Preexisting CD8+ T-cell immunity to the H7N9 influenza A virus varies across ethnicities

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The absence of preexisting neutralizing antibodies specific for the novel A (H7N9) influenza virus indicates a lack of prior human exposure. As influenza A virus–specific CD8+ T lymphocytes (CTLs) can be broadly cross-reactive, we tested whether immunogenic peptides derived from H7N9 might be recognized by memory CTLs established following infection with other influenza strains. Probing across multiple ethnicities, we identified 32 conserved epitopes derived from the nucleoprotein (NP) and matrix-1 (M1) proteins. These NP and M1 peptides are presented by HLAs prevalent in 16–57% of individuals. Remarkably, some HLA alleles (A*0201, A*0301, B*5701, B*1801, and B*0801) elicit robust CTL responses against any human influenza A virus, including H7N9, whereas ethnicities where HLA-A*0101, A*6801, B*1501, and A*2402 are prominent, show limited CTL response profiles. By this criterion, some groups, especially the Alaskan and Australian Indigenous peoples, would be particularly vulnerable to H7N9 infection. This dissection of CTL-mediated immunity to H7N9 thus suggests strategies for both vaccine delivery and development.

CD8 T cells | HLA types

Emerging unexpectedly in February 2013, the H7N9 influenza A virus (IAV) has thus far caused 137 human infections with 45 deaths (1). Clinical manifestations include major respiratory compromise, multiorgan failure, and exceedingly high serum cytokine and chemokine levels (2). Although May through September saw only five such cases, two more were recorded in October (1), indicating that H7N9 may return during the northern winter. Furthermore, the presence of a natural avian reservoir and the severity of the disease emphasized the need to focus on protective immunity. Most patients had contact with poultry within a week before clinical onset (2), suggesting that domestic birds are the source (2, 3). Even so, the potential for person-to-person spread is highlighted by ferret experiments (4) and instances of infection via close family contact (3). A very real concern is that further mutations may facilitate human-to-human transmission (5).

Evidence from animal (6) and human studies (7–9) suggests that, in the absence of neutralizing antibodies (NAbs), preexisting memory CD8+ T lymphocytes (CTLs) directed at conserved and/or cross-reactive IAV peptide + class I HLA (pHLA1) epitopes can diminish disease severity. The recall of IAV-specific CTLs promotes recovery manifested by milder symptoms, diminished virus shedding and transmission (6, 7). A comprehensive analysis of the 2009 pandemic H1N1 IAV (H1N1pdm-2009) indicated that CTL memory provided some protection for the antibody naïve (9). Thus, cross-reactive CTL memory generated after a prior encounter with seasonal or pandemic IAVs, or by a CTL-directed vaccine, could potentially limit the severity of an H7N9 pandemic.

The present analysis probes the extent of preexisting CTL immunity in populations that have not been exposed to the H7N9 virus. This potential for CTL recall is defined for HLA1s that are differentially prominent in various ethnicities. Using an evolutionary and immunological approach, we show substantial levels of immunogenic peptide conservation for nucleoprotein (NP) and matrix-1 (M1), with estimated coverage according to known HLA1 presentation profiles ranging between 16% and 57% of the global population. Overall, the findings support the view that it is important to consider developing vaccines with a T cell–based component that has the potential to protect against severe novel IAV infections. Furthermore, given that some ethnicities, including Australia's Indigenous and Alaskan people, show evidence of a diminished HLA1-related response capacity, it is essential that health policy development and planning gives such groups priority in IAV vaccination campaigns. The 2009 H1N1 pandemic caused higher attack rates and morbidity among Indigenous populations in the Americas, New Zealand, and Australia (10).

Results

Conservation of CTL Antigenic Regions in the Novel H7N9 Virus. The first step was to establish which known immunogenic IAV

Significance

The severity of the novel H7N9 influenza A virus (IAV) and the lack of neutralizing antibodies raise real pandemic concerns. In this scenario, CD8+ T lymphocytes (CTLs) may provide a layer of protection against the H7N9 virus. Our study dissects the extent of preexisting CTL immunity with the potential to respond to H7N9. We identified conserved immunogenic peptides with the capacity to elicit robust CTL responses against any human IAV, including the H7N9 virus, as well as the mutations that abolish CTL recognition. The human leukocyte antigen class I molecules that present these peptides vary in prevalence depending on the ethnicity. Such analyses found that the Alaskan and Australian Indigenous people may be particularly vulnerable to the H7N9 influenza disease.

