Conservation and Diversity of Influenza A H1N1 HLA-Restricted T Cell Epitope Candidates for Epitope-Based Vaccines

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Abstract

Background: The immune-related evolution of influenza viruses is exceedingly complex and current vaccines against influenza must be reformulated for each influenza season because of the high degree of antigenic drift among circulating influenza strains. Delay in vaccine production is a serious problem in responding to a pandemic situation, such as that of the current H1N1 strain. Immune escape is generally attributed to reduced antibody recognition of the viral hemagglutinin and neuraminidase proteins whose rate of mutation is much higher than that of the internal non-structural proteins. As a possible alternative, vaccines directed at T cell epitope domains of internal influenza proteins, that are less susceptible to antigenic variation, have been investigated.

Methodology/Principal Findings: HLA transgenic mouse strains expressing HLA class I A*0201, A*2402, and B*0702, and class II DRB1*1501, DRB1*0301 and DRB1*0401 were immunized with 196 influenza H1N1 peptides that contained residues of highly conserved proteome sequences of the human H1N1, H3N2, H2N2, H5N1, and avian influenza A strains. Fifty-four (54) peptides that elicited 63 HLA-restricted peptide-specific T cell epitope responses were identified by IFN-γ ELISpot assay. The 54 peptides were compared to the 2007–2009 human H1N1 sequences for selection of sequences in the design of a new candidate H1N1 vaccine, specifically targeted to highly-conserved HLA-restricted T cell epitopes.

Conclusions/Significance: Seventeen (17) T cell epitopes in PB1, PB2, and M1 were selected as vaccine targets based on sequence conservation over the past 30 years, high functional avidity, non-identity to human peptides, clustered localization, and promiscuity to multiple HLA alleles. These candidate vaccine antigen sequences may be applicable to any avian or human influenza A virus.

Introduction

Influenza A viruses are major pathogens of avian origin with global spread and rapid mutational change, some of which also infect humans and other mammals. Of particular concern are the several ways a human influenza pandemic could emerge. One is through the occurrence of a novel and highly pathogenic zoonotic strain capable of infecting humans, such as the H5N1 avian pathogen. Another possibility is through mutation from a mild to a more pathogenic human transmissible strain, such as mutation to the current H1N1 strain. The most threatening is mutations giving rise to a new highly transmissible-and-pathogenic human strain, as occurred with the original 1918 Spanish influenza. In any event, history teaches us that a vaccine to prevent a new influenza A pandemic must be effective against all future forms of the virus. Influenza A viruses are single stranded, negative-sense RNA viruses belonging to the family Orthomyxoviridae. The genome is composed of 8 RNA strands of about 13,500 bases, encoding at least ten viral proteins. The viral envelope is a lipid bilayer, consisting of the interior matrix protein 1 (M1) and three exterior transmembrane proteins: hemagglutinin (HA), neuraminidase (NA), and matrix protein 2 (M2). The viral core contains viral ribonucleoprotein complex particles, consisting of viral RNA, nucleoprotein (NP), and three polymerase proteins (PB1, PB2, and PA). Mutation in the viral RNA genome occurs by two mechanisms, known as antigenic drift and antigenic shift.
Antigenic drift is the frequent occurrence of point mutations resulting from defects in RNA replication mechanisms, while antigenic shift is less frequent, involving re-assembly of the RNA segments arising from exchanges between different strains in host cells infected by multiple viruses.

Protection by current human influenza vaccines is achieved by use of inactivated or attenuated forms of the corresponding pathogen and appears to require the function of neutralizing antibodies against the external HA and NA glycoproteins. However, these glycoproteins mutate rapidly through antigenic drift and current vaccines become ineffective as mutational differences accumulate in the circulating strains. In order to overcome the antigenic variability of influenza external glycoproteins, new vaccine strategies are increasingly directed at conserved regions of the viral proteins for production of T cell epitope-based vaccines. The goal is to identify conserved sequences that function as epitopes recognized by human leukocyte antigen (HLA) molecules for presentation to CD8+ and CD4+ T cells that provide immunity against all influenza A virus subtypes and obviate the need for yearly vaccine update. Several animal model studies taking this approach have reported T cell responses that reduce morbidity and promote recovery in mouse models of influenza challenge [1–4]. Both CD8+ and CD4+ T cell responses are required; CD8+ T cells to kill infected cells [5,6] and CD4+ T cells for the development of an effective immune response and immune memory [7–9]. A complication of cellular immunity is that T cell responses are dependent upon antigen presentation by highly polymorphic HLA molecules that vary greatly among human populations. However, the limited population coverage of some HLA alleles may be alleviated by focusing on T cell epitopes recognized by HLA supertypes that bind largely overlapping peptide repertoires on the basis of the specificity for the main anchor positions of the presented peptides [10,11]. We previously reported a detailed study of evolutionarily conserved sequences of all human and avian influenza A viruses that were recorded over the past 30 years (36,343 sequences) [12]. Fifty-four (54) sequences, ranging from 9 to 58 amino acids (aa) of the PB2, PB1, PA, NP, and M1 sequences were conserved in at least 80%, and in most cases 95–100% of all recorded human H1N1, H3N2, H1N2, and H5N1, and avian subtypes. These sequences have remained evolutionarily stable for all recorded human and avian influenza A viruses during the past decades, and are thus prime candidates for the development of T cell epitope-based vaccines against multiple influenza strains. However, the function of these conserved sequences as HLA-restricted T cell epitopes and the incidence of variant sequences in association with the conserved sequences were not known. Herein, we have focused on the identification and characterization of peptides of influenza virus A/New York/348/2003 (H1N1) that contain conserved sequences and elicit HLA-restricted T cell responses. HLA transgenic mice (HLA-A2, -A24, -B7, -DR2, -DR3, and -DR4) were immunized with the selected peptides. The peptides that elicited T cell activation by IFN-γ ELISpot assay and thus contained T cell epitopes were selected and analyzed for properties relevant to vaccine development, including evolutionary conservation and diversity, and correspondence to the 2007–2009 human H1N1 sequences.

