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# Cross-immunity Against Avian Influenza A(H7N9) Virus in the Healthy Population Is Affected by Antigenicity-Dependent Substitutions

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***Background.*** The emergence of infections by the novel avian influenza A(H7N9) virus has posed a threat to human health. Cross-immunity between A(H7N9) and other heterosubtypic influenza viruses affected by antigenicity-dependent substitutions needs to be investigated.

***Methods.*** We investigated the cellular and humoral immune responses against A(H7N9) and 2009 pandemic influenza A (H1N1) virus (A[H1N1]pdm09), by serological and T-cell–specific assays, in a healthy population. The molecular bases of the cellular and humoral antigenic variability of A(H7N9) were illuminated by structural determination.

***Results.*** We not only found that antibodies against A(H7N9) were lacking in the studied population, but also revealed that both $CD4^+$ and $CD8^+$ T cells that cross-reacted with A(H7N9) were at significantly lower levels than those against the A(H1N1)pdm09 peptides with substitutions. Moreover, individual peptides for A(H7N9) with low cross-reactivity were identified. Structural determination indicated that substitutions within these peptides influence the antigenic variability of A(H7N9) through both major histocompatibility complex (MHC) binding and T-cell receptor docking.

***Conclusions.*** The impact of antigenicity-dependent substitutions on cross-reactivity of T-cell immunity against the novel influenza virus A(H7N9) in the healthy population benefits the understanding of immune evasion of influenza viruses and provides a useful reference for universal vaccine development.

**Keywords.** antigenic variation; avian influenza A(H7N9) virus; cross-reactivity; structure; T-cell response.

Since the first occurrence of human infections by a novel avian-origin influenza A(H7N9) virus, in southeast China during February 2013 [1–4], the virus has reemerged every year and spread to additional regions. Early phylogenetic analyses based on genome sequencing revealed that the virus is a recent reassortment, with the H7 and N9 gene segments coming from, possibly, migratory birds and all of the other 6 internal genes from local chickens in the influenza A(H9N2) virus lineage [5]. Although several human signature mutations have been reported in the virus [1, 2], most of the human infections are believed to arise from contact with poultry [1–3, 6], and limited human-to-human transmission has also been observed [7], indicating the severe continuous threat of this novel avian influenza to humans.

Although antibodies cross-reactive to the hemagglutinin (HA) protein of A(H7N9) can be induced in mice by immunization with other H7 subtypes (eg, the North American H7 lineages) [8], no A(H7N9)-specific antibody titers were detected in 1544 serum samples collected between January 2012 and November 2012 from poultry workers in eastern China [9]. This not only indicates that there is no evidence for subclinical human infection by H7 virus before 2013, but also points out that cross-reactive serological immunity against this novel A(H7N9) strain does not preexist in healthy populations. Although Boni et al detected a low level of antibodies against A(H7N9) by protein microarray of serum samples of a healthy population collected in Vietnam [10], the implications of the results of this assay must be further evaluated.

T-cell responses play pivotal roles in alleviating disease severity and clearance of influenza virus [11]. Both $CD4^+$ and $CD8^+$ T cells elicited by one strain of influenza A virus have remarkable cross-protective function against heterosubtypic influenza A viruses, such as 2009 pandemic influenza A(H1N1) virus (A[H1N1]pdm09) [12–16]. Heterosubtypic T-cell reactivity to A(H7N9) by seasonal influenza viruses were also reported. However, the impact of antigenicity-dependent substitutions on

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the cross-reactivity of T-cell–mediated immunity against the novel A(H7N9) in the healthy population is still largely unknown.

In this study, we investigated baseline immunity against A(H7N9) as compared to A(H1N1)pdm09 in a healthy population. The molecular bases of the immune variation between the different virus subtypes were determined. These data reveal the immune profile specific for the novel A(H7N9) strain in the population and may provide references for vaccine development.

## MATERIALS AND METHODS

### Subject Information

A cohort of healthy volunteers (Supplementary Table 1) from Beijing, China, was recruited and provided specimens in May 2013 for laboratory analysis. Sera were obtained from all 53 volunteers (Z1–Z25 and Y1–Y28). Peripheral blood mononuclear cells (PBMCs) were collected from 25 donors (Z1–Z25). PBMCs were also available from 1 survivor of A(H7N9) infection 595 days after recovery. None of the subjects showed symptoms of influenza virus infection during the sampling period and declared no A(H7N9) infection history. All subjects confirmed that they were not inoculated with an influenza-related vaccine in the past 5 years (2009–2013). All participants provided informed, written consent for the studies performed on their samples and the publication of their cases. The study was approved by the Ethics Review Committee of Chinese Center for Disease Control and Prevention. The study was conducted in accordance with the principles of the Declaration of Helsinki and the standards of good clinical practice (as defined by the International Conference on Harmonization).

### Antibody Titers

Microneutralization (MN) and hemagglutination inhibition (HAI) assays were performed using A/California/7/2009 (H1N1) and A/Anhui/1/2013(H7N9) virus antigens, in accordance with World Health Organization protocols [9]. Titers of ≥1:80 for the MN assay and ≥1:40 for the HAI assay defined seropositivity.

### Design of T-Cell Peptide Pools

Protein sequences of A/Anhui/1/2013(H7N9) and A/California/7/2009(H1N1) were used to analyze peptides related to the T-cell response. Fifteen to 18-mer peptides overlapping by 11 amino acid residues and spanning the M1 proteins of A(H7N9) and A(H1N1)pdm09 were designed and synthesized (Supplementary Table 2). The peptides with the same sequences between A(H7N9) and A(H1N1)pdm09 were classified into the M1 (conserved) peptide pool, whereas the non-conserved peptides were divided into the respective A(H7N9) and A(H1N1)pdm09 peptide pools.

Previously identified HLA class I–restricted epitopes (8–11 residues) of influenza virus were retrieved from published data [17, 18]. Conserved peptides between A(H7N9) and A(H1N1)pdm09 were mixed into a conserved peptide pool (this pool was similar to the conserved pool reported by Liu et al [12], except for peptide NP[44-52], which is variable between A[H7N9] and A[H1N1]pdm09). Mutated HLA class I–restricted peptides were considered as A(H7N9)- and A(H1N1)pdm09-specific peptide pools (Table 1).

### Enzyme-Linked Immunospot (ELISPOT) Assay

The cytotoxic T-lymphocyte epitope–specific response was measured by performing interferon γ (IFN-γ) ELISPOT assays as described previously [17]. The number of spots was determined using an automatic ELISPOT reader and image analysis software (Cellular Technology Limited).

### Intracellular Cytokine Staining (ICS) and Flow Cytometry

The influenza virus peptide–specific ICS was performed as previously described [19], using anti–IFN-γ, anti-CD3, anti-CD8, and anti-CD4 surface markers (BD Bioscience).