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peptides are conserved within H7N9, using IAVs that caused major human pandemics or epidemics as a reference (Table S1). Included were the pandemic H1N1-1918 A/Brevig Mission/1, H2N2-1957 A/Japan/305, H3N2-1968 A/Hong Kong/16 and H1N1-2009 A/Auckland/viruses, and seasonal H1N1 IAVs from 1933, 1983, and 2006. Our focus was on peptides from the prominent NP and M1 (11, 12) proteins, as identified in the Immune Epitope Database (www.immuneepitope.org). We found a substantial level of conservation within NP (Fig. 1A) and M1 (Fig. 1B) with respect to H7N9-derived pHLA1 epitopes (Fig. 1). Analysis of 76 NP peptides established that 12 were conserved (Table S2), whereas 18 were unique to H7N9 and had not been found previously (Table S3). The remaining 46 were classified as variable (Fig. 1A), meaning that they are shared by at least one IAV isolated before the advent of H7N9-2013 (Table S4). Evidence of such conservation was even higher for M1 (Fig. 1B). Of 39 peptides, 20 were conserved, 9 were unique to H7N9, and 10 variable (Tables S5–S7). Remarkably, all 14 immunogenic peptides within $M1_{47–88}$ have been conserved over the last century, suggesting that this M1 region could be a target for a universal CTL vaccine.

Mapping Conserved NP and M1 Peptides Across All IAV Lineages. To confirm the conservation of the NP and M1 peptides across all human IAVs, including H1N1 (1918–1957, 1977–2009, and 2009–2013), H2N2 (1957–1968), and H3N2 (1968–2013), we identified the established, nonsynonymous amino acid (aa) changes (Fig. 2, gray bars). Then, immunogenic CTL peptides were deposited onto this map, according to the conserved, unique, and variable nomenclature (Tables S2–S7). The epitopes within NP and M1 that do not fall on the variable bars show regions that have not evolved in human IAVs over the last century, indicating that they are functionally important for virus survival. Interestingly, this confirms that the conserved epitopes, identified in Fig. 1, have not changed in other human viruses (except for NP_{259}; in green). Conversely, the variable (red) and unique (blue) epitopes are found predominantly in the variable-gray regions (Fig. 2). Phylogenetic analysis highlights the avian origins of the H7N9 NP and M1 genes (Fig. S1). Interestingly for M1, H7N9 is closely related to the H1N1pdm-2009 IAVs that are well established in humans (Fig. S1B), indicating a higher prevalence of shared peptides than for NP. The close phylogenetic relationship to avian IAVs further suggests that CTL epitope–based vaccines designed for H7N9 might confer protection against other avian IAVs (H5N1 and H9N2) that occasionally infect humans.

Recall Potential of Memory CTLs Specific for Conserved H7N9 Peptides. Based on the conservation analysis (Figs. 1 and 2), we dissected human CTL immunity toward the H7N9 IAV by probing reactivity

to conserved, unique, and selected variable immunogenic peptides. We first characterized the recall potential of preexisting memory pools specific for conserved H7N9 peptides. The analysis focused predominantly on NP, the major target for immunodominant CTL responses (11) and the highly conserved, immunodominant A*0201-restricted $M1_{58–66}$. The conserved NP epitopes included $A*0301-NP_{265–273}$, $B*2705-NP_{383–391}$, $B*5701-NP_{199–207}$, $B*1801-NP_{219–228}$, $B*0801-NP_{225–233}$, $B*0702-NP_{172–181}$, and $A*2402-NP_{39–47}$ (Fig. 3). We classified the WT form of the $NP_{383–391}$ peptide (found in H7N9) that binds B*2705 as conserved, although an escape mutant is prominent in H3N2 strains (13). To unravel the recall potential of preexisting CTL memory to the H7N9 virus, peripheral blood mononuclear cells (PBMCs) obtained from healthy adults expressing a spectrum of HLAs were stimulated with the relevant conserved antigenic peptides for 10 d. The presence and frequencies of peptide-specific CTLs across multiple donors were then determined by an IFN-γ/TNF-α production (Fig. 3).

Our data show CTL responses to six of eight conserved epitopes: $A*0301-NP_{265}^+$, $A*0201-M1_{58}^+$, $B*2705-NP_{383}^+$, $B*5701-NP_{199}^+$, $B*1801-NP_{219}^+$, and $B*0801-NP_{225}^+$ (Fig. 3 A–F). For these immunogenic peptides, all donors ($n = 42$) displayed specific CTL responses. In contrast, we did not detect any CTLs specific for $B*0702-NP_{172}$ or $A*2402-NP_{39}$ (Fig. 3 G and H), both classified as conserved. This suggests that, although the peptides may be conserved, these are weak epitopes that do not elicit CTL reactivation. At least for B*0702, this could reflect preferential presentation of immunodominant (but highly variable) variants of NP_{418} (14) (see below). As a consequence, $B*0702^+NP_{172}$ may be subdominant and unlikely to play a major role in IAV-specific CTL immunity.

