium (RPMI 1640 with 10% pooled human serum), seeded in 48-well plates at 2.5×10^6 cells/well (in 1.0 ml) and stimulated with peptide pools containing 5 peptides each at 2 µg/ml. Starting at day 7, cells were split into two wells and fed with fresh human T cell medium containing 20 U/ml of human IL-2 (Hemagen, Columbia, MA) and were re-fed with medium and IL-2 every 2 to 3 days.

2.4 Tetramer expression, preparation and Tetramer-Guided Epitope Mapping (TGEM)

HLA-DR0401 monomer was expressed, purified and biotinylated as described previously [33,34]. Tetramers for screening peptide pools and mapping individual epitopes were generated and stained as previously described [33,34].

For phenotypic analysis of H5N1 specific T cells, enriched CD4⁺ T cells were labeled with anti-CD45RA-FITC, CD4-PerCP-Cy5.5 and CD3-APC, and sorted into CD3⁺CD4⁺CD45RA⁺ (phenotype of naïve T cells) and CD3⁺CD4⁺CD45RA[−] (phenotype of memory T cells) fraction by cell sorter (FACSVantage). Sorted CD3⁺CD4⁺CD45RA⁺ and CD3⁺CD4⁺CD45RA[−] T cells were seeded (2.5×10^6) in individual wells into 48-well plate wells which were pre-coated for 2 hours with autologous adherent cells and washed with media. Adherent cells and sorted CD4⁺ T cells were stimulated with H5HA peptides and cultured for 14 days and assayed with tetramer staining.

2.5 T cell lines and protein stimulation proliferation assays

H5HA specific T cell lines were grown from tetramer positive T cells sorted with a FACS Vantage and expanded in 48-well plate in the presence of 2.5×10^6 irradiated (5000 rads, Cs-137 source) allogeneic PBMC and 2 µg/ml phytohemagglutinin (PHA, Remel Inc. Lenexa, KS). 16 days after expansion, T cells were stained with tetramer to evaluate the tetramer specificity of cloned T cell lines. T cell lines with more than 70% tetramer positive were used in protein stimulation assay.

For protein stimulation proliferation assays, tetramer sorted T cell lines were stimulated with irradiated HLA-DR0401 positive monocytes primed with H5HA recombinant protein. Briefly, HLA-DR0401 positive monocytes were enriched from 150×10^6 PBMC with anti-CD14-microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturer's instruction. 20×10^6 CD14⁺ monocytes were resuspended in 100 µg/ml of H5HA protein in T cell culture medium in a total volume of 100 µl at 37°C for 2–3 hours. The protein-primed and non-primed monocytes were irradiated, washed, resuspended and mixed at ratios of 1:0, 1:5, 1:25 and 0:1, and seeded in round-bottom 96-well plate wells at 10^5 cells/well with 1:1 T cell to monocyte ratio. 48 hours after stimulation, 1 µCi of ³H-thymidine (Amersham Biosciences, Piscataway, NJ) was added to each well. At 72 hours cells were harvested on Harvester 96 Mach III M

(Tomtec, Hamden, CT) and the incorporations of ^3H -Thymidine were read on Microbeta Trilux scintillation counter (Perkin Elmer, Shelton, CT).

2.6 H5HA protein and H5N1 subvirion immunization of HLA-DR0401 transgenic mice

I-Ab^{0/0} DR0401-IE mice (7–8 week old) (Taconic Farms, Hudson, NY) were immunized subcutaneously at the base of the tail with either 20 μg H5HA protein or 5 μg H5N1 subvirion vaccine in 100 μl 50% PBS/Complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Control mice were immunized with 50% PBS/Complete Freund's adjuvant only. Each group comprised 3 mice. On day 10, mice were boosted with 100 μl containing either 20 μg H5HA protein or 5 μg H5HA subvirion vaccine in 50% PBS/Incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). Control mice were boosted with 100 μl 50% PBS/Incomplete Freund's adjuvant. Spleens were harvested from mice on day 21 and single cell suspended by gently pressing through a 0.45 μm filter in Hank's buffered salt solution. Mouse red blood cells were lysed using ACK lysis buffer.

For recall proliferation assays, 0.5×10^6 splenocytes were cultured with peptides in 96 well round bottom plate wells in a volume of 100 μl . DMEM-10 (DMEM containing 100 $\mu\text{g}/\text{ml}$ Penicillin, 100U/ml Streptomycin, 2 mM glutamine, 1 mM Na-Pyruvate, 50 mM β 2-Mercaptoethanol, and 10% FBS). At 72 hours 1 μCi of ^3H -thymidine was added to plates. After overnight culture, T cell proliferation was assayed by scintillation counting as described above. All animal work was approved by the Benaroya Research Institute Institutional Animal Care and Use Committee and animals were housed in the BRI AAALAC-accredited Specific Pathogen Free animal facility.