Materials and Methods

Ethics Statement

Mice were maintained in a pathogen-free facility at the Johns Hopkins University according to IACUC guidelines.

Influenza Peptides

Peptide arrays of PB2 (BEI Cat.: NR-2616), PB1 (NR-2617), PA (NR-2618), NP (NR-2611), and M1 (NR-2613) of influenza virus A/New York/348/2003 (H1N1) were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (BEI). A total of 196 peptides (all 17 aa long except PB2368–383 and 393–408) were selected to fully cover all highly conserved sequences under study. Where these sequences spanned two or more 17 aa peptides, the consecutive peptides were overlapped by 11 aa (Figure S1). Two immunization peptide pools for immunization were formed: one composed of 84 PB2 and 13 M1 peptides (Table S1), and the second composed of 48 PB1, 23 PA, and 28 NP peptides (Table S2). Each of the 196 peptides was dissolved in 100% DMSO and constituted to 20% with sterile filtered water. The final concentration of each peptide was 2 µg/µl. The dissolved peptides were stored at −20°C.

HLA Transgenic Mice

Six different strains of HLA transgenic mice were used to cover HLA alleles of class I and class II supertypes. These six alleles were selected based on their prevalence in the Caucasian population and the availability of HLA transgenic mouse strains in our laboratory. The HLA class I supertypes studied were HLA-A2 (A*0201) mice expressing a chimeric heavy chain with murine α3 domain and human β2m. Both H-2Db and murine β2m genes were disrupted by homologous recombination [13]. HLA-A24 (A*2402) mice express a chimeric heavy chain and human β2m; the H-2Kb, H-2Db, and murine β2m genes were disrupted by homologous recombination (Lemonnier et al., unpublished), HLA-B7 (B*0702) mice express a chimeric heavy chain with the HLA-B*0702 α1 and α2 domains and the H-2Kb murine α3 domain [14]. The H-2Kb and H-2Db genes in HLA-B7 mice were inactivated by homologous recombination. The HLA-A2 and -B7 transgenic mice were kind gifts from Steve Pascolo (Institut Pasteur, France) and Pierre-Simon Rohrlich (Institut Pasteur, France), respectively.

The HLA class II supertypes studied herein were DR2 (DRB1*1501), DR3 (DRB1*0301), and DR4 (DRB1*0401) where HLA-DR2, -DR3, and -DR4 transgenic mice were kind gifts from Lars Fugger (Weatherall Institute of Molecular Medicine, Oxford, UK) and Arthur Vanderark (Oregon Health and Science University, Portland), Chella S. David (Mayo Clinic, Rochester), and Grete Sonderstrup (Stanford University School of Medicine), respectively. The peptide-binding domain of HLA-DR2 transgenic mice is encoded by human sequences, while the membrane proximal portion containing the CD14-binding domain is encoded by mouse sequences [DRA1*0101: I-Ez and DRB1*1501: I-EB] [15]. HLA-DR3 transgenic mice express HLA-DRA*0101 and -DRB1*0301 [16]. HLA-DR4 transgenic mice express HLA-DRA*0101, -DRB1*0401, and human CD4 [17]. The derivation and validation of the above transgenic mice has been described in the cited publications.

Immunization

Mice were immunized with the selected 196 peptides in 2 pools by use of a protocol which had been validated for T cell studies [18] and adapted for these transgenic mice studies. Peptides were pooled in matrices as described [19] and injected in groups of 9 mice of each transgenic strain; two for matrix array screening, two for identifying individual peptides, four for characterizing apparent functional avidity of T cells to positive peptides at three titration points: 10, 1, and 0.1 µg/ml peptide concentrations, and one as a control (adjuvant alone). Mice were injected subcutaneously at the base of tail with 100 µl of the immunization peptide.
IFN-γ ELISpot Assay

Harvested spleens from immunized transgenic mice were selectively depleted of CD8^+ or CD4^+ T cells by use of anti-CD8 or anti-CD4 antibody-coated immunomagnetic beads with LD columns (Milteny Biotec, Auburn, CA) according to the manufacturer’s protocol. The resulting CD8^+ or CD4^+ depleted splenocytes were tested individually by IFN-γ ELISpot assays against the 196 influenza peptides arranged in two 10×10 matrix arrays, resulting in 40 peptide pools, where each peptide was present in two different pools, as described [19]. Peptides identified as immunogenic in the matrix array screen were restested individually in a confirmatory assay and a peptide titration assay. Thus, each ELISpot positive response was detected three times: by matrix array screening, individually by confirmatory assay, and by peptide titration.