Most of these memory CTL responses to the conserved pHLA1s, $A*0301^+NP_{265}$, $A*0201^+M1_{58}$, $B*2705^+NP_{383}$, $B*5701^+ NP_{199}$, $B*1801^+NP_{219}$, and $B*0801^+NP_{225}$ (Fig. 3) displayed a robust functional potential and were detected at frequencies comparable to those found for the prominent $A*0201^+M1_{58–66}$ CTL epitope (Fig. 3B). This indicates that a substantial proportion (16–57%; Table 1) of the human population should have preexisting CD8+ CTLs that can respond to the H7N9 IAV. With ethnic differences in mind, we estimated population coverage based on the HLA types that present known, conserved immunogenic H7N9 NP and M1 peptides. Clearly, the extent of such CTL immunity to H7N9 varies considerably across ethnicities (African, 37%; Caucasoid, 57%; Oriental, 37%; Amerindian, 36%; Indigenous Alaskans and Indigenous Australian, 16%). This suggests that both the potential to recruit established CTL immunity and disease severity could show a clear ethnic bias in the face of an H7N9 pandemic.
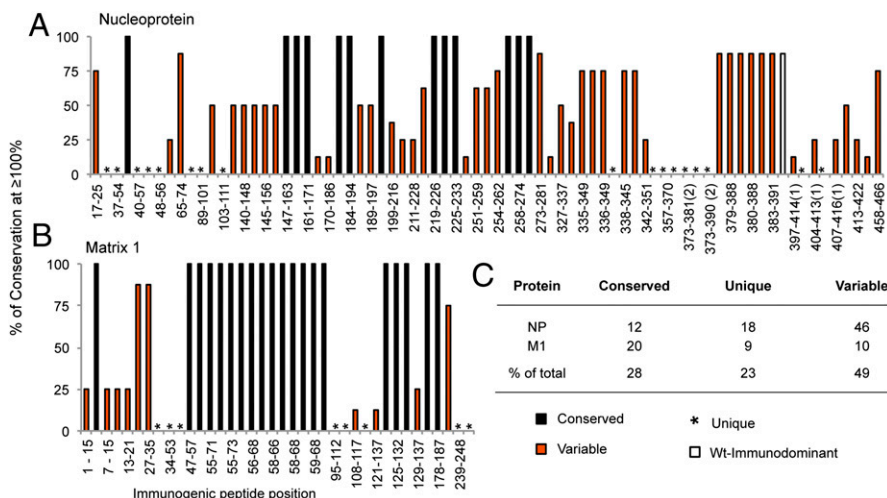

Fig. 1. High level of conservation for H7N9 CTL peptides. CTL antigenic peptides within (A) NP and (B) M1 were obtained from the Immune Epitope Database (IEDB, www.iedb.org; April 2013) and analyzed using the IEDB's Epitope Conservancy Analysis tool (http://tools.immuneepitope.org/tools/conservancy/iedb_input). (C) Summary of numbers and percentages of conserved, unique and variable epitopes within NP and M1. Conservation at 100% match was determined by comparing the corresponding CTL peptides in H7N9 to those of representative strains that have circulated in the human population (Table S1). Black, CTL peptides conserved over the last century; red, variable epitopes; *, unique CTL peptides for the H7N9 IAV; white, conserved $H7N9-NP_{383}$ peptide that binds to HLA-B*2705 (escape mutants were identified in H3N2 strains for NP_{383}).
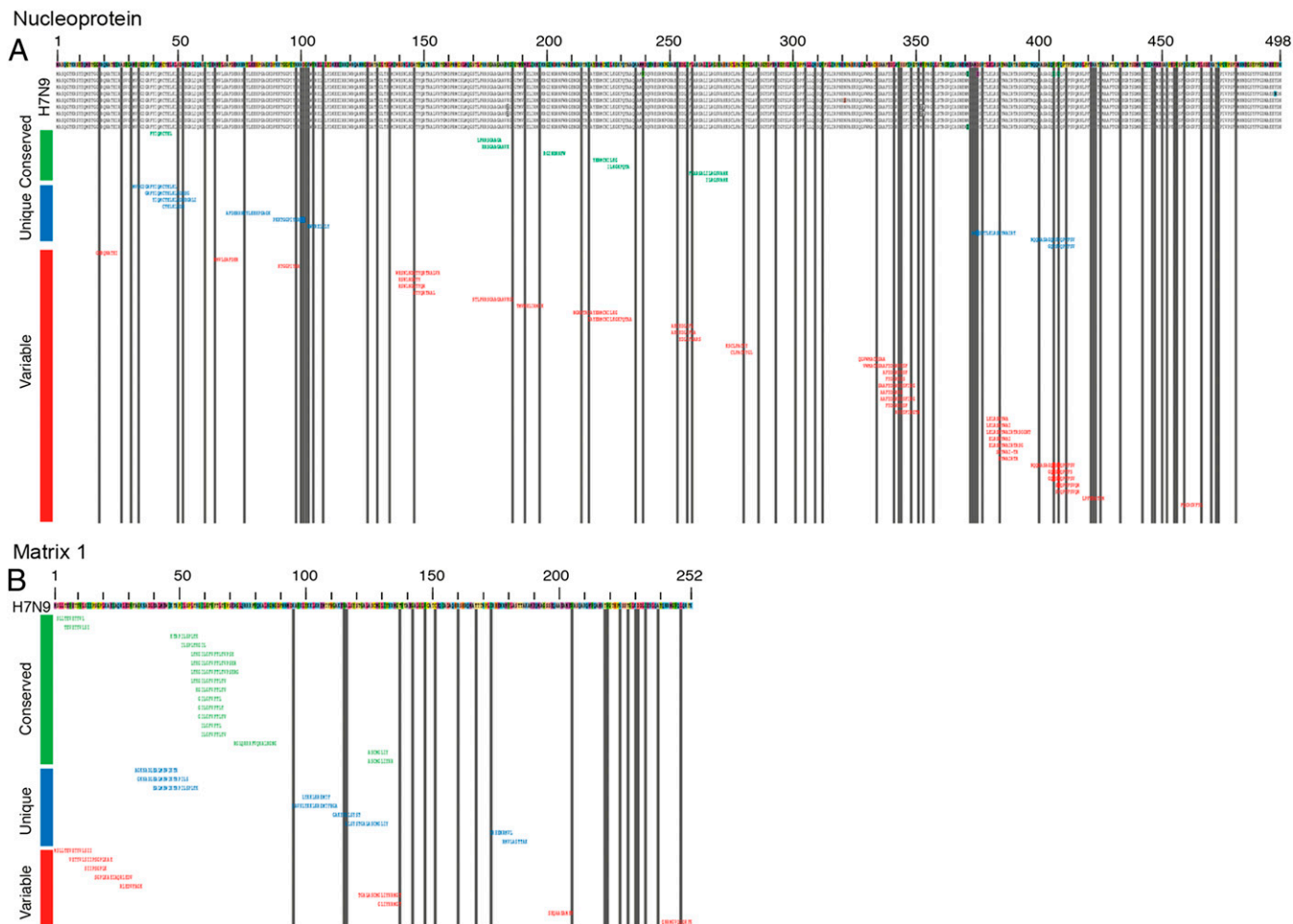
Fig. 2. CTL peptide map for NP and M1 across all human IAV lineages. The analyses spanned the full protein-coding region of the NP and M1 proteins to deduce changes in the conserved, unique and variable epitopes (Tables S2–S7). Green, blue, and red bars on the left of the peptides refer to conserved, unique, and variable CTL peptides, respectively. The horizontal gray bars throughout the alignments highlight the nonsynonymous substitutions established in H1N1, H2N2, and H3N2 viruses through their evolutionary history in human population. CTL peptides within (*A*) NP and (*B*) M1, which do not fall on the gray bars, show regions that have not changed in human influenza A viruses (the exception being NP_{259}), indicating lesser selection pressure on those sites.