2.7 Protein sequence homology analysis

Sequence homology analysis of H5HA (accession #: AAW80717), H1HA (accession #: CAC86622), H2HA (accession #: AAA64366), and H3HA (accession #: ABO37490) was performed by the Lipman-Pearson method using DNASTar software (<http://www.dnastar.com/>).

3.0 Results

3.1 Identification of antigenic epitopes in H5HA antigen

We used the TGEM approach [33,34] to identify CD4+ T cell epitopes within the H5HA protein of Influenza A/Vietnam/1203/04 (H5N1) strain restricted by HLA-DR0401. CD4+ enriched PBMC responder cells were stimulated with 14 peptide pools (5 peptides per pool) encompassing H5HA protein. Peptides encompassing the H5HA protein are listed in Table 1. The outgrowth of peptide responsive T cells was detected with HLA-DR0401 tetramers loaded with peptide pools as described in Materials and Methods. Representative tetramer staining results from one subject (subject 300) are shown in Figure 1. Positive staining was observed for peptide pools #1, #4, #10 and #12 for DR0401 (Figure 1A). The positive staining observed in pool #14 likely resulted from non-specific tetramer staining as both CD4+ and CD4- cells were stained at a similar ratio by the DR0401/pool#14 tetramer. Positive tetramer staining results in pool #2 and #5 were also detected in other DR0401 subjects (data not shown). Cells from tetramer positive wells were subjected to a second round of tetramer staining with DR0401 tetramers loaded with individual peptides from their corresponding peptide pool. The positive tetramer staining results for the single-peptide loaded tetramers are summarized in Figure 1B. H5HA₁₇₋₃₆ (H5p33 from pool #1), H5HA₅₇₋₇₆ (H5p38 from pool#2), H5HA₁₂₁₋₁₄₀ (H5p46 from pool #4), H5HA₁₆₉₋₁₈₈ (H5p52 from pool #5), H5HA₃₇₇₋₃₉₆ (H5p78 from pool #10) and H5HA₄₄₁₋₄₆₀ (H5p86 from pool #12) were identified as antigenic epitopes. Table 2 summarizes the frequency of epitopes identified in five subjects. Epitope

specific T cells for H5HA₄₄₁₋₄₆₀ were detected in all subjects while other epitopes were only detected in one or two subjects (Table 2).

3.2 Determining naturally processed epitopes in H5HA protein

To evaluate how many epitopes identified with synthetic peptides could be processed and presented by antigen-presenting cells from whole protein; tetramer positive T cells for these epitopes were sorted out, expanded as cell lines and stimulated with recombinant H5HA protein with freshly isolated CD14⁺ monocytes as antigen presenting cells. As shown in Figure 2, 5 out of 6 epitopes: H5HA₁₇₋₃₆, H5HA₅₇₋₇₆, H5HA₁₂₁₋₁₄₀, H5HA₃₇₇₋₃₉₆ and H5HA₄₄₁₋₄₆₀, showed a dose dependent response to stimulation by antigen presenting cells loaded with recombinant H5HA protein. One epitope, H5HA₁₆₉₋₁₈₈ did not show a dosage dependent response and served as a negative control for the specificity of assay. This epitope appears to antigenic but is likely not naturally processed in this assay. Other non-specific T cells lines were also used as negative controls (data not shown). Therefore, it seemed that majority of MHC II epitopes identified by TGEM represent naturally processed epitopes.

3.3 Determination of immunodominant epitopes in vitro and in vivo

To determine immunodominant epitopes, primary CD4⁺ T cells were in vitro stimulated with 100µg/ml of recombinant H5HA protein at 37°C for 150 minutes in the presence of antigen presenting cells. After 14 days in vitro expansion, the cells were detected with tetramers loaded with peptides identified from the previous experiments. As shown in Figure 3A, antigen-specific T cells for only two epitopes, H5HA₅₇₋₇₆ and H5HA₄₄₁₋₄₆₀, were clearly detectable. H5HA₅₇₋₇₆ specific T cells were detected in 2 out of 4 subjects and H5HA₄₄₁₋₄₆₀ specific cells were detected in all 4 of the subjects tested.