The ELISpot assays were performed using mouse IFN-γ ELISpot sets from BD Biosciences (San Jose, CA) according to the manufacturer’s protocol. Briefly, the ELISpot plates were coated with anti-IFN-γ at 5 μg/ml and incubated at 4°C overnight. The plates were blocked with RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg of streptomycin/ml, and 100 U of penicillin for 2 h at room temperature, and either CD8^+ or CD4^+ depleted splenocytes (0.5 × 10^6 cells/well) were then added for assays of class II and I T cell epitopes, respectively. The cells were cultured at 37°C in 5% CO_2 in the presence of peptide pools (final concentration of each peptide was 10 μg/ml) or individual peptides at final concentrations of 10 μg/ml, 1 μg/ml, and 0.1 μg/ml. Wells with medium alone served as background; Concanavalin A (2.5 μg/ml, Sigma-Aldrich, St. Louis, MO) was used as a polyclonal stimulator; and known HLA-restricted peptides from Dengue serotype 3 were included in each assay as positive controls. After 16 h of culture, the plates were washed and incubated with biotinylated anti-IFN-γ for 2 h at room temperature, followed by HRP-conjugated streptavidin for 1 h at room temperature. Reactions were developed with AEC substrate (Calbiochem-Novobiochem, San Diego, CA). Final enumeration of IFN-γ spot-forming cells (SFC) was performed using the Immunospot Series 3B Analyzer ELISPOT reader (Cellular Technologies, Shaker Heights, OH) with aid of the Immunospot software version 3.0 (Cellular Technologies), indicating the number of SFC/10^6 cells. The results were considered positive if the number of SFC subtracted by those in the background (culture with medium alone) was above 10 and the number of SFC was higher than the background plus two standard deviations. The results shown are SFC minus background (culture with medium alone) was above 10 and the number of SFC was higher than the background plus two standard deviations. The results shown are SFC minus back- ground (culture with medium alone) was above 10 and the number of SFC was higher than the background plus two standard deviations. The results shown are SFC minus background (culture with medium alone) was above 10 and the number of SFC was higher than the background plus two standard deviations. The results shown are SFC minus background (culture with medium alone) was above 10 and the number of SFC was higher than the background plus two standard deviations.
Table 1. HLA-A24, -B7, -DR2, -DR3, and -DR4 restriction of 54 peptides of influenza proteins M1, NP, PA, PB1, and PB2 that contain conserved sequences of 9 or more amino acids.

| Protein | ELISpot positive 17 aa peptide* | A24# | B7 | DR2 | DR3 | DR4 |
|---------|---------------------------------|------|----|-----|-----|-----|
| M1      | 169 TNQIHRENIRXVASTT 185        | -    | -  | 56.5 (0.1) | 120.4 (0.1) | -   |
|         | 175 HENRMYLASTKAMEQ 191         | -    | -  | -   | -   | 165.1 (0.1) |
|         | 181 LASTKAMEQMGSSSE 197         | -    | -  | -   | 115.21 (1) | -   |
| NP      | 7 KRSGIGFQFYMCTEL 47            | 45.5 (0.1) | - | - | - | - |
|         | 31 RMIGGIGFQFYMCTEL 47          | -    | -  | -   | -   | 52.29 (0.1) |
|         | 37 GRFQFYMCTELKNYDE 53          | -    | -  | 66.7 (1) | -   | -   |
|         | 73 ERNQKYLEEHPASQGKD 89         | -    | -  | -   | -   | 121.1 (0.1) |
|         | 103 KKVRELVLVDKEEIRRI 119       | -    | -  | 61.42 (0.1) | -   | -   |
|         | 109 VLYDKEEIRRIWRQANN 125       | -    | -  | 501.42 (0.1) | -   | -   |
|         | 133 LTHIMIWHSNLNDTYQ 149        | 238.59 (0.1) | - | - | - | - |
|         | 402 SAGOISTQPTFSVQRNL 418       | 207.3 (0.1) | - | - | - | - |
|         | 408 TQPTFSVQRNLPFQDTEK 424      | 110.14 (1) | 41.2 (10) | - | - | - |
| PA      | 42 LEVCFMYSDFHFINFEQQ 58        | -    | 64.11 (1) | - | - | - |
|         | 126 EVHYYLEKANKIKSEQU 142       | -    | -  | 37.11 (0.1) | -   | -   |
|         | 132 LEKANKIKSEQUHIHIH 148       | -    | -  | 41.10 (0.1) | -   | -   |
|         | 558 SRPMFLYVTRNTGSKSK 574       | -    | -  | 114.24 (0.1) | -   | -   |
| PB1     | 31 SHGTGTGYTDMTVPNTH 47         | -    | -  | -   | 106.1 (0.1) | -   |
|         | 37 GYTMĐTVPNTHQYSERG 53         | -    | -  | -   | 125.11 (0.1) | -   |