Lack of Established CTL Responses to Unique H7N9 Peptides. We then examined CTL responses to the antigenic peptides unique to the H7N9 virus (Table S3). Although human populations that have not previously encountered H7N9 would likely see these pHLA1s as novel, there is also the possibility that there could be some "plasticity" in the cross-recognition of antigenic variants (15, 16).

We thus screened unexposed individuals for responses to unique H7N9 epitopes, $A*0101-NP_{44–52}$ (Fig. 4 *A* and *B*), $A*6801-NP_{89–101}$ (Fig. 4*G*), and the $NP_{37–54}$, $NP_{373–390}$, and $NP_{397–414}$ presented by $B*1501$ (Fig. S2). PBMCs expressing a spectrum of HLAs were cultured with either the H7N9 peptide(s) or peptides from other human IAVs. Analysis of the normally immunodominant

Table 1. Estimation of the population coverage according to the HLA restriction of conserved epitopes in H7N9

Peptide(s)	Restriction	Population coverage across ethnicities						
		Caucasoid*	North American natives[†]	Oriental*	African*	Amerindian*	Alaskan Yupik[‡]	Australian Aboriginals[§]
$M1_{58–66}$	HLA-A2	25	21.66	27.17	15.76	24.78	2.3	12.7
$NP_{265–273}$	HLA-A3	11.9	6.6	3.26	6.48	3.98	0.1	1.4
$NP_{383–391}$	HLA-B27	3.71	8.5	3.62	1.46	4.98	13.28	0.1
$NP_{199–207}$	HLA-B57	2.91	3	1.33	3.96	0.68	0	0.5
$NP_{219–228}$	HLA-B18	6.31	2	0.92	4.62	0.5	0.6	0.2
$NP_{225–233}$	HLA-B8	7.41	3.7	1.4	4.83	1.1	0.4	1.2
	Total	57.24	45.46	37.7	37.11	36.02	16.7	16.1