To further confirm that the immunodominant H5HA protein epitopes identified by in vitro protein stimulation and tetramer staining are indeed naturally processed epitopes in an in vivo immune response, I-Ab^{0/0} HLA-DR0401 transgenic mice were immunized with either recombinant H5HA protein or H5N1 subvirion. These mice have been shown to express robust levels of human DR0401 protein (data not shown). After primary and boost immunizations, splenocytes were stimulated with H5HA peptides, H5HA₁₇₋₃₆, H5HA₅₇₋₇₆, H5HA₁₂₁₋₁₄₀, H5HA₁₆₉₋₁₈₈, H5HA₃₇₇₋₃₉₆, and H5HA₄₄₁₋₄₆₀, to induce a recall response. As shown in Figure 3B, both H5HA₅₇₋₇₆ and H5HA₄₄₁₋₄₆₀ peptides induced a clear recall response in H5N1 subvirion immunized mice but not from control immunized mice. The recall response to H5HA₅₇₋₇₆ was seen in 3 out of 3 immunized mice, while recall response to H5HA₄₄₁₋₄₆₀ was seen in 2 out of 3 immunized mice. Similar immunodominant recall responses were observed in an identical experiment on DR0401 mice immunized with H5HA protein (data not shown). Other epitopes H5HA₁₂₁₋₁₄₀ and H5HA₃₇₇₋₃₉₆ also showed a recall response but at a much lower magnitude. The results of the in vitro and in vivo methods indicate that both H5HA₅₇₋₇₆ and H5HA₄₄₁₋₄₆₀ peptides correspond to naturally processed and immunodominant epitopes.

3.4 H5HA₅₇₋₇₆ epitope is a unique epitope for H5N1 while H5HA₄₄₁₋₄₆₀ is a cross-reactive epitope for other Influenza A strains

Since the subjects recruited in this study have no known exposure history to H5N1 avian flu virus, we were curious about the phenotypes of the H5HA reactive T cells, especially for these potentially immunodominant epitopes. To address this question, CD4⁺ T cells from healthy donors were sorted into CD4⁺CD45RA⁺ (mostly comprised by naïve CD4⁺ T cells) and CD4⁺CD45RA⁻ (mostly comprised by memory CD4⁺ T cells) fractions and stimulated with H5HA₁₇₋₃₆, H5HA₅₇₋₇₆, H5HA₁₂₁₋₁₄₀ and H5HA₄₄₁₋₄₆₀ peptides, respectively. 14 days after expansion, the presence of epitope specific T cells were detected by tetramer staining. As shown in Figure 4, H5HA₁₇₋₃₆, H5HA₅₇₋₇₆ and H5HA₁₂₁₋₁₄₀ specific T cells were only detectable in

CD45RA⁺ fraction, not in CD45RA⁻ fraction. In contrast, H5HA₄₄₁₋₄₆₀ specific T cells were detected in both fractions (Figure 4). Similar results were observed in a second subject (data not shown). These results inferred that T cells specific for H5HA₁₇₋₃₆, H5HA₅₇₋₇₆ and H5HA₁₂₁₋₁₄₀ were predominantly contained in naïve T cell pool, while H5HA₄₄₁₋₄₆₀ specific T cells were present in both naïve and memory pools. We suspected that the H5HA₄₄₁₋₄₆₀ specific T cells present in the memory pool were cross reactive T cells also responsive to other influenza A subtypes (for example H1HA protein which is one of the dominant subtypes included in the Flu Vaccines of recent years) because there is only one amino acid difference (H5HA_{448M} vs. H1HA_{445L}) between H5HA and H1HA in H5HA₄₄₁₋₄₆₀ region. To investigate this possibility, we stimulated CD4⁺ T cells with either H5HA₄₄₁₋₄₆₀ or H1HA₄₃₈₋₄₅₇ (the corresponding region to H5HA H5HA₄₄₁₋₄₆₀) peptide, and detected with DR0401/H5HA₄₄₁₋₄₆₀ and DR0401/H1HA₄₃₈₋₄₅₇ tetramers for each stimulation condition. As shown in Figure 5A, we were able to detect DR0401/H5HA₄₄₁₋₄₆₀ tetramer positive cells from CD4⁺ T cells stimulated with H1HA₄₃₈₋₄₅₇ peptide, or vice versa. In contrast, H5HA₅₇₋₇₆ is a unique immunodominant epitope for H5HA antigen. Indeed, sequence homology analysis revealed that the H5HA₅₇₋₇₆ sequence region has seven amino acid differences to corresponding regions in H1HA and H2HA, and no homology to H3HA (Figure 5B) from human influenza A H1N1, H2N2 and H3N2 subtypes. The differences are significant enough to disrupt the T cell cross reactivity. As shown in Figure 5C, cloned H5HA₅₇₋₇₆ specific T cells responded to H5HA₅₇₋₇₆ stimulation robustly but not to the H1HA and H3HA stimulation (Figure 5C). Taking together, these data indicate that H5HA₄₄₁₋₄₆₀ is a cross-reactive epitope, H5HA₅₇₋₇₆ is a unique, novel immunodominant epitope specific for H5HA antigen but not H1HA, H2HA or H3HA.