|         | 120 DKLQGQRTYDVWTLNRN 136       | -    | -  | 142.6 (0.1) | -   | -   |
|         | 126 ROTYDWTNLNRQPAAATA 142      | -    | -  | 78.0 (0.1) | -   | -   |
|         | 328 NQPEWFNRILSIAPIMF 344       | -    | 60.8 (10) | - | - | - |
|         | 340 APIMFSNKNMLRLKGKYM 356      | -    | -  | 175.0 (0.1) | -   | -   |
|         | 352 GGKYMFFESMKLMTRQI 368       | -    | -  | 52.2 (1) | -   | -   |
|         | 358 EKSKMLTRTIQPAEMA 374        | 84.20 (0.1) | - | - | - | - |
|         | 465 REYRTCKLGINMSKKK 481        | 231.73 (1) | - | - | - | - |
|         | 471 KLGINMSKKKSYINISRQ 487      | -    | -  | 116.10 (0.1) | -   | -   |
|         | 489 TFETSFYRYGFVANV 505         | 213.9 (0.1) | - | - | - | - |
|         | 495 FFFYRGFVAVNFSEMPLS 511      | 210.25 (0.1) | - | - | - | - |
|         | 507 MelpsFGVFVYHESDM 523        | -    | -  | 274.15 (0.1) | -   | -   |
|         | 519 ESADMSIGVTVKNNM 535         | 75.10 (0.1) | - | - | - | - |
|         | 525 IGTVIKMNINNDLG 541          | 159.53 (0.1) | - | - | - | - |
|         | 537 NDLGPATAOMALQFIK 553        | 92.2 (1) | - | - | - | - |
|         | 548 LQLFKIDYRYYRCHRCHQ 564      | 61.2 (1) | 230.23 (0.1) | 97.30 (0.1) | - | - |
|         | 554 DYYRYYYRCHRQGTQIQT 570      | 109.13 (1) | 166.22 (0.1) | 76.2 (0.1) | - | - |
|         | 560 RCHRGDTQIQRTSRSEF 576       | 194.47 (1) | - | - | - | - |
|         | 650 GPCLKMEYDAVATTHSW 666       | 142.45 (0.1) | 41.9 (0.1) | - | - | - |
|         | 656 EYDAVATTHSPWPKERN 672       | -    | -  | 59.2 (0.1) | -   | -   |
|         | 680 RGILEDEQMYQRCCNLF 696       | 78.4 (0.1) | - | 181.10 (1) | - | - |
| PB2     | 42 NPSLRLMKWMAMKYPIT 58         | -    | -  | 166.3 (0.1) | -   | -   |
|         | 48 KWWWMMAMKYPITADKRIT 64       | -    | -  | -   | 161.18 (0.1) | -   |
|         | 54 KYPPITADKRMVPER 70           | -    | -  | 499.24 (0.1) | -   | -   |
|         | 126 KHTFGPVHFRQVKIR 142         | -    | -  | 316.20 (0.1) | -   | -   |
|         | 132 PVHRNQVRKRDRDIN 148         | -    | -  | 311.37 (0.1) | -   | -   |
|         | 256 DOSLIIARINIVRRAAV 272       | -    | -  | 169.12 (1) | -   | -   |
|         | 369 RATAILIKKATRRLIQQI 385      | -    | -  | 54.2 (0.1) | -   | -   |
|         | 434 LLLHFGKDAVFLNLNWG 450       | -    | -  | 444.14 (0.1) | -   | -   |
|         | 458 MGMILGTDMPSTEMS 474         | -    | -  | 238.5 (0.1) | -   | -   |
|         | 464 LFDMTPDSTEMSMTVGRV 480      | -    | -  | 324.28 (0.1) | -   | -   |
|         | 500 RFLRVRDORQNVLSPE 516        | 184.3 (0.1) | - | - | - | - |
Immunization of the HLA transgenic mice with the 196 H1N1 peptides was carried out with 2 pools of about 100 peptides each, with groups of 9 mice of each transgenic strain. Interferon-γ (IFN-γ) ELISpot assays for HLA-restricted class I and class II responses were performed with splenocytes of the immunized mice that were depleted of CD4+ and CD8+ T cells, respectively, to identify the responding T cell subset. The initial assays contained matrix arrays of peptide pools followed by validation assays with individual peptides [19]. Of the 196 peptides, 54 contained T cell epitopes that elicited 63 ELISpot responses (8 A24, 2 B7, 16 DR2, 17 DR3, and 20 DR4) (Table 1). None of the 196 peptides tested induced T cell responses in mice expressing the HLA-A2 allele. Forty-seven (47) of the 54 epitopes were restricted by one HLA allele; eight by class I HLA-A24 and -B7, and 39 by class II HLA-DR2, -DR3, and -DR4. The remaining 7 peptides contained epitopes that were presented by at least two HLA alleles of distinct supertypes i.e. they contained multiple or promiscuous T cell epitopes. Epitopes of PB1200–206 and PB2240–2564 were presented by both HLA class I and II alleles. Sixteen (16) pairs of consecutive peptides were restricted by the same HLA allele, possibly because there were identical epitopes in the overlapping 11 aa sequence shared by the 2 adjacent peptides. Clusters of 2 or more T cell epitopes with at least 16 conserved aa were M1123–137 had three overlapping epitopes (123 ALASCMGLIY 132 was restricted by A1; 125 ASCMGLIY 132 by B35; and 129 GLIYNRMGA 137 by A2) [22,24]. Thus, the highly conserved sequences contained common epitopes shared by pathogenic influenza strains and could be restricted by a broad range of HLA alleles.