### Expansion of A(H1N1)pdm09-Specific T Cells In Vitro

PBMCs from donors were incubated with peptide pools in Roswell Park Memorial Institute 1640 medium (Gibco) containing 10% fetal bovine serum (Hyclone), at 37°C with 5% $CO_2$, at a density of $2.5 \times 10^6$ cells/mL in 24-well plates. On day 3 of incubation, 20 U/mL recombinant human interleukin 2 was added to the medium. Half of the medium was then changed on day 7, with supplementation by recombinant human interleukin 2. Cells were harvested and tested for the presence of influenza virus–specific T cells on day 9.

### Preparation of Peptide-HLA Complexes

As previously described [20, 21], heavy chains of HLA and $\beta_2$m were expressed in *Escherichia coli* as inclusion bodies and subsequently refolded in vitro in the presence of different peptides.

### X-ray Crystallography, Structure Determination, and Refinement

Crystal screening of the HLA-A*1101/H1-22 and HLA-A*1101/H7-22 complexes was performed at a concentration of 15 mg/mL by the sitting-drop vapor-diffusion method at 4°C. Single crystals grew in 0.1 M ammonium acetate, 0.1 M BIS-Tris (pH 5.5), and 17% (w/v) polyethylene glycol 10 000 in 14 days. Crystallographic data were collected at 100 K in-house on a Rigaku MicroMax007 rotating-anode X-ray generator, operated at 40 kV and 20 mA (Cu Kα; λ = 1.5418 Å), equipped with an R-AXIS VII++ image-plate detector. Data were indexed and scaled using DENZO and the HKL2000 software package (Supplementary Table 3). The structures were determined using molecular replacement with the structure of HLA-A*1101 (Protein Data Bank accession code 1QVO) as the model and refined as described previously [19]. Structure-related figures were generated using PyMOL (available at: http://www.pymol.org/).

### Statistics

Differences in mean values were evaluated for statistical significance ($P < .05$, $P < .01$, or $P < .001$) by the Student *t* test. Data were

**Table 1. Nonconserved CD8$^+$ T-Cell Epitopes Composing the Influenza A(H7N9) Virus and 2009 Pandemic Influenza A(H1N1) Virus (A[H1N1]pdm09) Pools**

| Name | A(H7N9) Specific[a] | Name | A(H1N1)pdm09 Specific | Protein (Position) | HLA Restriction | Anchor Residues[b] | Exposed Residues |
|---|---|---|---|---|---|---|---|
| H7-7 | **A**MDSNTLEL | H1-7 | TMDSNTLEL | NP (373–381) | A2 | – | + |
| H7-8 | CTELKLSD**N** | H1-8 | CTELKLSDY | NP (44–52) | A1 | + | – |
| H7-9 | **AIVDKNITL**[c] | H1-9 | **AIMEKNIVL** | NS1 (122–130) | A2 | + | + |
| H7-11 | MLLRTA**V**GQV | H1-11 | MLLRTAIGQV | PA (548–557) | A2 | + | – |
| H7-12 | R**T**MAWTVVNSI | H1-12 | RIMAWTVVNSI | PA (84–93) | A2 | + | – |
| H7-13 | FVE**A**LARSI | H1-13 | FVETLARSI | PB1 (254–282) | A2 | – | + |
| H7-14 | LLFLK**V**P**V** | H1-14 | LLFLKIPA | PB1 (7–14) | A2 | + | + |
| H7-20 | RLE**D**VFAGK | H1-20 | RLESVFAGK | M1 (27–35) | A3[d] | – | + |
| H7-21 | ESMREEY**R**Q | H1-21 | ESMREEYQQ | M2 (66–74) | A3[d] | – | + |
| H7-22 | **TMVMELIRMIK**[c] | H1-22 | **TIAMELIRMIK** | NP (188–198) | A3[d] | + | – |
| H7-23 | **RVSSFIRGTR**[c] | H1-23 | **RVSSFIRGKK** | NP (342–351) | A3[d] | + | + |
| H7-25 | LYKKLKRE*M*T*F*[c] | H1-25 | LYKKLKREITF | M1 (99–109) | A24 | – | + |
| H7-26 | TFHGAKEV**A**L | H1-26 | TFHGAKEVSL | M1 (108–117) | A24 | – | + |
| H7-27 | AYQ**N**RMGVQ**L** | H1-27 | AYQKRMGVQM | M1 (239–248) | A24 | + | + |
| H7-28 | DTVNRTH**K**Y | H1-28 | DTVNRTHQY | PB1 (41–49) | A26 | – | + |
| H7-29 | FLKDVM**D**SM | H1-29 | FLKDVMESM | PB1 (166–174) | A2 | – | + |
| H7-30 | **A**LARSICEK | H1-30 | TLARSICEK | PB1 (257–255) | A3[d] | – | + |
| H7-31 | KYTKT**T**YWW | H1-31 | KYTKTIYWW | PB1 (430–438) | A24 | + | – |
| H7-32 | PVAGGT**S**SVYI[e] | H1-32 | PVAGGTGSVYI | PB2 (219–229) | A2 | – | + |
| H7-33 | I**VD**KNI**T**LKA | H1-33 | IMEKNIVLKA | NS1 (123–132) | A2 | + | – |

[a] The mutated sites in the sequences of the A(H7N9)-specific peptides as compared to the A(H1N1)pdm09-specific peptides are underlined and in bold.

[b] The locations of mutated residues in the peptides between A(H7N9) and A(H1N1)pdm09 were deduced on the basis of previously determined structures of HLAs. The residues within the P2 (position 2 from the N terminus), P3 and Pc (C terminus of the peptide) and P5 for 8-mer, P6 for 9-mer, and P7 for 10-mer peptides are defined as anchor residues. Residues in other positions are defined as exposed residues. Peptides with mutations at the corresponding positions are indicated by "+"; those without mutations are indicated by "–."

[c] The peptides in bold indicate the A(H7N9)-specific T-cell epitopes identified in this study that have decreased antigenicities in healthy donors.

[d] Indicates the A3 supertype that includes HLA-A11, HLA-A33, HLA-A31, HLA-A68, and HLA-A03.

[e] All of the A(H7N9) peptides are conserved between different A(H7N9) strains, except peptide H7-Mu32 in Hangzhou/1 is PVAGG**S**SSVYI.

assembled and statistically calculated using a spreadsheet program (Excel; Microsoft).

### Accession Code

The coordinates and structure factors of peptide H1-22 and H7-22 complexed to HLA-A*1101 were deposited in the Protein Data Bank under accession codes 4MJ5 and 4MJ6, respectively.