4.0 Discussion

Newly emerging and re-emerging infectious diseases pose a continuous threat to the health of our society. Studies of host immune responses against these microbes provide insights on both the pathogenic mechanisms of the organisms and new approaches in vaccine development. Identification of immunodominant T cell epitopes within the infectious organism is one of the key initial steps that are essential for understanding host cellular immune responses. Since CD4⁺ T cells play a central role in the regulation of the adaptive immune response against viruses, it is particularly important to identify HLA-class II restricted epitopes for studying cellular immunity to influenza A. In this study, we applied TGEM technology to identify H5HA specific antigenic epitopes prior to a potential H5N1 epidemic-pandemic shift.

An inherent caveat in the TGEM approach is that identified T cell responding epitopes by tetramer staining may not be naturally processed. We were pleased to see that at least 4 out of 6 epitopes we identified are naturally processed from whole protein by both in vitro and in vivo methods. It should be mentioned that, while our results indicate that these 4 H5HA peptides correspond to naturally processed epitopes, the precise length of the naturally processed protein fragment could differ from those of the synthetic peptides. Although TGEM is a synthetic peptide based assay system, the HLA class II restricted epitopes identified by TGEM were highly correlated to naturally processed epitopes. This may reflect that epitopes identified by TGEM technique have high affinity to ‘empty’ HLA class II molecules, because tetramer preparation relies on the affinity of peptide loading onto ‘empty’ HLA class II monomer. Peptides with higher affinity compete better to bind in HLA class II and therefore have a better chance to be presented on cell surface in vivo. These aspects of the tetramer methodology lead to a second caveat in the TGEM approach. Because the assay relies on high affinity peptide binding, the assay may fail to detect low affinity peptides that are legitimate epitopes. Our cumulative results over the past several years indicate that the TGEM method detects epitopes with binding affinities as low as 10–20 μ M. Therefore, it is possible that there are additional

low affinity epitopes (below 10–20 μ M) or epitopes with low naïve frequencies within H5HA. These epitopes could play an important role in natural immunity.

Influenza A virus has 3 surface proteins (HA, NA and M2) and 8 internal proteins (NP, PA, PB1, PB2, M1, NS1, NS2 and PB1-F2) [15,16]. The internal proteins and MP2 are relatively conserved between viral subtypes. Therefore, cellular immunity against epitopes derived from these proteins are often cross reactive because the epitopes are actually identical or only slightly different among different Influenza A viral strains [35,36]. Since the surface glycoprotein HA is subject to antigenic shift and drift due to host immunological defense pressures, novel HA T cell epitopes are constantly emerging from most new influenza A strains. Identification of H5N1 unique, specific, immunodominant CD4 T cell epitopes is of particular interest for 1) tracking viral strain specific T cell immunity, 2) evaluation of strain specific vaccine efficacy, and 3) development of peptide-based strain specific vaccines. In addition, some studies have suggested that antibody-producing B cells may favor T-cell help towards T cells recognizing the same protein antigen toward which the antibody is produced to bind to [37] [38]. Therefore, CD4+ T cell immunity against H5HA may be best suited to provide help to H5HA specific B cells in antibody production.

In this study, we identified two immunodominant epitopes, H5HA₅₇₋₇₆ and H5HA₄₄₁₋₄₆₀ (Figure 3A and B). The H5HA₅₇₋₇₆ epitope is a novel strain specific epitope that has very little homology to other human tropic influenza strains (Figure 5B). T cells specific to the H5HA₅₇₋₇₆ epitope were only found in the naïve (CD45RA+) T cell population (Figure 4). However despite its uniqueness among influenza strains, H5HA₅₇₋₇₆ epitope is a highly conserved among H5HA clades or subclades. Actually, it is completely identical in all candidate H5N1 vaccine reference viruses recommended by the World Health Organization [39]. Therefore, it could be a very useful T cell epitope to evaluate specific H5N1 vaccine efficacy. In addition, it could also be an important epitope as a peptide vaccine candidate. First, our in vitro experiment demonstrated that H5HA₅₇₋₇₆ epitope could be efficiently processed and presented by antigen-presenting cells (Figure 3A). Second, when HLA-DR0401 mice were immunized with H5N1 subvirion vaccine or H5HA recombinant protein, T cell reactivity towards H5HA₅₇₋₇₆ epitope was most frequent and strongest (Figure 3B). T cell immunity against H5HA₅₇₋₇₆ epitope was detected in 3 out of 3 H5N1 subvirion immunized mice. Third, epitope mapping data showed H5HA₅₇₋₇₆ epitope was only identified in 1 out of 5 subjects (Table 2) indicating that there is a lack of T cell immunity towards H5HA₅₇₋₇₆ epitope in the general HLA-DR0401+ population. Collectively, we conclude that H5HA₅₇₋₇₆ epitope could be a necessary and efficient epitope as a peptide based vaccine candidate.