Percentages based on HLA coverage for the relevant HLA supertype.
*From ref. 31.
[†]From ref. 9.
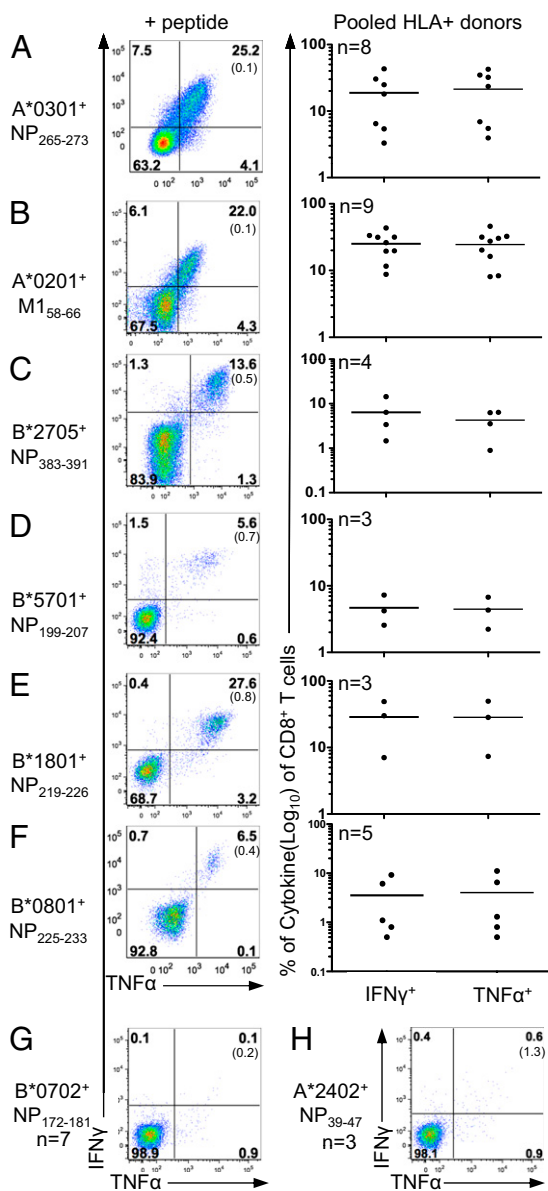[‡]From ref. 7.
[§]From ref. 32.

Fig. 3. CTL responses to conserved immunodominant H7N9 peptides. PBMCs from healthy donors were peptide-stimulated and cultured for 10 d. CTL responses were determined by an IFNγ/TNFα intracellular cytokine staining (ICS). Representative FACS plots for (*A*) A*0301+NP$_{265}$, (*B*) A*0201+M1$_{58}$, (*C*) B*2705+NP$_{383}$, (*D*) B*5701+NP$_{199}$, (*E*) B*1801+NP$_{219}$, (*F*) B*0801+NP$_{225}$, (*G*) B*0702+NP$_{172}$, and (*H*) A*2402+NP$_{39}$ are shown. Values for no peptide are in brackets. Graphs show pooled data from multiple donors. Background (no peptide controls) was subtracted.

A*0101-NP$_{44–52}$ showed that the H7N9-specific substitution at p9 (Y9N) was not recognized by CTLs that respond to either H1N1pdm-2009 NP$_{44}$ or to the seasonal NP$_{44}$-S7N variant (Fig. 4 *A* and *B*). Furthermore, preestablished HLA-A*0101+NP$_{44}$-specific memory CTLs do not recognize the Y9H variant of H3N2, indicating that that any mutation at p9 leads to immune escape.

To understand the molecular basis of the cross-reactivity (with WT) of H7N9-NP$_{44}$-S7N vs. evasion by the H7N9-NP$_{44}$-Y9N and H7N9-NP$_{44}$-Y9H variants, we analyzed thermal stability and crystal structures for HLA-A*A0101 in complex with NP$_{44}$ variants (Fig. 4 *C–F*). The NP$_{44–52}$ (WT) and NP$_{44}$-S7N are comparable in their capacity to stabilize the HLA-A*0101 molecule, with a thermal melt point of 58.2 °C and 59.5 °C (Fig. 4*C*). However, the p9 mutations reduced the stability of the pHLA1 by 10 °C

(Fig. 4*C*), most likely as a result of the large, aromatic Tyr being replaced with a smaller Asn or a charged His. In addition, the refold yield of HLA-A*0101 in the presence of NP$_{44}$-Y9H and NP$_{44}$-Y9N was decreased by 50×, thereby precluding structural studies. Thus, the reduced thermal stability of the NP$_{44}$-Y9N (H7N9) and NP$_{44}$-Y9H (H3N2) complexes likely results in reduced peptide presentation and immunogenicity. Further determination of HLA*A0101 crystal structures allowed precise definition of the cross-reactivity between the NP$_{44}$-WT and the seasonal NP$_{44}$-S7N. Structural analysis (Table S8) of NP$_{44}$-WT and NP$_{44}$-S7N bound to HLA-A*0101 (resolution of 2.4 and 2.0 Å) show that NP$_{44}$-WT adopts a classical extended conformation in the antigen-cleft of HLA-A*0101 (Fig. 4*D*) (17). The P2-Thr and P9-Tyr are buried, acting as anchor residues along with the P6-Leu. The P3-Glu is partially buried in the D pocket and forms a salt bridge with the Arg156. The P4-Leu, P5-Lys, and P8-Asp are solvent exposed and represent potential contact points for the T cell receptor (TCR) (Fig. 4*D*). The substitution at p7 Ser→Asn did not affect the conformation of the peptide within the antigen-binding cleft (Fig. 4*E*). Overall, analyzing stability and structure for the NP$_{44}$ variants shows that, although NP$_{44}$-S7N does not change either parameter when bound to HLA-A*0101, the variations at p9 within NP$_{44}$-Y9N-H7N9 or NP$_{44}$-Y9H-H3N2 drastically decrease the stability of the pHLA1 complex, with a consequent loss of T-cell recognition.