## RESULTS

### Lack of Humoral Responses Against A(H7N9) in the Population

Thus far, it has been determined that humoral immunogenic sites on the HA of influenza A virus are mainly distributed over 5 conformational sites (Figure 1*A* and Table 2). The Sa and Sb sites are located proximal to the receptor-binding site, whereas Ca and Cb lie on the vestigial esterase domain. Furthermore, a subdominant B-cell epitope is displayed on the stem of the HA. Structure-based conservancy analysis of the epitopes from A(H7N9) [27] revealed that the major surface of 4 humoral antigenic sites on the head of HA were significantly distinct from that of A(H1N1)pdm09 [28]. The epitope on the stem of HA was comparatively conserved but still contained mutations that protruded toward the antibody-targeted interface. Influenza virus–specific humoral immune responses determined by both HAI and MN assays against A(H7N9) and A(H1N1)pdm09 demonstrated that none of A(H7N9)-specific antibody titers could be detected among the study subjects. In contrast, humoral responses against A(H1N1)pdm09 were detected in 54.7% of subjects (29 of 53) with double-positive HAI titers of ≥40 and MN titers of ≥80 (Figure 1*B*), indicating a high ratio of previous A(H1N1)pdm09 exposure of the population.

### Limited T-Cell Cross-reactivity Against A(H7N9) Detected by Overlapping Peptides

T-cell immunogenicities of A(H7N9) and A(H1N1)pdm09 were analyzed through conservancy evaluation of 116 previously identified immunogenic overlapping peptides from the whole proteome of the influenza virus [29]. Overall, 31% of the immunogenic peptides were conserved in A(H7N9), with all of them derived from internal proteins (Supplementary Table 4). Specifically, the M1 protein contained the highest density of immunogenic peptides in the sequence (calculated as the number of immunogenic peptides per 100 amino acids). Furthermore, the magnitude of T-cell responses against M1 harbors a dominant role in the T-cell responses against the whole proteome of the influenza virus [29, 30]. Thus, T-cell immune responses against the overlapping peptides from the M1 proteins of A(H7N9) and A(H1N1)pdm09 were evaluated to investigate the cross-reactivity of A(H1N1)pdm09-specific T cells against A(H7N9) among the subjects.

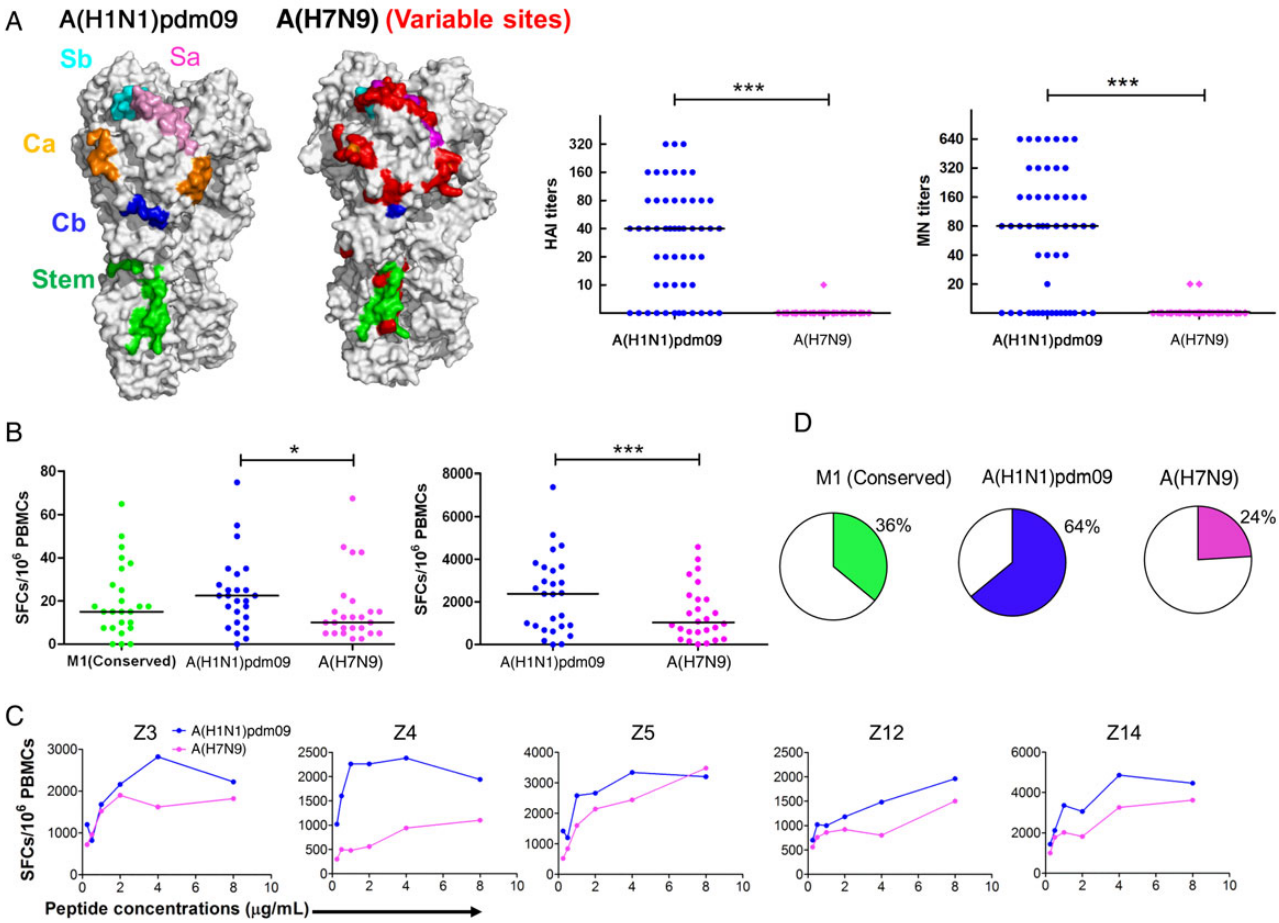


**Figure 1.** Detection of serologic and cellular immune responses to influenza A(H7N9) virus. *A*, Left panels, The location of the major antigenic sites is shown for antibody binding of hemagglutinin (HA) protein, based on the structure of 2009 pandemic influenza A(H1N1) virus (A[H1N1]pdm09) HA (Protein Data Bank accession code 3AL4) and the structure of A(H7N9) HA (Protein Data Bank accession code 4KOL). The variable residues between A(H7N9) and A(H1N1)pdm09 in the B-cell epitopes are shown in red. Right panels, Sera from normal individuals (n = 53) were tested for A(H1N1)pdm09 and A(H7N9) antibodies by hemagglutination inhibition (HAI) and microneutralization (MN) assays. *B*, Cellular immunity to A(H1N1)pdm09 and A(H7N9) in healthy donors. Left, T-cell responses were investigated using freshly isolated peripheral blood mononuclear cells (PBMCs) from 25 individuals, using interferon γ enzyme-linked immunospot (ELISPOT) assays with pools of overlapping peptides derived from influenza virus M1 protein that are either absolutely conserved or not conserved between A(H1N1)pdm09 and A(H7N9) (Supplementary Table 2). Right, A(H1N1)pdm09-specific T cells in PBMCs from 25 individuals were expanded with stimulation by the A(H1N1)pdm09 M1 peptide pool for 9 days. The cross-reactivity to peptides in the A(H7N9)-specific pool was tested using ELISPOT assays. *C*, T-cell responses to A(H1N1)pdm09 and A(H7N9) detected by peptide pools at different concentrations for individual peptides. Data from 5 representative donors are presented. *D*, The ratios of populations with positive responses to different peptide pools (green, M1 conserved pool; blue, A[H1N1]pdm09-specific pool; and purple, A(H7N9)-specific pool), using fresh PBMCs, were analyzed based on the results in panel *C* (ex vivo ELISPOT). Horizontal lines indicate median values. Each dot represents an individual donor, and the HAI data are representative of 2 independent tests. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