Analysis of the Presence of Human Peptide Sequences in Influenza Peptides

Each of the 196 influenza 17 aa peptides used in this study was compared with human proteome sequences to investigate the presence of reported human T cell epitopes in the conserved sequences of influenza A proteins. Numbers represent aa positions. Highly conserved aa are shown as grey boxes. T cell epitopes were restricted by HLA-DR4 (black boxes), -DR3 (blue boxes), -DR2 (brown boxes), -A24 (green boxes), and -B7 (orange boxes).

Figure 1. Localization of HLA-restricted T cell epitopes of conserved sequences of influenza polymerases, NP, and M1 proteins. Numbers represent aa positions. Highly conserved aa are shown as grey boxes. T cell epitopes were restricted by HLA-DR4 (black boxes), -DR3 (blue boxes), -DR2 (brown boxes), -A24 (green boxes), and -B7 (orange boxes).
possibility of identity to human self-antigens that could trigger an autoimmune response to immunization. Specifically, we screened
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# Table 2. Presence of reported human influenza A T cell epitopes in 21 highly conserved sequences of A/New York/348/2003 (H1N1).

| Highly conserved 17 aa peptide* | HLA allele this work* | Published HLA alleles | Influenza strain |
|---------------------------------|-----------------------|-----------------------|------------------|
| M1 1 MSLTEVETYLSVPV 17          | -                     | A2                    | A/Puerto Rico/8/34 (H1N1) |
| M1 121 AGALASCMGLYRMGA 137      | -                     | A1, A2, B35, DRB1*0404 | A/Vietnam/1203/2004 (H5N1), Influenza A (H3N2) |
| M1 169 TNPLIRHENRMVLASTT 185    | DR2, DR3              | B39, DR2, DRB1*0103, DRB1*1101, DRB1*0701, DRB5*0101 | A/Vietnam/1203/2004 (H5N1), Influenza A |
| M1 175 HENRMLVLASTTAKAMEQ 191   | DR4                   | A3, A11, DRB1*0701    | A/Puerto Rico/8/34 (H1N1), A/Vietnam/1203/2004 (H5N1) |
| NP 61 LTIERMWLSAFDERRRNK 77     | -                     | A3                    | Influenza A |
| NP 67 VLSAFDERRNLYLEEHP 83      | -                     | DRB1*0101             | A/Vietnam/1203/2004 (H5N1) |
| NP 73 ERRNKPYLEEHPASGDOP 89     | DR4                   | DR1, DRB1*0101        | A/NT/60/68 (H3N2), A/Vietnam/1203/2004 (H5N1) |
| NP 91 KTTGPPVRKVQGKVWRE 107     | DR3                   | A68                   | A/Texas/1/77 (H3N2) |
| NP 109 VLYDEKEERRIRWQQANN 125   | DR3                   | DRB1*1101             | A/Vietnam/1203/2004 (H5N1) |
| NP 402 SAGQSTQPTSFVQRNL 418     | DR2                   | DRB1*0101, DRB1*0404  | A/Vietnam/1203/2004 (H5N1) |
| PA 42 LECVCYMDPHFNEQG 58        | DR2                   | A2                    | A/Puerto Rico/8/34 (H1N1) |
| PB1 1 MDWPNTLFLKQLYQA 17        | -                     | A2                    | Influenza A |
| PB1 37 GYTMDFVWNTHQYSE 53       | DR4                   | A26                   | Influenza A |
| PB1 346 NAMARLKGYPFESKSM 362    | -                     | B62, B27              | Influenza A |
| PB1 352 GKGYMFSKMKRTQI 368      | DR2                   | B44                   | Influenza A |
| PB1 489 TTEFTSFRRYGFVANF 505    | A24                   | A1, B44               | Influenza A |
| PB1 501 FVANIFSMELPSGFGSVG 517  | -                     | A2                    | Influenza A |
| PB1 537 NDLGPAQAMQLFK 553       | A24                   | B7                    | Influenza A |
| PB1 560 RCHRGDTQIQTRRSF 576     | DR2                   | B62                   | Influenza A |
| PB1 566 TQQTTSFERNLWQD 582      | -                     | B27                   | Influenza A (H3N2) |
| PB2 48 KIWMAAMKYPPDATKR 64      | DR4                   | A2                    | A/Puerto Rico/8/34 (H1N1) |

*Conserved aa are boldface. Published HLA epitopes were extracted from the IEDB. HLA class I epitopes are italicized. HLA class II epitopes longer than 17 aa are represented only by the corresponding residues in the 17 aa peptides of A/New York/348/2003 (H1N1).

1) Seventeen (17) T cell epitope sequences of the 2003 strain (11 PB1, 4 PB2, and 2 M1) were conserved in at least 88% of all recorded human and avian influenza strains (Table 4). In particular, PB1499–505 was 100% conserved in all H1N1 viruses. Several variant sequences within this group were recorded, but these were mostly single conservative amino acid substitutions representing a small fraction (less than 5%) of all the recorded 1977–2006 virus sequences. The major change in 2009 was the apparent complete replacement of 2 previous consensus sequences by variant sequences, each with 1 mutated aa (PB2132–148, 630–646).

2) A group of 9 PB1 and PB2 T cell epitopes of the A/New York/348/2003 H1N1 strain was variants of the 1977–2006 total recorded influenza A virus population at a single mutated aa position (Table 5). These variant A/New York/348/2003 strain sequences represented less than 15% of the consensus sequences of the entire 1977–2006 avian and human virus population. One of these, PB1507–523, became the H1N1 consensus sequence of 2007–2009. For the others, a single aa modification to the BEI peptide would result in 96–100% representation in the 2009 human H1N1 population.

3) The remaining 28 peptides were each represented in the dataset by 2 to 7 variant sequences with multiple mutations (Table S3). The A/New York/348/2003 sequences were the predominant form in only 13 of the 28 peptides and at reduced representations of 6 to 72% of the recorded viruses. As the variant forms contained a mixture of conserved sequences and variable amino acids, it is not possible to predict the immunogenicity of the variant sequences represented in nature and their use as vaccine sequences.
These data demonstrated that when T cell epitopes contain mixtures of conserved and non-conserved aa, the occurrences of mutated sequences in a subsequent influenza A strain are greatly enhanced.

Discussion

An enigma of the immunobiology of influenza A is that vaccines fail to provide long term protection against infection and natural infection does not prevent reinfection. The rapid mutation of the viral proteins, particularly the external HA and NA proteins that are targets for neutralizing antibodies, is credited with a significant role in this loss of immunity. Defective adaptive immunity is also observed with several RNA viruses (including HIV-1 and dengue viruses) [26–29]. Howev-

Table 3. Determination of human self-peptides in representative influenza 17aa peptides.