To understand whether CTLs can recognize any of the other, unique H7N9 peptides, we analyzed those with the capacity to bind A*6801 and B*1501. It seems that, although CTLs are induced by a spectrum of IAV NP$_{89}$ variants, the A*6801-NP$_{89}$-H7N9 is not recognized by these memory sets (Fig. 4*G*). Thus, H7N9-NP$_{89}$ is an escape variant in the 1–9% of the population that expresses HLA-A*6801. Similarly, the NP$_{37}$, NP$_{373}$, and NP$_{397}$ presented by HLA-B*1501 did not induce any responses (Fig. S2), although IAV-specific CTLs directed at other immunodominant epitopes, HLA-A*0301-NP$_{265}$ (Fig. S2; D15-1 and D15-2) and HLA-A*0201 M1$_{58}$ (Fig. S2; D15-3), were readily detected.

Overall, our analysis of the potentially immunogenic NP and M1 peptide variants unique to H7N9 indicates that individuals expressing HLA-A*0101, HLA-A*6801, and HLA-B*1501 will lack preexisting memory CTLs capable of recognizing epitopes defined by those HLA1 types. The H7N9-NP$_{44}$-Y9N variant is within an immunodominant epitope in HLA-A*0101+ individuals, with this immunoevasion affecting the 1–14% of individuals with that HLA type, depending on ethnicity. In contrast, the nonantigenic A*6801-NP$_{89}$ H7N9 variant would affect 1–9% of the population expressing A*6801. We found no CTL responses to any of the B*1501 variants tested (Fig. S2).

CTL Cross-Reactivity for the Variable B*3501- and B*0702-NP$_{418}$ Epitope.
Having assessed preexisting CTL immunity to conserved and unique (to H7N9) epitopes, we then analyzed the peptides that can be shared with H7N9 and are variably expressed in different IAVs (Table S4). The extent of any such cross-reactivity would depend on the influenza infection history. Interestingly, the variable peptides within H7N9 most closely resemble those of the pandemic H1N1-1918 virus (Table S4). This is evident from the minimal aa differences in key peptides from the H7N9 and H1N1-1918 strains (Fig. S3). Indeed, the H7N9 variant of the immunodominant NP$_{418}$ peptides presented by the large B7 family (15, 16) was identical to that within the 1918-H1N1 virus and closely resembled that from H1N1pdm-2009 (Table S4). In agreement with our previous data (16), this H7N9-NP$_{418}$ variant is not recognized by memory CTL specific for the various seasonal influenza types from the last decades (Fig. S4*B*). Tetrameric complexes of HLA-B*0702 and HLA-B*3501 with different NP$_{418}$ peptides were used to stain PBMCs stimulated in vitro with a pool of 12 NP$_{418}$ variants. The H7N9 variant of the B*3501-NP$_{418}$ tetramer showed none to minimal cross-reactivity for CTLs stimulated with the NP$_{418}$ peptides expressed by recently circulating IAVs (Fig. S4). Prior exposure to the pandemic H1N1pdm-2009 may, however, give some protection to B*0702+ individuals (Fig. S4*A*).
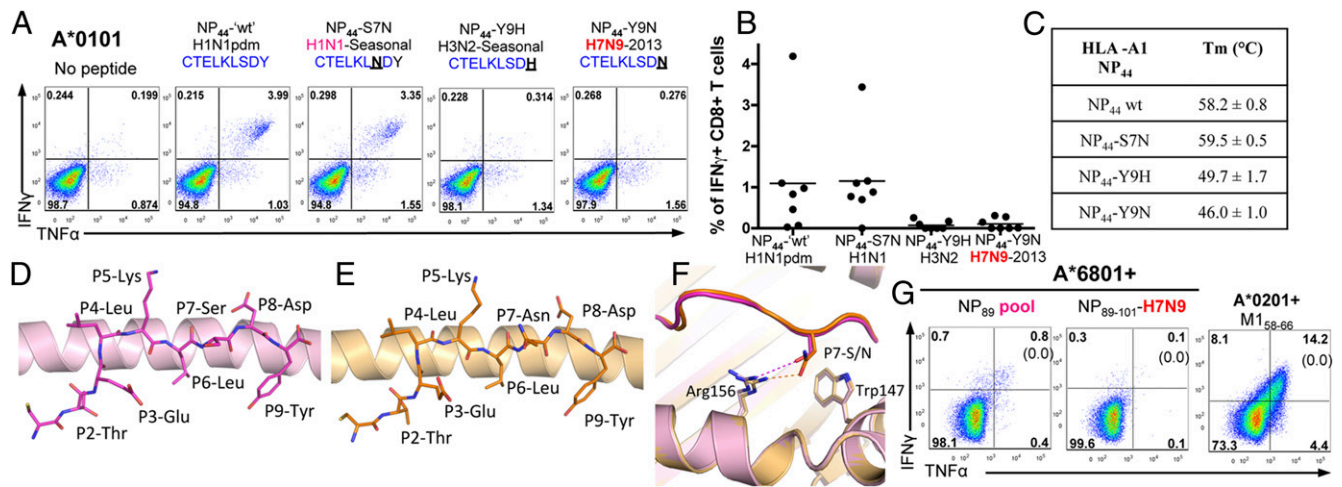
Fig. 4. H7N9 escape mutants for A*0101-NP$_{44}$ and A*6801-NP$_{89}$. (*A–F*) The Y9N mutation in the immunodominant H7N9 NP$_{44}$ peptide abrogates CTL recognition by reducing thermal stability. (*A*) Representative FACS plots for CTL responses to different A*0101-NP$_{44}$ variants. (*B*) CTL responses (IFNγ ICS, $n = 7$) against four NP$_{44}$ variants. (*C*) Thermal stability for the A*0101-NP$_{44}$ variants. (*D* and *E*) Crystal structures of HLA-A*0101 (cartoon) bound to the NP$_{44}$-WT peptide (pink) and to the NP$_{44}$-S7N peptide (orange), respectively. Only the α1-helix of the HLA is shown for clarity. (*F*) Superposition of the HLA-A*0101 binding cleft to NP$_{44}$-WT (pink) and NP$_{44}$-S7N (orange), with the Arg156 and Trp147 of the HLA represented in stick; the H bond shown as dashed lines. (*G*) An ICS response to a unique H7N9 peptide NP$_{89}$ restricted by HLA-A*6801, following stimulation with the NP$_{89}$-H7N9 variant or a pool of NP$_{89}$ seasonal and pandemic variants (Table S4). Confirmation of prior IAV exposure was determined by assessing the reactivity to A*0201-M1$_{58}$.