Twenty-five subjects were recruited to investigate the T-cell responses against the M1 proteins from A(H1N1)pdm09 and A(H7N9). The results demonstrated that the conserved peptide pool (Supplementary Table 2) displays a certain magnitude of response (mean, 20.2 spot-forming cells [SFCs]/$10^6$ PBMCs), which represents the existence of cross-reactivity against A(H7N9) (Figure 1*B*). However, for the variable peptide pools, which harbor the majority of all of the overlapping peptides (24 of 39), the magnitude of the responses against the A(H7N9)-specific pool were significantly lower (mean, 16.2 SFCs/$10^6$ PBMCs) than that against the A(H1N1)pdm09 pool (mean, 23.5 SFCs/$10^6$ PBMCs; $P < .05$). The proportion of the population with positive responses (≥20 SFCs/$10^6$ PBMCs) against the A(H7N9)-specific peptides was 24% (Figure 1*D*), which was much lower than the proportion with positive responses against A(H1N1)pdm09 (64%). The A(H1N1)pdm09-specific T cells from the PBMCs of the healthy donors were expanded in vitro to assess possible low-frequency T cells and, subsequently, to investigate cross-reactivity against A(H7N9). Similar to the ex vivo results, a low cross-reactivity against the A(H7N9) pool (mean, 1464.0 SFCs/$10^6$ PBMCs) as compared to the one against A(H1N1)pdm09 (mean, 2415.2 SFCs/$10^6$ PBMCs) was still observed after the in vitro expansion of A(H1N1)pdm09-specific T cells ($P < .001$; Figure 1*B*). We compared the cross-reactivity between A(H7N9) and A(H1N1)pdm09 by the ELISPOT assay

**Table 2. Antigenic Variable Sites in Influenza A(H7N9) Virus Aligned With 2009 Pandemic Influenza A(H1N1) Virus**

| Antigenic Site, Virus | Domain | Sequence |
|---|---|---|
| Sa | | |
| H1N1/California/7 | HA1 | $^{128}$PN $^{156}$KKGNS $^{162}$PKLSKS |
| H7N9/Anhui/1 | HA1 | SG SNTDNAA PQMTKS |
| Sb | | |
| H1N1/California/7 | HA1 | $^{187}$TSADQQSLYQNA |
| H7N9/Anhui/1 | HA1 | STAEQTKLYGSG |
| Ca | | |
| H1N1/California/7 | HA1 | $^{140}$PHAGAK $^{169}$INDKG |
| H7N9/Anhui/1 | HA1 | RRSGSS KNTRK |
| H1N1/California/7 | HA1 | $^{206}$SSR $^{224}$RD $^{238}$EPG |
| H7N9/Anhui/1 | HA1 | SSN NG NPN |
| Cb | | |
| H1N1/California/7 | HA1 | $^{79}$LSTASS |
| H7N9/Anhui/1 | HA1 | FLEFSA |
| Stem | | |
| H1N1/California/7 | HA1 | $^{38}$LLEDK $^{291}$AIN |
| H7N9/Anhui/1 | HA1 | LVERT TII |
| H1N1/California/7 | HA2 | $^{19}$DGW $^{38}$LKSTQNAIDEITNKVNSVI |
| H7N9/Anhui/1 | HA2 | DGW YKSTQSAIDQITGKLNRLI |

The positions of the antigenic sites are analyzed on the basis previous studies [22–26].

under different peptide concentrations. We found that T-cell responses to both A(H7N9) and A(H1N1)pdm09 increased with the concentration of the stimulus, but A(H7N9)-specific T-cell responses were still lower than those to A(H1N1)pdm09 under different concentrations of the peptide pools (Figure 1*C*).

Characterization by ICS assays revealed that both $CD8^+$ (Figure 2*A*) and $CD4^+$ (Figure 2*B*) T-cell responses were involved. A lower frequency of both A(H7N9)-specific $CD8^+$ (Figure 2*C*) and $CD4^+$ (Figure 2*D*) T cells was detected as compared to peptides from A(H1N1)pdm09 ($P < .01$). We also analyzed the cross-reactive $CD8^+$ and $CD4^+$ T cells to A(H1N1)pdm09 in the PBMCs of a survivor of A(H7N9) infection 595 days after disease onset. Through the ICS assay, the A(H7N9)-specific $CD8^+$ and $CD4^+$ T-cell frequencies were 3.21% and 0.48%, respectively, after the in vitro expansion of the PBMCs under the stimulation of A(H7N9) M1 protein–derived peptides. In addition, the cross-reactive A(H1N1)pdm09-specific $CD8^+$ and $CD4^+$ T-cell frequencies were 1.45% and 0.40%, respectively, which were lower than those of the A(H7N9)-specific T cells (Figure 3).

### Cross-reactive $CD8^+$ T-Cell Responses Against A(H7N9)

To efficiently determine the cross-reactive $CD8^+$ T-cell responses to A(H7N9) and examine the HLA restriction of the responses, a panel of previously defined HLA class I–restricted peptides located within the whole proteome of influenza A virus were analyzed and divided into conserved or variable peptides between A(H1N1)pdm09 and A(H7N9) [17]. Similar to the responses against the overlapping long peptides of influenza A virus M1 protein, certain T-cell responses against the conserved $CD8^+$ T-cell epitope pool were detected (Figure 4*A*). Moreover, $CD8^+$ T-cell responses against mutated peptides from A(H7N9) (Table 1) could be detected from both freshly isolated PBMCs and in vitro–expanded polyclonal T-cell lines among the subjects (Figure 4*A*). However, the magnitude of the response against the epitopes from A(H7N9) was much lower than that against the epitopes from A(H1N1)pdm09 ($P < .01$). A rare population demonstrated positive responses against A(H7N9) peptides (4%), lower than the population positive against A(H1N1)pdm09 (28%; Figure 4*B*). In contrast, for A(H7N9)-specific peptide pool–cultured polyclonal T-cell lines, the $CD8^+$ T-cell responses against mutated peptides from A(H7N9) were higher than the responses to A(H1N1)pdm09 (Figure 4*A*), which also indicated a disaccord between the antigenicities of the epitopes derived from A(H1N1)pdm09 and A(H7N9). ICS assays for 2 representative subjects, Z13 and Z21, also demonstrated a significantly lower frequency of A(H7N9)-specific $CD8^+$ T cells as compared to that against A(H1N1)pdm09 (Figure 4*C*). The IFN-γ–secreting cells were mostly CD8 positive, indicating $CD8^+$ T-cell specificity of the short peptides.