| Viral peptide* | Human peptide | Human protein name | GenPept ID |
|----------------|---------------|--------------------|------------|
| M1 169 TNPLRNHENMRMVLASTT 185 | 26 MVLAST 31 | Ring finger protein 220 | NP_060620 |
| M1 175 HENMRMVLASTTAKAME 191 | 140 TAKAME 145 | Mediator of cell motility 1 | NP_057039 |
| M1 181 LASTTAKAMEEQMAGSSE 197 | 1387 EOMAGS 1392 | MYST histone acetyltransferase 3 | NP_001092882 |
| NP 7 KRSYEQMETDOGERQAT 23 | 582 KRSYEQ 587 | Metastasis associated protein | NP_004680 |
| NP 103 KKWRLVLYDKEEIRRI 119 | 121 EERRI 126 | Annexin IV | NP_001144 |
| NP 402 SAGQISTQPTFSQVRNL 418 | 80 PTFSQV 85 | Mucin 6, gastric | NP_005952 |
| NP 408 TQPTFSQVRNLQDFTTT 424 | 1805 QPTFSV 1810 | Chromodomain helicase DNA binding protein 9 | NP_079410 |
| PA 126 EHHYYLEKANMKSEK 142* | 1266 YELEKANK 1272 | Dystrophin Dp427c isoform | NP_000100 |
| | 1274 YELEKANK 1280 | Dystrophin Dp427m isoform | NP_003997 |
| | 1151 YELEKANK 1157 | Dystrophin Dp427l isoform | NP_003998 |
| | 1270 YELEKANK 1276 | Dystrophin Dp427pl isoform | NP_004000 |
| PB1 31 SHGTTGTYMDTVNRTH 47 | 3151 GYMTDM 3156 | Polydom | NP_699197 |
| PB1 31 SHGTTGTYMDTVNRTH 47 | 2141 GYMTDM 2146 | Multiple EGF-like-domains 8 | NP_001401 |
| PB1 471 KLLGINMSSSKXYINT 487 | 609 MSKKS 614 | Suppressor variegation 4–20 homolog 1 isoform 1 | NP_060105 |
| PB1 489 TEFTSSFYRFYGVAF 505 | 561 SFFYRF 566 | Phosphatidylinositol glycan anchor biosynthesis | NP_036459 |
| PB1 537 NDLGPATQAQLMAQLFQ 553 | 919 PATAQM 924 | Rho GTPase-activating protein | NP_055530 |
| PB1 548 QLFLKFDYRYTRCHRG 564 | 231 DYRYT 236 | Syntaxin binding protein 5 isoform a | NP_640337 |
| PB2 256 DOSLIAIAARRHVRAAV 272 | 725 ARRAV 730 | Akt substrate AS250 | NP_065076 |
| PB2 256 DOSLIAIAARRHVRAAV 272 | 1301 IARRN 1306 | ATP-binding cassette, sub-family A, member 6 | NP_525023 |
| PB2 458 MGMIGILPDNTPTSEMS 474 | 1964 DMTPST 1969 | Voltage-gated sodium channel Type II, isoform 1 | NP_66287 |
| PB2 458 MGMIGILPDNTPTSEMS 474 | 1964 DMTPST 1969 | Voltage-gated sodium channel Type II, isoform 2 | NP_001035233 |

*Conserved aa are boldface.

1PA131–137 shared 7 aa identity with human Dystrophin Dp427 isoform proteins.

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and restricted by a broad range of HLA class I and II alleles. Thus, it is reasonable to expect that the conserved peptides identified here can elicit human T cell responses in the context of several HLA alleles and HLA-supertypes [38] and that the memory T cells can cross-react with epitopes from H1N1, H3N2, and H5N1 [27,39,40]. The class I alleles described herein HLA-A*0201, -A*2402, and -B*0702 belong to the distinct supertypes A2, A24, and B7, respectively [10,11]. HLA class II supertypes are not as well documented but the 3 alleles of the transgenic mice of this study are assigned to supertypes DR1, DR3, and DR4 [41] based on similar protein and three-dimensional structures. Thus, fewer HLA alleles within the supertypes could be selected to protect a broad population in contrast to selection of individual alleles.

Analysis of the conservation and mutational variants of these H1N1 HLA-restricted epitopes was revealing of the marked effect that single aa mutations may have on the representation of T cell epitopes in evolving virus populations. Over the 3 years interval (2007 to 2009) between the database records analyzed by Heiny et al. (2006) to the current 2009 H1N1 sequence analysis, only 8 of the 54 highly conserved T cell epitope sequences were without mutational change. These 8 peptides (M1175–191, 181–197, PB131–47, 120–136, 126–142, 489–505, 495–511, and 548–564) were representative of almost complete conservation, 95–100%, during the previous recorded history of human H1N1 virus sequences. All others of the identified HLA-restricted T cell epitopes contained at least 1 aa substitution, primarily but not exclusively, of the non-conserved aa of the H1N1 peptides. Our data suggest that the most favorable sequences for a T cell epitope-based vaccine are the 17 H1N1 T cell epitopes of the PB1, PB2, and M1 proteins (Table 4). We are presently investigating the protective effect of these sequences against live influenza challenge. These 17 T cell epitopes were highly conserved over the 33 years (1977–2009) of the examined database records, representing 88 to 100% of all

| Protein | A/New York/346/2003 H1N1 ELISpot positive peptides | 1977--2006 Influenza A* | 2007 human H1N1* | 2008 human H1N1* | 2009 human H1N1+ |
|---------|-------------------------------------------------|------------------------|-----------------|-----------------|-----------------|
| PB1     | 31 SHGCTGTYMTDVNKR 136                           | 99                     | 100             | 100             | 100             |
|         | 129 DTKQGRTVDWTLLN 136                           | 97                     | 100             | 100             | 100             |
|         | 126 RQTYDWTLLNQRPA 142                           | 99                     | 100             | 100             | 100             |
|         | 340 APIFNSNMARLQGYM 356                          | 96                     | 98              | 100             | 92              |
|         | --------R---                                    | 2                     | 2               | -               | 8               |
|         | 489 TTEFTSFYRFG0ANF 505                          | 100                   | 100             | 100             | 100             |
|         | 495 FYRFG0ANFSLPES 511                           | 99                     | 100             | 100             | 100             |
|         | 519 ESAMDGTVKNNN 535                             | 97                     | 100             | 100             | 99              |
|         | --------T                                        | #                     | -               | -               | 1               |
|         | 525 IGTVKNNKINNDLGP 541                          | 97                     | 100             | 100             | 99              |
|         | 537 NDLGPAQMALQLPX 553                           | 98                     | 100             | 100             | 99              |
|         | S--------                                       | 0.11                  | -               | -               | 1               |
|         | 548 LQLPIXDYRNYRCHRG 564                         | 98                     | 100             | 100             | 100             |
|         | 554 DRYTRYRCHRGDQTIQT 570                        | 98                     | 100             | 100             | 99              |
|         | --------A                                        | 0.04                  | -               | -               | 1               |
|         | --------Y                                        | #                     | -               | -               | 1               |
|         | --------S                                        | #                     | -               | -               | 1               |
|         | --------O                                        | 0.14                  | 3               | 100             | -               |
|         | 132 PVHFRQVKIRRVDIN 148                          | 88                     | 100             | 100             | -               |
|         | --------T                                        | 4                     | -               | -               | 100             |
|         | 500 RFLRVCDQRGNYLLSZ 516                         | 92                     | 100             | 100             | 100             |
|         | 630 RMQSSLTVNVRGSMR 646                          | 97                     | 100             | 100             | -               |
|         | --------L                                        | 1                     | -               | -               | 100             |
| M1      | 175 HENRVLASTAKAMEQ 191                          | 98                     | 100             | 100             | 100             |
|         | 181 LASTTAKAMEQAGSSE 197                         | 95                     | 100             | 100             | 100             |