Thus, our data suggest that there is the potential for a rapidly spreading H7N9 IAV to recall robust, immunodominant, CD8$^+$ CTL memory in substantial numbers of people (average of 35% across multiple ethnicities, range 16–57%; Table 1). Even so, although that may ameliorate influenza disease and reduce viral spread for some individuals and groups, it is also the case that such cross-reactivity is not found for certain HLA types that are prevalent in what may be extremely vulnerable populations.

Discussion

Although human cases of the newly emerged H7N9 virus are thought to have resulted primarily from contact with infected birds, the genetic characteristics of this virus raise real concerns that a mutant could readily emerge to cause efficient human-to-human spread. As we have no prior history with this pathogen, individuals of all ages would be susceptible. In the absence of protective NAbs, evidence from both human studies and animal experiments suggests that IAV-specific CD8$^+$ CTL immunity promotes more rapid recovery and milder disease (6–9). Hence, cross-reactive CTL memory pools generated by previous infection with seasonal or pandemic IAVs could potentially provide some protection against an H7N9 pandemic. The present analysis focuses on the capacity of CD8$^+$ T cells primed by infection with currently circulating and past H1N1 and H3N2 IAVs to respond to peptides that are shared with, or unique to, the H7N9 virus. Our sequence, phylogenetic, and immunologic analyses show that 28% of the CTL peptides within the immunogenic NP and M1 proteins are conserved between the IAVs of interest, 49% are found variably in that spectrum, and 23% are unique to H7N9. Studies are underway to understand whether the stability of certain regions within the viral NP and M1 results from ineffective CD8$^+$ T-cell immunity or the functional necessity for the influenza virus.

Perhaps of greater significance is the recognition that some HLA1 alleles present conserved IAV peptides. Strong representation of HLA A*0201, A*0301, B*5701, B*1801, and B*0801 in any ethnic group is predictive of preexisting CTL memory, and thus protection, following challenge with a novel IAV. Conversely, individuals with HLA-A*0101, A*6801, B*1501, and A*2402 may have little, if any, evidence of established CTL immunity to, for example, the H7N9 virus. Interestingly, HLA1 has been the one parameter repeatedly associated with HIV control, with B57 and B27 being the most protective, whereas the converse is true for

B*35 and B*53 (18). Although we have tested preexisting CD8$^+$ T-cell immunity to H7N9 in a vast number ($n = 59$) of samples ($n = 42$ for Fig. 3; $n = 10$ for Fig. 4; $n = 7$ for Figs. S1–S4), the donors displayed a number of HLAs, which made the sample size for each HLA smaller. Further studies need to dissect influenza-specific CTLs in a larger number of donors corresponding to specific HLAs across different ethnicities.

The present analysis thus adds to other insights indicating that the impact of established IAV CTL immunity should be analyzed across different ethnicities (19). Thus far, H7N9 infection has been limited to the ethnic Chinese population. The overall conservation of CTL antigenic peptides within H7N9 is 35%, ranging between 57% (Caucasoid), 38% (Oriental), 37% (African), 16% (Australian Aboriginals), and 16% (Alaskan Natives), making the latter two groups most vulnerable to H7N9 challenge. This is consistent with the high adult mortalities (up to 100%) for isolated Alaskan villages in the 1918–1919 pandemic (20). Similarly, the Indigenous Australians were highly susceptible to the A/H1N1 pandemic viruses in 1918 (21) and 2009 (10). As many as 10–20% died in 1919 (21) vs. <1% of other (predominantly Caucasian) Australians. Hospitalization and morbidity rates for the Indigenous were also greatly increased in the recent 2009 A/H1N1 pandemic (22, 23), with 16% of hospitalized patients being from those communities. Although this may reflect a combination of factors, including household crowding, a high prevalence of comorbidities, and difficulties in accessing healthcare, the relative lack of HLA1s that present conserved IAV peptides may also be a contributing factor.