Individual functioning peptides (Figure 4*D*) from A(H1N1)pdm09 and A(H7N9) were further identified to evaluate the T-cell cross-reactivity in an HLA-restricted manner and to elucidate the mechanisms of the less efficient cross-reactivity. One HLA-A2–restricted peptide (H1-9) and 2 HLA-A3 supertype allele–restricted peptides (H1-22 and H1-23) have a dominant role in A(H1N1)pdm09-specific $CD8^+$ T-cell responses. However, the mutated peptides H7-9, H7-22, and H7-23 from A(H7N9) only induced lower responses than the corresponding peptides from A(H1N1)pdm09 in the subjects with specific HLA alleles.

### Molecular Basis of the Antigenic Variability of A(H7N9)-Specific Peptides

Among the 20 nonconserved short peptides analyzed herein (Table 1), 7 had amino acid substitutions at the anchoring positions (primary anchoring positions P2 and Pc, secondary anchoring position P3, and P5 for 8-mers, P6 for 9-mers, and P7 for 10-mers), whereas 9 had amino acid substitutions at the exposed positions that may be directly recognized by T-cell receptors (TCRs), with 4 remaining peptides mutated at both the anchoring and exposed residues. This indicates that substitutions both at the exposed and the anchoring positions of the peptides contribute to the variable antigenicity of the nonconserved peptides in A(H7N9).

To interpret the T-cell immunogenicity transition of A(H7N9) in detail, we determined the crystal structure of HLA-A*1101 in complex with the peptide H1-22 or H7-22 (Figure 5*A* and Supplementary Table 3). Although only 2

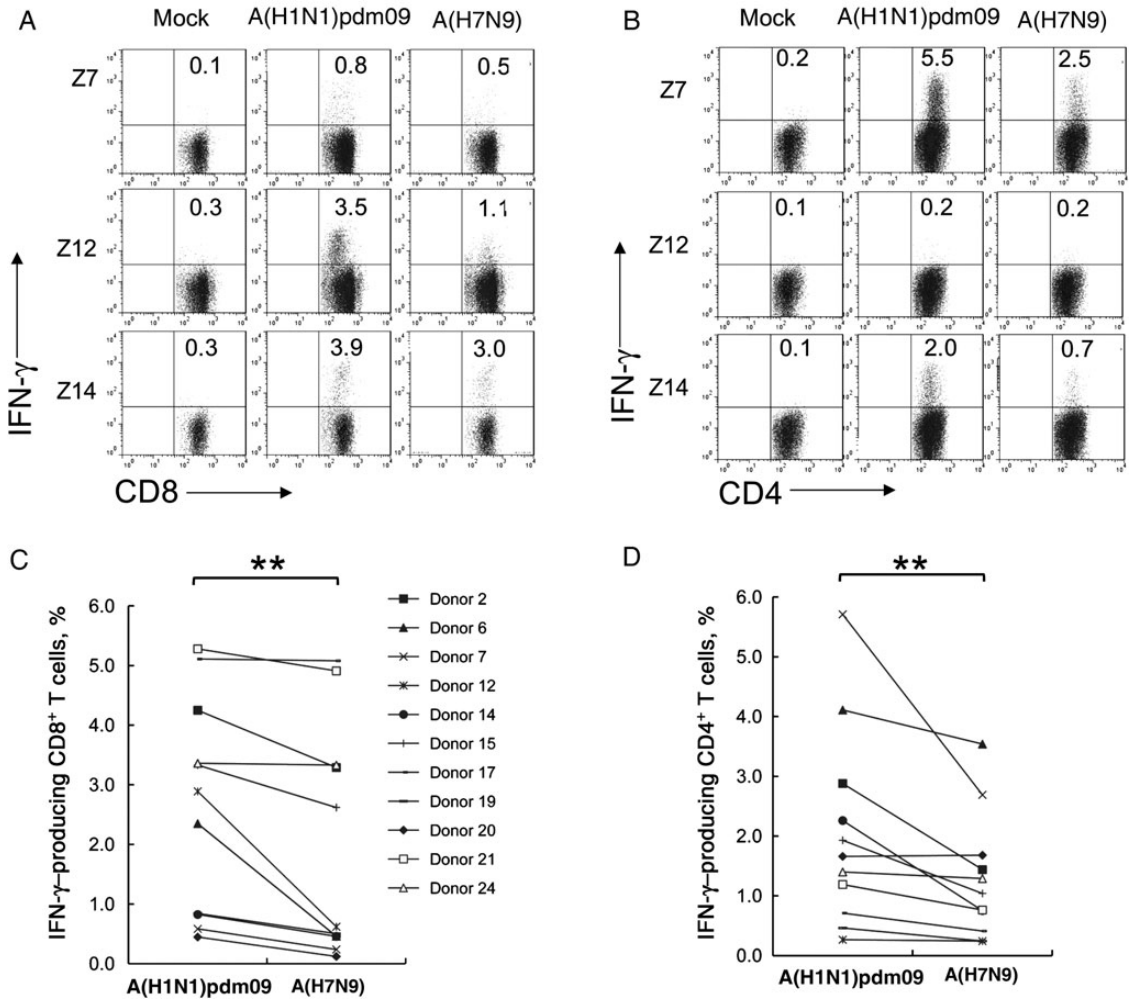


**Figure 2.** Influenza virus–specific interferon γ (IFN-γ)–secreting cells among $CD8^+$ and $CD4^+$ T cells. 2009 Pandemic influenza A(H1N1) virus (A[H1N1]pdm09)–specific T cells in peripheral blood mononuclear cells (PBMCs) from the individuals were expanded with the stimulation of the A(H1N1)pdm09-specific M1 peptide pool for 9 days. Subsequently, the ratios of the IFN-γ–producing cells in $CD8^+$ (*A*) and $CD4^+$ (*B*) T cells were tested using intracellular cytokine staining (ICS) and flow cytometry. ICS was performed using cells from 11 donors who had high numbers of spot-forming cells in enzyme-linked immunospot assays. Donors Z7, Z12, and Z14 were 3 representatives whose IFN-γ–producing cells were primarily $CD4^+$ T cells, $CD8^+$ T cells, or both $CD4^+$ T cells and $CD8^+$ T cells, respectively. The lower responses against influenza A(H7N9) virus as compared to that against A(H1N1)pdm09 of the $CD8^+$ (*C*) and $CD4^+$ (*D*) T cells in each individual are shown in 2 linked points. $^{**}P < .01$.

residues at the N-terminus of H1-22 are mutated as compared to that of H7-22 (I2M and A3V), the conformation of the middle portion of the peptide, which is involved in TCR contact, displayed a dramatic change as a result of the substitutions. The B pocket of HLA-A*1101 is too small to accommodate the large residue Met, the P2 anchor in A(H7N9) peptide H7-22 (Supplementary Figure 1), which makes the P1 anchor Thr of H7-22 insert into the B pocket. The molecular conformation changes of the H7-22 peptide as compared to that of H1-22 represents the structural basis of the antigenic variation of A(H7N9), compared with A(H1N1)pdm09. Thus, taking these data together, the mutations in A(H7N9) both influence HLA-binding and modulate the antigenicity of the peptide by altering TCR recognition. This may illustrate a common mode of antigenic variation of nonconserved peptides in A(H7N9), which only induces a limited scale of cross-reactive T-cell responses.