*Highly conserved aa of 1977–2006 influenza A subtypes are boldface.

**3175 PB1, 3144 PB2, and 3781 M1 human H1N1, H3N2, H1N2, H5N1, and avian H5N1 and other avian subtypes sequences circulating between 1977 and 2006 were extracted from NCBI GenBank and GenPept databases as of September 2006. Sequences representing less than 1% were not included unless they were also represented in the 2007–2009 strains. All human PB1, PB2, and M1 H1N1 sequences from 2007 to 2009 were extracted from the Influenza Virus Resource on Jun 17, 2009.

†31 PB1, 31 PB2, and 393 M1 human H1N1 2007 sequences.

### Table 4. Representation of 17 H1N1 T cell epitope sequences among all influenza A 1977–2006 strains and H1N1 strains 2007–2009 that corresponded to the most frequent sequences with at least 88% conservation.
recorded avian and human influenza A viruses, including the H1N1 isolates. Further, they are clustered and have distinct advantages in the design of an epitope-based genetic vaccine, including the retention of native sequences for the function of transporters associated with antigen processing (TAPs) [42] and for the flanking sequences that are reported to modulate epitope processing and function in the selection of immunodominant epitopes [43]. Each of these 17 sequences, except M1181–197 and PB1537–553, was also characterized by high apparent functional avidity at the lowest peptide concentration of 0.1 μg/ml in the IFN-γ ELISpot assay. Several studies showed that high avidity CD8+ T cells were more effective in limiting viral replication in vitro [44–46]. Further, the 17 T cell epitopes had no identity of 8 or more continuous aa to human peptides that might trigger onset of human autoimmune diseases by priming autoreactive T cells. It is also noteworthy that several of the epitopes are located in described functional domains: PB1 518–575 in the interacting domain of PB1 with PB2 (PB1506–659) [47]; and the overlapping PB2126–142 and PB2132–148 in the PB1- and NP-binding domain of PB21–269 [48]. T cell epitopes within functional domains would remain conserved over time as viral mutations useful towards immune escape may disrupt the function of the domains and affect viral fitness unless compensated functionally by multiple co-mutations [49,50]. Thus, a T cell-based vaccine including these 17 highly conserved T cell epitopes, as described in this study, may possibly greatly reduce, if not eliminate, the incidence of variant amino acids of the corresponding T cell epitopes of any future influenza A pathogen.

**Table 5.** Representation of 9 H1N1 T cell epitope sequences with single amino acid substitutions from the most frequent sequences (≥ 80% conservation) among all influenza A 1977–2006 strains and H1N1 strains 2007–2009.

| Protein | A/New York/348/2003 H1N1 ELISpot positive peptide | 1977--2006 | 2007 human H1N1 | 2008 human H1N1 | 2009 human H1N1 |
|---------|---------------------------------------------------|-------------|----------------|----------------|----------------|
| PB1     | --------K-- | 86 | - | - | 99 |
|         | --------R--K-- | 13 | 99 | 84 | - |
|         | --------H-- | # | - | - | 1 |
|         | --------I-- | 89 | 1 | - | - |
|         | MELPSFGVSG÷NESADM | 523 | 10 | 99 | 100 | 100 |
|         | --------L | 86 | - | - | 99 |
|         | RCHRGDQTQITRRSFEI | 576 | 11 | 100 | 100 | - |
|         | --------L | 0.04 | - | - | 1 |
|         | --------L | 84 | - | - | 100 |
| PB2     | MEYDAVATTHSW | 666 | 12 | 99 | 97 | - |
|         | --------I-- | 0.68 | - | 3 | - |
|         | --------T-- | 0.42 | 1 | - | - |
|         | --------I-- | 87 | - | - | 96 |
|         | EYDAVATTHSW÷PERNR | 672 | 11 | 100 | 100 | - |
|         | --------T-- | 0.76 | - | - | 4 |
|         | --------K-- | 85 | - | - | 100 |
|         | RÇLGEDEQMYQRCCNLF | 696 | 10 | 98 | 87 | - |
|         | --------V-- | 0.23 | 1 | 10 | - |
|         | --------L-- | # | - | 3 | - |
| PB2     | --------Q-- | 89 | - | - | 100 |
|         | LRLHPQXDKAVLFNLWG | 450 | 7 | 97 | 100 | - |
|         | --------R-- | 0.03 | 1 | - | - |
|         | --------I-- | 0.03 | 1 | - | - |
|         | --------V-- | 90 | - | - | 99 |
|         | MWEINGPESVLINTYQW | 552 | 8 | 100 | 100 | 1 |
|         | --------V-- | 84 | - | - | 99 |
|         | PESVLINTYQWIIRNWE | 558 | 8 | 99 | 100 | 1 |

*Highly conserved aa of 1977–2006 influenza A subtypes are boldface.