In contrast to H5HA₅₇₋₇₆, sequence homology analysis revealed that the H5HA₄₄₁₋₄₆₀ epitope is highly conserved in HA proteins among H1N1 and H2N2 viral strains, two human tropic influenza subtypes (data not shown) [20,21]. Therefore, T cell responses to H5HA₄₄₁₋₄₆₀ epitope could result from a cross (Figure 5A) or a recall response from annual immunization of Influenza vaccine comprised of the H1N1 subtypes. Indeed, when CD4+ T were sorted into CD45RA+ (naïve phenotype) and CD45RA- (memory phenotype) fractions, H5HA₄₄₁₋₄₆₀ response T cells were clearly detected in CD45RA- T cell pool (Figure 4). Furthermore, T cell reactivity towards H5HA₄₄₁₋₄₆₀ epitope showed a consistently potent response throughout the epitope mapping experiments in all the subjects used in this study (Table 2). Again, like H5HA₅₇₋₇₆, H5HA₄₄₁₋₄₆₀ was confirmed as a naturally processed immunodominant epitope in immunized mice in vivo. Collectively, these data strongly argued that H5HA₄₄₁₋₄₆₀ is a cross-reactive epitope for H1N1, H2N2 and H5N1 strains. The strong H5HA₄₄₁₋₄₆₀ epitope specific reactivity is likely a record of vaccination or exposure outcome of current flu-vaccines.

Besides H5HA₅₇₋₇₆ and H5HA₄₄₁₋₄₆₀, the remaining 3 naturally processed epitopes: H5HA₁₇₋₃₆, H5HA₁₂₁₋₁₄₀ and H5HA₃₇₇₋₃₉₆, H5HA₁₂₁₋₁₄₀ and H5HA₃₇₇₋₃₉₆ showed only

moderate immunogenicity in the immunized transgenic mice (Figure 3B). Therefore, although these epitopes could be naturally processed by antigen-presenting cells, they may not be immunodominant epitopes.

In summary, our study indicated that 1) novel HLA class II restricted epitopes can emerge from a novel influenza antigen, i.e., H5HA antigen, 2) novel epitopes could be effectively identified by our TGEM technology, 3) T cells specific for these novel epitopes exist in antigen unexposed individuals in the naïve cell population, 4) T cell responses to these novel epitopes may be suboptimal in the unexposed population, 5) knowledge of epitopes could be used to further determine the immunodominant epitopes using a convenient in vitro protein stimulation and tetramer staining process, 6) immunodominant epitopes defined from this in vitro assay could fully mirror in vivo study results. We successfully identified two immunodominant H5HA epitopes, one is unique for H5N1 subtypes but conserved within H5N1 clades and subclades, the other is a cross-reactive epitope for multiple subtypes. The H5HA₅₇₋₇₆ immunodominant epitopes should be a valuable marker for tracking H5N1 specific immunity possible in the development of peptide-based vaccines.

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References

1. WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO.
http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_06_19/en/index.html
2. Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, et al. Avian influenza A (H5N1) infection in humans. *N Engl J Med* 2005;353(13):1374–85. [PubMed: 16192482]
3. de Jong MD, Hien TT. Avian influenza A (H5N1). *J Clin Virol* 2006;35(1):2–13. [PubMed: 16213784]
4. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza A virus in wild birds. *Science* 2006;312(5772):384–8. [PubMed: 16627734]
5. Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. *Emerg Infect Dis* 2006;12(1):3–8. [PubMed: 16494709]
6. Wong SS, Yuen KY. Avian influenza virus infections in humans. *Chest* 2006;129(1):156–68. [PubMed: 16424427]
7. Woo PC, Lau SK, Yuen KY. Infectious diseases emerging from Chinese wet-markets: zoonotic origins of severe respiratory viral infections. *Curr Opin Infect Dis* 2006;19(5):401–7. [PubMed: 16940861]
8. Fauci AS. Pandemic influenza threat and preparedness. *Emerg Infect Dis* 2006;12(1):73–7. [PubMed: 16494721]
9. Webby RJ, Webster RG. Are we ready for pandemic influenza? *Science* 2003;302(5650):1519–22. [PubMed: 14645836]
10. Webster RG, Govorkova EA. H5N1 influenza--continuing evolution and spread. *N Engl J Med* 2006;355(21):2174–7. [PubMed: 17124014]
11. Cross KJ, Burleigh LM, Steinhauer DA. Mechanisms of cell entry by influenza virus. *Expert Rev Mol Med* 2001;3(21):1–18. [PubMed: 14585145]
12. Lewis DB. Avian flu to human influenza. *Annu Rev Med* 2006;57:139–54. [PubMed: 16409141]
13. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* 2000;69:531–69. [PubMed: 10966468]
14. Steinhauer DA. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 1999;258(1):1–20. [PubMed: 10329563]

15. Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand J Immunol* 2004;59(1):1–15. [PubMed: 14723616]
16. Tamura S, Tanimoto T, Kurata T. Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. *Jpn J Infect Dis* 2005;58(4):195–207. [PubMed: 16116250]
17. Wilson IA, Cox NJ. Structural basis of immune recognition of influenza virus hemagglutinin. *Annu Rev Immunol* 1990;8:737–71. [PubMed: 2188678]
18. Tamura S, Kurata T. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn J Infect Dis* 2004;57(6):236–47. [PubMed: 15623947]
19. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* 2005;79(5):2814–22. [PubMed: 15709000]
20. Connor RJ, Kawaoka Y, Webster RG, Paulson JC. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 1994;205(1):17–23. [PubMed: 7975212]
21. Marozin S, Gregory V, Cameron K, Bennett M, Valette M, Aymard M, et al. Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. *J Gen Virol* 2002;83(Pt 4):735–45. [PubMed: 11907321]
22. Swain SL, Agrewala JN, Brown DM, Jelley-Gibbs DM, Golech S, Huston G, et al. CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol Rev* 2006;211:8–22. [PubMed: 16824113]
23. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 2006;12(1):48–54. [PubMed: 16494717]
24. Constant SL, Bottomly K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol* 1997;15:297–322. [PubMed: 9143690]
25. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 2003;421(6925):852–6. [PubMed: 12594515]
26. Ramsburg EA, Publicover JM, Coppock D, Rose JK. Requirement for CD4 T cell help in maintenance of memory CD8 T cell responses is epitope dependent. *J Immunol* 2007;178(10):6350–8. [PubMed: 17475864]
27. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 2003;300(5617):339–42. [PubMed: 12690202]
28. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol* 2004;5(9):927–33. [PubMed: 15300249]
29. Seo SH, Webster RG. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. *J Virol* 2002;76(3):1071–6. [PubMed: 11773383]
30. Swain SL, Dutton RW, Woodland DL. T cell responses to influenza virus infection: effector and memory cells. *Viral Immunol* 2004;17(2):197–209. [PubMed: 15279699]
31. Riberdy JM, Christensen JP, Branum K, Doherty PC. Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(–/–) mice. *J Virol* 2000;74(20):9762–5. [PubMed: 11000251]
32. Meyer, D.; Singe, RM.; Erlich, H.; Fernandez-Vina, M.; Thomson, G. Single Locus Polymorphism of Classical HLA Genes. In: Hansen, JA., editor. *Immunobiology of the Human MHC: Proceedings of the 13th International Histocompatibility Workshop and Conference*. Seattle, WA: IHWG Press; 2007. p. 653-704.
33. Yang J, Danke NA, Berger D, Reichstetter S, Reijonen H, Greenbaum C, et al. Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD4+ T cells in human subjects. *J Immunol* 2006;176(5):2781–9. [PubMed: 16493034]
34. Yang J, James EA, Huston L, Danke NA, Liu AW, Kwok WW. Multiplex mapping of CD4 T cell epitopes using class II tetramers. *Clin Immunol* 2006;120(1):21–32. [PubMed: 16677863]
35. Gioia C, Castilletti C, Tempestilli M, Piacentini P, Bordi L, Chiappini R, et al. Cross-subtype Immunity against Avian Influenza in Persons Recently Vaccinated for Influenza. *Emerg Infect Dis* 2008;14(1):121–8. [PubMed: 18258091]

36. Roti M, Yang J, Berger D, Huston L, James EA, Kwok WW. Healthy Human Subjects Have CD4+ T Cells Directed against H5N1 Influenza Virus. *J Immunol* 2008;180(3):1758–68. [PubMed: 18209073]
37. Sette A, Moutaftsi M, Moyron-Quiroz J, McCausland MM, Davies DH, Johnston RJ, et al. Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. *Immunity* 2008;28(6):847–58. [PubMed: 18549802]
38. Marshall D, Sealy R, Sangster M, Coleclough C. TH cells primed during influenza virus infection provide help for qualitatively distinct antibody responses to subsequent immunization. *J Immunol* 1999;163(9):4673–82. [PubMed: 10528164]
39. WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines.
http://www.who.int/csr/disease/avian_influenza/guidelines/summaryH520070403pdf

Figure 1.