To characterize the binding of peptides H1-9, H1-22, and H1-23, derived from A(H1N1)pdm09 and their A(H7N9) variants with the corresponding HLA alleles, refolding of these peptides with HLA-A*0201 and HLA-A*1101 was performed (Figure 5*B*). The data indicated that peptide H7-23 has dramatically decreased binding by HLA-A*1101, owing to the mutation (K10R) in the primary anchor residue as compared to that of the wide-type peptide H1-23 from A(H1N1)pdm09. Despite the different conformations, peptides H1-22 and H7-22 have a similar binding affinity to HLA-A*1101, indicating a similar efficacy for both P2-Ile of H1-22 and P1-Thr of H7-22 as the P2 anchor for HLA-A*1101–binding peptides. For the HLA-A2–restricted peptide H7-9 from A(H7N9), a similar

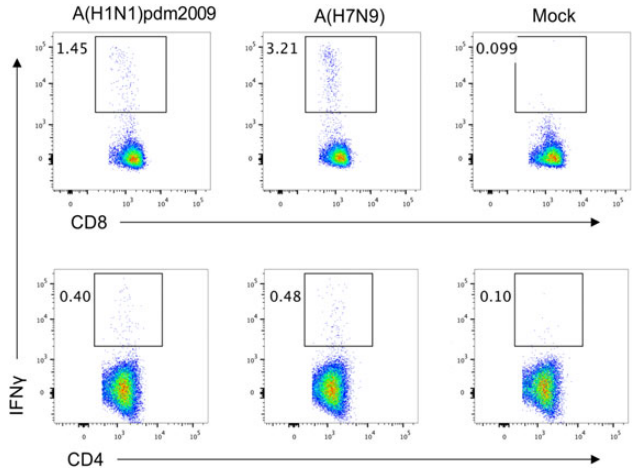


**Figure 3.** Influenza virus-specific $CD8^+$ and $CD4^+$ T-cells in a H7N9 survivor. Influenza virus-specific T-cells in peripheral blood mononuclear cells from the individuals were expanded with the stimulation of the H7N9-specific M1 peptide pool for 9 days. The T-cell lines were collected and the ratios of the interferon γ (IFN-γ)-producing $CD8^+$ and $CD4^+$ T-cells were tested using intracellular cytokine staining under the stimulation of 2009pH1N1 and H7N9-specific M1 peptides or medium (Mock). Subsequently, the cells were harvested and stained with anti-CD3, anti-CD8, anti-CD4 surface markers and anti-IFN-γ and the analyzed on a FACS flow cytometer.

efficiency in the production of soluble peptide/HLA complex was found as compared to that of the wide-type peptide H1-9 from A(H1N1)pdm09, indicating the insignificant impact of the M3V substitution on HLA-A2 binding.

## DISCUSSION

The novel avian influenza virus A(H7N9) harbors not only mammalian-adapted amino acid substitutions related to its jump from avian to human hosts [1, 2], but also epitope mutations that are new for human immune system recognition and clearance of the virus [31]. Herein, we investigated the impact of antigenicity-dependent substitutions on the cross-reactivity of T-cell immunity against the novel influenza virus A(H7N9) in the healthy population, which may benefit the understanding of the immune evasion of influenza viruses and provide useful reference for universal vaccine development.

Since the outbreak of A(H1N1)pdm09 infection in spring 2009 [32], the prevalence of the virus has been continuously reported during the following seasons (2009–2013) and has become one of the major epidemic strains of influenza virus in northern China [33]. We determined a high level of A(H1N1)pdm09-specific antibodies among healthy donors, implying a high exposure rate in the population [34–36]. The HA acts as the major antigen to induce the production of protective antibodies against influenza virus, in which 5 major B-cell epitopes have been reported [33]. As we analyzed the 5 corresponding potential B-cell epitopes on A(H7N9) HA, 4 epitopes on the globular head domain, which plays a dominant role in HA antigenicity [37], differed as compared to that of A(H1N1)pdm09. Variable residues also occur in the epitope on the stem region of the HA [22]. In agreement with these structural analyses, our experimental data indicated that no preexisting antibodies against A(H7N9) were present in the healthy population, including elderly individuals, contemporary to the time of the outbreak.

In contrast to B-cell epitopes, T cells recognize epitopes that are mainly derived from relatively conserved internal proteins of influenza virus. Influenza virus–specific, cross-reactive $CD4^+$ and $CD8^+$ T cells broadly exist among the population, providing protection from subsequent infections by heterotypic viruses [13, 16, 29, 38, 39]. Previous studies indicate that the relatively mild clinical outcomes of A(H1N1)pdm09 infection can be partially explained by the protective efficacy of cross-reactive T-cell responses against A(H1N1)pdm09 elicited by seasonal influenza virus infection or vaccination [13, 15, 40]. The cross-reaction of memory T cells against avian influenza A(H5N1) virus established by seasonal human influenza A virus infection also exists in healthy individuals [29, 39]. Recent studies also indicated that seasonal influenza can prime hosts for $CD4^+$ and $CD8^+$ T-cell immunity to A(H7N9) [41–43]. Herein, we reveal that a certain level of cross-reactive T-cell responses against A(H7N9) exists among different individuals, which is predominantly contributed to by the conserved T-cell epitopes. This is in accordance with our previous findings of the predominant role of the conserved T-cell epitopes among the anti–influenza virus responses [17].

Recent studies have also demonstrated the complexity of cross-reactive $CD8^+$ T cells among humans and animal models. The recognition of A(H7N9) by memory cytotoxic T lymphocytes established by other influenza virus strains is dependent on different HLA I alleles featured across distinct ethnicities [44]. It is also indicated that protective immunity can be contributed to by several immunodominant epitopes that are conserved during heterosubtypic influenza virus infection [45]. However, few studies have focused on how substitutions on the nonconserved epitopes impact the heterosubtypic T-cell reactivity and potential influenza virus evasion of T-cell recognition [46]. In our present study, we observed a significantly low level of cross-recognition of A(H7N9)-specific nonconserved peptides by A(H1N1)pdm09-specific $CD4^+$ and $CD8^+$ T cells, incurred by few antigenicity-dependent substitutions. Considering the large number of mutated epitopes in A(H7N9), the limited cross-reactivity against the virus may pose a challenge for viral clearance in infected humans, which is associated with the poor outcome of the disease.