Close to 50% of the immunogenic H7N9 NP peptides are found with variable prevalence in other IAVs known to have established CTL memory in human populations. With both the H1N1pdm-2009 virus and H7N9, it is intriguing that the variable CTL peptides more closely resemble those from the pandemic H1N1-1918 than from recently circulating H1N1 (before 2009) and H3N2 influenza strains. Sequence analysis of the "resurrected" H1N1-1918 influenza virus indicates that this pathogen was, indeed, avian derived. It also seems that the 1918 NP survived and remained stable in the swine influenza reservoir, to emerge again in the H1N1pdm-2009 virus. Similarly, evolutionary analysis of H7N9 shows that all gene segments are of avian origin (24).

From our analysis, it seems that memory CTLs specific for prominent variable peptides (like NP$_{418}$) from recently circulating

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seasonal strains would not recognize a large proportion of the variable H7N9-derived peptides in any pandemic situation. A major variant that does not stimulate preexisting CTLs is the H7N9-Y9N substitution of HLA-A*0101-NP$_{44}$. This mutation to an anchor residue at peptide P9 greatly destabilizes the pHLA complex, compromises CTL binding/accessibility, and can lead to viral escape, similar to what occurs in a mouse model (25).

Overall, the level of CTL peptide conservation within the H7N9 NP and M1 proteins appears to be lower than the 70–80% found previously for the swine-derived H1N1pdm-2009 (26). Lower CTL epitope conservation may partly explain the relative severity of H7N9 influenza in ethnic Chinese, with 37% population coverage of cross-reactive HLA types. Infection outcomes in known H7N9 cases were far from uniform. Some recovered within a few days, whereas others required steroid treatment, intensive care unit admission (75%), and mechanical ventilation (86%) (27). In all, 34% of hospitalized patients ultimately died. It is highly possible that some of the difference in outcomes was influenced by the extent of cross-reactive CTL memory.

Materials and Methods

Donors and PBMC Isolation. PBMCs were obtained from 52 donors: HLA-A*0101$^+$($n = 7$ donors), A*0201$^+$ ($n = 9$ donors), A*0301$^+$ ($n = 8$ donors), B*2705$^+$ ($n = 4$ donors), B*0702 ($n = 7$ donors), B*5701 ($n = 3$ donors), B*1801 ($n = 3$ donors), B*0801 ($n = 5$ donors), A*6801 ($n = 3$ donors), B*2402 ($n = 3$ donors), and B*15:01 ($n = 3$ donors) healthy individuals, after informed consent was obtained. HLA genotyping was done at the Victorian Transplant and Immunogenetics Service (West Melbourne, Australia). The experiments were conducted according to the Australian National Health and Medical Research Council Code of Practice and approved by the University of Melbourne Human Ethics Committee.

Epitope Conservation Analysis. H7N9 NP and M1 proteins sequences from the Global Initiative on Sharing All Influenza Data (GISAID, www.gisaid.org) and the epitope data from the Immune Epitope Database (www.immuneepitope.org, accessed July 2013) were used to map antigenic CTL regions within the immunogenic internal influenza proteins NP (76 epitopes) and M1 (39 epitopes), as described in *SI Materials and Methods*.

T-Cell Restimulation, Intracellular Cytokine Assay, and Tetramer Staining. PBMCs were stimulated with NP- and M1-derived peptides for 10 d, followed by the analysis of influenza-specific CTLs by a 5-h ICS or tetramer staining, as described in *SI Materials and Methods*. Based on the conservation sequence analysis (Tables S2–S7), CTL reactivity to H7N9 was assessed to a total of 39 NP and 1 M1 immunogenic peptides: the conserved M1$_{58}$, 8 conserved NP peptides, 7 H7N9 unique, and 26 variable NP peptides.

Protein Expression, Purification, Crystallization, and Thermal Stability. HLA-A*0101–soluble HLA1 heterodimers containing NP$_{44}$ peptides were prepared, crystallized, and structures solved as described in *SI Materials and Methods*. The coordinates have been submitted to the Protein Data Bank (PDB) (ID codes 4NQV for NP44-A1 and 4NQX for NP44-S7N. Molecular graphics representations were created using PyMol (28). To assess the effect of peptide mutations, we tested the stability of each pHLA complex (29) using a thermal shift assay (*SI Materials and Methods*).

Phylogenetic Analysis and Deduction of Ancestral Nonsynonymous Substitutions. Maximum likelihood analysis was performed for the full protein coding genes of the NP and M1 using the general time reversible substitution model with the γ-shaped rate variation in RaXML v7.7 (30). Ancestral nonsynonymous substitutions along the branches of the NP and M1 protein gene trees were deduced using the baseml program in PAML.

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