Identification of HLA-DR0401 restricted H5HA antigenic epitopes by TGEM. (A) Pooled mapping results. All the FACS data come from subject 300. The pound symbol (#) followed by a number represents the peptide pool number. (B) Fine mapping results. A representative negative staining result is shown at the last panel of (B) as negative control of tetramer staining.

Figure 2.

Naturally processed H5HA CD4⁺ T cell epitopes. H5HA specific T cell lines were stimulated with mixture of recombinant H5HA protein primed or non-primed monocytes at 1:0, 1:5, 1:25 and 0:1 ratios. The response of epitope specific T cell lines to protein stimulation were evaluated by a proliferation assay based on the capacity of ^3H -Thymidine incorporation. The non responsiveness of H5HA₁₆₉₋₁₈₈ specific T cell line to the stimulation served as negative control of the assay.

Figure 3.

Identification of H5HA immunodominant epitopes. (A) Detection of antigen specific T cells following in vitro recombinant H5HA protein stimulation of enriched CD4⁺ T cells. Freshly isolated CD4⁺ T cells were stimulated and expanded in vitro with recombinant H5HA protein (100μg/ml). Amplification of epitope specific T cells was determined by tetramer staining at day 14. DR0401/H5p52 (H5HA₁₆₉₋₁₈₈) tetramer was expected as negative and served as negative control of the assay. (B) H5HA peptide recall response of splenocytes from DR0401 mice immunized with H5HA protein. Splenocytes were stimulated with H5HA peptides, H5HA₁₇₋₃₆, H5HA₅₇₋₇₆, H5HA₁₂₁₋₁₄₀, H5HA₃₇₇₋₃₉₆, H5HA₄₄₁₋₄₆₀, or a control peptide (IGRP₂₄₇₋₂₅₉) for a proliferation assay. Closed bars: H5N1 subvirion immunized mouse.

Opened bars: adjuvant immunized mouse. Student *t* test was used in the statistical analysis. *: $p < 0.05$; **: $p < 0.01$.

Figure 4.

CD45RA phenotype of H5HA specific T cells. FACS sorted CD4⁺CD45RA⁺ and CD4⁺CD45RA⁻ T cells were stimulated by H5HA peptides in the presence of antigen presenting cells. 14 days after stimulation, the presence of epitope specific T cells were detected by tetramer staining.

Figure 5.

Cross reactivity studies of H5HA₄₄₁₋₄₆₀ and H5HA₅₇₋₇₆ epitopes. (A) Cross staining of H5HA₄₄₁₋₄₆₀ and H1HA₄₃₈₋₄₅₇ peptide stimulated cells. Cells were stimulated with either H5HA₄₄₁₋₄₆₀ or H1HA₄₃₈₋₄₅₇ peptide and then stained with DR0401/H5HA₄₄₁₋₄₆₀ or DR0401/H1HA₄₃₈₋₄₅₇ tetramers. (B) Homology analysis of H5HA₅₇₋₇₆ with H1HA, H2HA and H3HA. '*' refers to an identical residue and '-' refers to a gap in homology. One letter amino acid codon was used. (C) Proliferation assay of an H5HA₅₇₋₇₆ specific T cell line in response to the H5HA₅₇₋₇₆, H1HA₅₆₋₅₇ and H3HA₅₁₋₆₉ peptides.

Table 1

Sequences of the H5HA peptide library. Bolded text identifies peptides identified as antigenic epitopes.