As previously determined in structural studies, mutational escape within T-cell epitopes contributes to the antigenic disaccord between the epitopes derived from different influenza virus strains [14, 47]. Gras et al [14] investigated the cross-reactive T-cell responses against the HLA-B7 supertype–restricted variable epitope NP418–426 in humans. They elucidated that

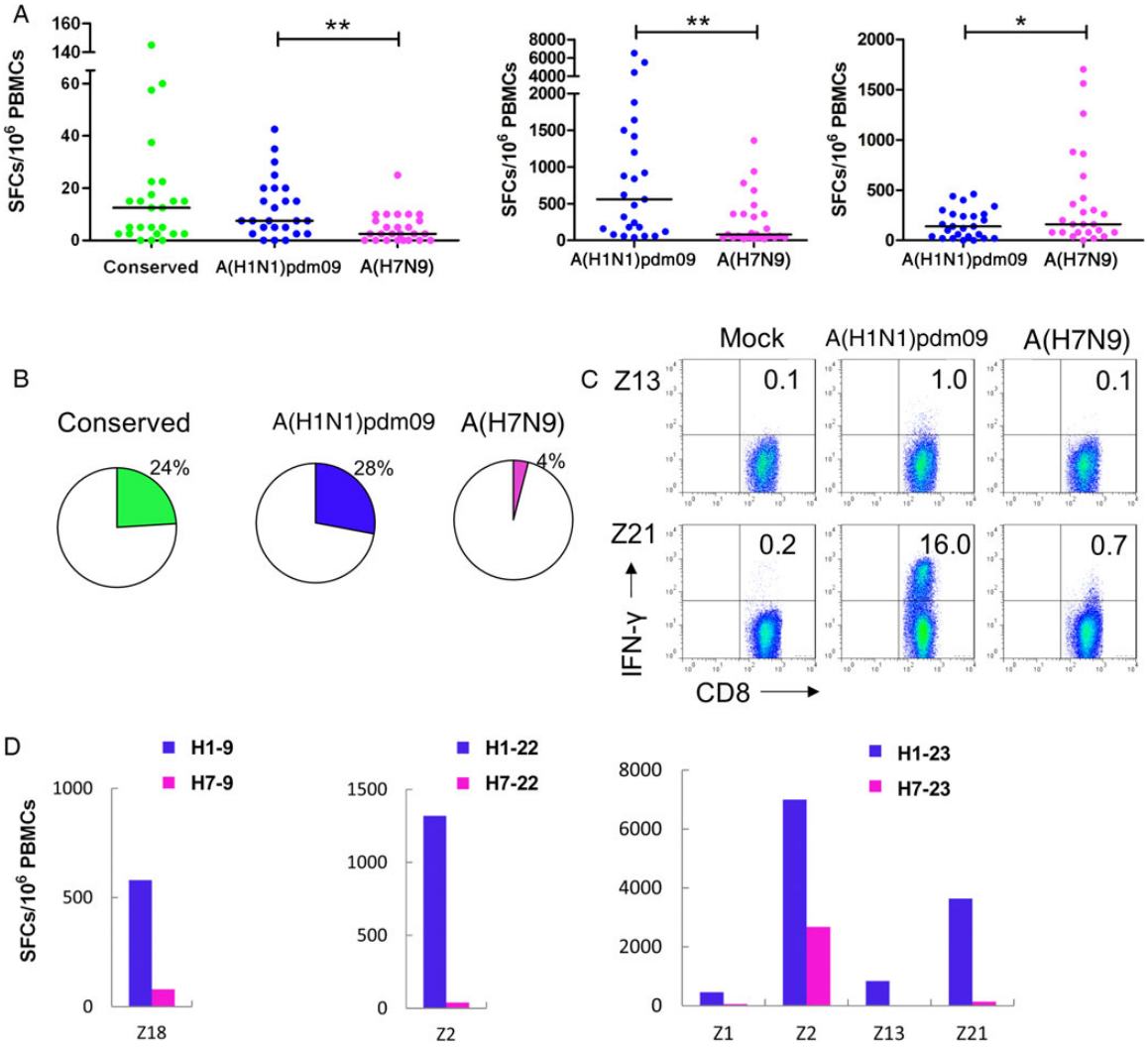


**Figure 4.** Investigation of cross-reactive CD8$^+$ T-cell responses against influenza A(H7N9) virus, using short peptides. *A*, CD8$^+$ T-cell peptide–specific T-cell responses to 2009 pandemic influenza A(H1N1) virus (A[H1N1]pdm09) and A(H7N9). Left, A(H1N1)pdm09- and A(H7N9)-specific CD8$^+$ T-cell responses were tested through ex vivo enzyme-linked immunospot (ELISPOT) assays using CD8$^+$ T-cell peptide pools (Table 1) as stimulus. Middle, A(H1N1)pdm09-specific T cells in peripheral blood mononuclear cells (PBMCs) from 25 individuals were expanded via stimulation of the CD8$^+$ T-cell peptide pool with A(H1N1)pdm09 specificity for 9 days. A(H1N1)pdm09- and A(H7N9)-specific CD8$^+$ T-cell responses were tested by ELISPOT. $^{**}P < .01$. Right, A(H1N1)pdm09- and A(H7N9)-specific T-cell responses in PBMCs expanded via the stimulation of the A(H7N9) peptide pool. $^{*}P < .05$. *B*, The ratios of populations with positive responses to different peptide pools (green, conserved CD8$^+$ T-cell peptide pool; blue, 2009-specific pool; and purple, A(H7N9)-specific pool), using fresh PBMCs were analyzed based on the results in the graph at left in panel *A* (ex vivo ELISPOT). *C*, A(H1N1)pdm09- and A(H7N9)-specific interferon γ (IFN-γ)–secreting PBMCs from donors 13 and 21 were assessed after in vitro culturing with A(H1N1)pdm09-specific CD8$^+$ T-cell peptides. *D*, Individual CD8$^+$ T-cell epitopes that have antigenic variation between A(H1N1)pdm09 and A(H7N9) were determined among different donors with the corresponding HLA-A restrictions. PBMCs from different donors were tested via the IFN-γ ELISPOT assay by stimulation of individual peptides with A(H1N1)pdm09- and A(H7N9)-signature sequences, respectively. Abbreviation: SFC, spot-forming cell.

cross-reactive CD8$^+$ T-cell immunity does not exist between A(H1N1)pdm09 and recent seasonal influenza viruses, owing to structural variation of the solvent-exposed residues in T-cell epitopes that can be recognized by TCRs. Moreover, mutations of anchor residues (or partially solvent-exposed secondary anchors) can also dramatically decrease CD8$^+$ T-cell responses and result in delayed viral clearance [48]. The A(H7N9)-derived peptide H7-22 was determined in our study to have low T-cell cross-reactivity among the HLA-A11$^+$ donors. The structure of the HLA-A*1101/peptide clearly showed that the A(H7N9) variant possessed mutations in both the TCR-interacting and HLA-anchoring positions. Thus, this indicates that T-cell recognition of the mutant viral epitopes is significantly decreased as compared to that of A(H1N1)pdm09, owing to the poor T-cell activation threshold and disrupted peptide-HLA interactions, although low cross-reactivity of the variable peptides was also observed in our study, which may contribute to the TCR conformational plasticity [49]. Notably, although H7-22 has a dramatically different conformation as compared to H1-22 when presented by HLA-A*1101, the binding abilities of

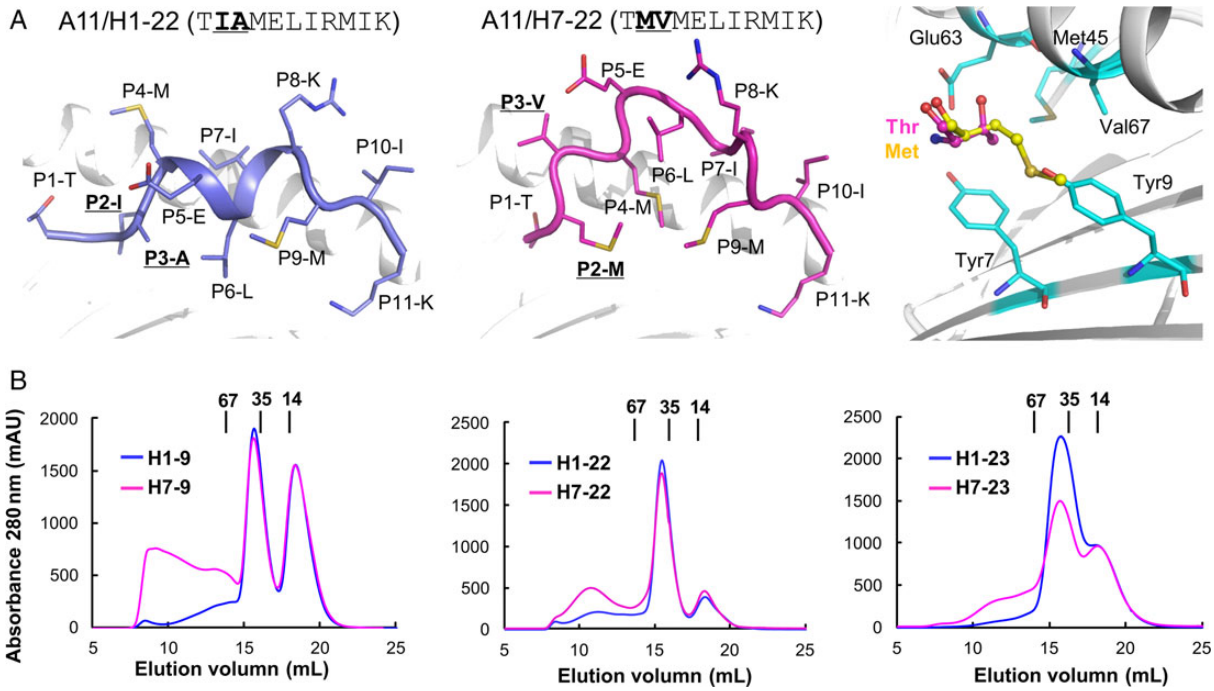


**Figure 5.** Molecular basis of the low cross-reactive T-cell responses to influenza A(H7N9) virus. *A*, Structures of HLA-A*1101 complexed with 2009 pandemic influenza A (H1N1) virus (A[H1N1]pdm09)–derived peptide H1-22 (left) and A(H7N9)-derived H7-22 (middle) were determined by X-ray crystallography. The names of mutated residues P2-I/P2-M and P3-A/P3-V are underlined. The B pocket of HLA-A*1101 cannot accommodate the large residue Met of the H7N9-derived peptide (right). The residues Tyr7, Try9, Met45, Glu63 and Val67 (in cyan sticks) of HLA-A*1101 form a small B pocket which can accommodate the P2 anchors (eg, Ile in peptide H1-22 or Thr [in purple sticks and spheres] in peptide H7-22). However, the P2 anchor Met (in yellow sticks and spheres) in peptide H7-22, cannot insert into the B pocket due to steric hindrance with residues (eg, Tyr9 in the B pocket). *B*, The capability of peptides to help HLA heavy chain and $\beta_2$m renature in vitro. The peaks of the monomer HLA complexes with the expected molecular mass of 45 kDa were eluted at an estimated volume of 16 mL on a Superdex 200 column (GE Healthcare). The profile is marked with the approximate positions of the molecular mass standards of 67, 35, and 14 kDa. The results are representative of 2 independent tests.

both peptides to HLA-A*1101 are retained at the same level. Our previous study identified an HLA-A2–restricted epitope derived from hepatitis B virus with an unconventional P2 anchor Thr, which has a tight binding with Glu63 in the B pocket of HLA-A*0201 through a salt bridge [50]. In our current study, the P1 anchor Thr of H7-22 also forms a salt bridge with Glu63 of HLA-A*1101, contributing to the tight binding between the mutated peptide and HLA-A*1101.

In the present study, we found that A(H7N9)-specific antibodies were absent in the studied population, which is related to the different B-cell epitopes on A(H7N9) HA as compared to A(H1N1)pdm09. A certain level of cross-reactive T-cell responses directed toward conserved viral T-cell epitopes of A(H7N9) and A(H1N1)pdm09 exists. However, loss of T-cell recognition was observed for the mutated epitopes of A(H7N9), which also have a particular role in anti–influenza virus immunity. The T-cell antigenic variations between A(H7N9)- and A(H1N1)pdm09-specific epitopes were contributed to by antigenicity-dependent substitutions revealed by the major histocompatibility complex/epitope structures. Our study provides an important reference for current universal vaccine development for influenza viruses. The antigenic variability of major T-cell immunogens such as M1, NP, and PB1 should be carefully evaluated during the development of universal vaccines, considering that the variation in only a few amino acids would totally change the immunogenicity of the peptides, as indicated in the present study.

## Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

## Notes

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G. F. G, W. J. L., and S. T. designed the study. W. J. L., S. T., M. Z., C. Q., Y. B., and H. X. performed the experiments. J. Q. solved the structures. W. J. L., S. T., M. Z., and S. Z. analyzed the data. W. J. L., S. T., M. Z., Y. W., H. Z., J. Y., W. L., H. Y., Y. S., G. W., and G. F. G. interpreted the data. W. J. L., S. T., M. Z., and G. F. G. wrote the manuscript.

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***Potential conflicts of interest.*** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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