Name	Sequence	Amino acid
H5p31	MEKIVLLFAIVSLVKSDQIC	1-20
H5p32	AIVSLVKSDQICIGYHANN	9 - 28
H5p33	DQICIGYHANNSTEQVDTIM	17 - 36
H5p34	ANNSTEQVDTIMEKNVTVTH	25 - 44
H5p35	DTIMEKNVTVTHAQDILEKK	33 - 52
H5p36	TVTHAQDILEKKHNGKLCDL	41 - 60
H5p37	LEKKHNGKLCDLGDKPLIL	49 - 68
H5p38	LCDLDGVKPLILRDCSVAGW	57 - 76
H5p39	PLILRDCSVAGWLLGNPMCD	65 - 84
H5p40	VAGWLLGNPMCDDEFINVPEW	73 - 92
H5p41	PMCDDEFINVPEWSYIVEKAN	81 - 100
H5p42	VPEWSYIVEKANPVNDLCYP	89 - 108
H5p43	EKANPVNDLCYPGDFNDYEE	97 - 116
H5p44	LCYPGDFNDYEELKHLLSRI	105 - 124
H5p45	DYEELKHLLSRINHFELIKI	113 - 132
H5p46	LSRINHFELIKIIPKSSWS	121 - 140
H5p47	KIIPKSSWSSEASLGVS	129 - 148
H5p48	SWSSSEASLGVSACPYQKG	137 - 156
H5p49	LGVSSACPYQKGSSFFRNVV	145 - 164
H5p50	YQKGSSFFRNVVWLIKKNST	153 - 172
H5p51	RNVVWLIKKNSTYPTIKRSY	161 - 180
H5p52	KNSTYPTIKRSYNTNQEDL	169 - 188
H5p53	KRSYNTNQEDLLVLWGIHH	177 - 196
H5p54	QEDLLVLWGIHHPNDAAEQT	185 - 204
H5p55	GIHHPNDAAEQTKLYQNPTT	193 - 212
H5p56	AEQTKLYQNPTTYISVGTST	201 - 220
H5p57	NPTTYISVGTSTLNQRLVPR	209 - 228
H5p58	GTSTLNQRLVPRIATRSKVN	217 - 236
H5p59	LVPRIATRSKVNGQSGRMEF	225 - 244
H5p60	SKVNGQSGRMEFFWTILKPN	233 - 252
H5p61	RMEFFWTILKPNDAINFESN	241 - 260
H5p62	LKPNDAINFESNGNFIAPEY	249 - 268
H5p63	FESNGNFIAPEYAYKIVKKG	257 - 276
H5p60	APEYAYKIVKKGDSIMKSE	265 - 284
H5p65	VKKGDSIMKSELEYGNCNT	273 - 292
H5p66	MKSELEYGNCNTKCQTPMGA	281 - 300
H5p67	NCNTKCQTPMGAINSSMPFH	289 - 308
H5p68	PMGAINSSMPFHNHPLTIG	297 - 316
H5p69	MPFHNHPLTIGECPKYVKS	305 - 324
H5p70	LTIGECPKYVKSRLVLATG	313 - 332
H5p71	YVKSRLVLATGLRNSPQRE	321 - 340

Name	Sequence	Amino acid
H5p72	LATGLRNSPQRERRRKKRGL	329 - 348
H5p73	PQRERRRKKRGLFGAIAAGFI	337 - 356
H5p74	KRGLFGAIAAGFIEGGWQGMV	345 - 364
H5p75	AGFIEGGWQGMVDGWYGYHH	353 - 372
H5p76	QGMVDGWYGYHHSNEQSGY	361 - 380
H5p77	GYHHSNEQSGYAADKESTQ	369 - 388
H5p78	GSgyAADKESTQKAIDGVTN	377 - 396
H5p79	ESTQKAIDGVTNKVNSIIDK	385 - 404
H5p80	GVTNKVNSIIDKMNTQFEAV	393 - 412
H5p81	IIDKMNTQFEAVGREFNNLE	401 - 420
H5p82	FEAVGREFNNLERRIENLNK	409 - 428
H5p83	NNLERRIENLNKKMEDGFLD	417 - 436
H5p84	NLNKKMEDGFLDVWTYNAEL	425 - 444
H5p85	GFLDVWTYNAELLVLMENER	433 - 452
H5p86	NAELLVLMENERTLDFHDSN	441 - 460
H5p87	ENERTLDFHDSNVKNLYDKV	449 - 468
H5p88	HDSNVKNLYDKVRLQLRDNA	457 - 476
H5p89	YDKVRLQLRDNAKELGNGCF	465 - 484
H5p90	RDNAKELGNGCFEYHKCDN	473 - 492
H5p91	NGCFEYHKCDNECMESVRN	481 - 500
H5p92	KCDNECMESVRNGTYDYPQY	489 - 508
H5p93	SVRNGTYDYPQYSEEARLKR	497 - 516
H5p94	YPQYSEEARLKREEISGVKL	505 - 524
H5p95	RLKREEISGVKLESIGIYQI	513 - 532
H5p96	GVKLESIGIYQILSIYSTVA	521 - 540
H5p97	IYQILSIYSTVASSLALAIM	529 - 548
H5p98	STVASSLALAIMVAGLSLWM	537 - 556
H5p99	LAIMVAGLSLWMCSNGSLQC	545 - 564
H5p100	SLWMCSNGSLQCRICI	553 - 572

Table 2
DR0401 restricted H5HA T cell epitopes detected in human PBMC using TGEM

Subject Number	H5p33	H5p38	H5p46	H5p52	H5p78	H5p86
040					+	+
300	+		+		+	+
520		+				+
888	+		+			+
948				+		+