*3175 PB1 and 3144 PB2 human H1N1, H3N2, H1N2, H5N1, and avian H5N1 and other avian subtypes sequences circulating between 1977 and 2006 were extracted from NCBI GenBank and GenPept databases as of September 2006. Sequences representing less than 1% were not included unless they were also represented in the 2007–2009 strains.

All human PB1 and PB2 H1N1 sequences from 2007 to 2009 were extracted from the Influenza Virus Resource on Jun 17, 2009.

+168 PB1 and 171 PB2 human H1N1 2009 sequences.

+31 PB1 and 31 PB2 human H1N1 2008 sequences.

+314 PB1 and 314 PB2 human H1N1 2007 sequences.

#New sequence representation not found in the 1977–2006 influenza A subtypes sequences.

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Supporting Information

Figure S1 Selected 196 peptides of Influenza A/New York/348/2003 (H1N1) used for mapping T cell responses in 6 HLA transgenic mouse strains (A2, A24, B7, DR2, DR3, and DR4). Red bold amino acids are conserved residues. Numbers represent residue positions. Overlapping residues were aligned.

Table S1 The first immunization peptide pool consisted of 13 M1 and 84 PB2 peptides of A/New York/348/2003 (H1N1) containing the highly conserved aa.

Table S2 The second immunization peptide pool consisted of 28 NP, 23 PA, and 48 PB1 peptides of A/New York/348/2003 (H1N1) containing the highly conserved aa.

Table S3 Representation of 20 (9 NP, 4 PA, 9 PB2, 5 PB1, and 1 M1) T cell epitope peptides of A/New York/348/2003 (H1N1) among human H1N1, H3N2, H1N2, H5N1, and other avian subtypes circulating between 1977 to 2006.

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Author Contributions

Conceived and designed the experiments: PTJT JTA. Performed the experiments: PTJT JTA. Analyzed the data: PTJT JTA. Contributed reagents/materials/analysis tools: ATH OM JS FL. Wrote the paper: PTJT ETAM, Jr. JTA.

References

1. Epstein SL, Kong WP, Misljon JA, Lo CY, Tunney TM, et al. (2005) Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 23: 3404-3410.
2. Epstein SL, Tunney TM, Misljon JA, Lo CY, Cooper LA, et al. (2002) DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice. Emerg Infect Dis 8: 796-801.
3. Jimenez GS, Phanchon R, Wei Q, Rusalov D, Geall A, et al. (2007) Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. Hum Vaccin 3: 157-164.
4. Powell TJ, Strutt T, Reome J, Hol lenough JA, Roberts AD, et al. (2007) Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against superchalal infection with heterosubtypic virus. J Immunol 178: 1030-1038.
5. Epstein SL, Lo CY, Misljon JA, Bennink JR (1998) Mechanism of protective immunity against influenza virus infection in mice without antibodies. J Immunol 160: 322-327.
6. Hamada H, Garcia-Hernandez Mde L, Reome JB, Mira SK, Strutt TM, et al. (2009) Te17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. J Immunol 182: 3469-3481.
7. Brossen DM, Dieder AM, Meents DL, Swain SL (2006) CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. J Immunol 177: 2889-2898.
8. Mozurzakowska K, Furchner M, Zehrkova D, Feng J, Gerhard W (2005) Roles of CD4+ T-cell-independent and -dependent antibody responses in the control of influenza virus infection: evidence for noncoagulate CD4+ T-cell activities that enhance the therapeutic activity of antiviral antibodies. J Virol 79: 5943-5951.
9. Strutt TM, McKinstry KK, Swain SL (2009) Functionally diverse subsets in CD8 T cell responses against influenza. J Clin Immunol 29: 145-150.
10. Sette A, Sidney J (1999) Nine major HLA class I supertypes account for the vast immunity against influenza virus infection in mice without antibodies. J Immunol Methods 254: 59-66.
11. Peters B, Sidney J, Bourne P, Bui HH, Buss S, et al. (2005) The immune epitope database and analysis resource: from vision to blueprint. PLoS Biol 3: e91.
12. Heiny AT, Miotto O, Srinivasan KN, Khan AM, Zhang GL, et al. (2007) Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 81: 12241-12251.
13. Gianfrani C, Oerhoff C, Sidney J, Chesnut RW, Sette A (2000) Human memory CTL response specific for influenza A virus is broad and multispecific. Hum Immunol 61: 438-452.
14. Rohrlich PS, Cardinaud S, Firat H, Lamari M, Briand P, et al. (2003) HLA-DR4 and human CD4 transgenes in mice determine the variable region functional characterization in response to influenza virus. Int Immunol 15: 765-772.
15. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, et al. (1997) HLA-B*0702 transgenic, H-2KbDb double-knockout mice: phenotypical and functional characterization in response to influenza virus. J Virol 71: 127-133.
16. Maciel MJ Jr, Kellathur SN, Chakhlikar P, Dhalla R, Sidney J, et al. (2008) Comprehensive analysis of T cell epitope discovery strategies using 17DDD yellow fever virus structural proteins and BALB/c (H2d) mouse model. Virology 378: 195-203.
17. Peters J, Sidney J, Bourne P, Bui HH, Buss S, et al. (2005) The immune epitope database and analysis resource: from vision to blueprint. PLoS Biol 3: e91.
18. Tobery TW, Wang S, Wang XM, Neep J, Pispens M, Glenn J, et al. (2008) Immunicom analysis of the repertoire of T-cell specificities for influenza A virus in humans. J Virol 82: 12241-12251.
19. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, et al. (2007) Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 81: 5161-5166.
20. Peters B, Sidney J, Bourne P, Bui HH, Buss S, et al. (2005) The immune epitope database and analysis resource: from vision to blueprint. PLoS Biol 3: e91.
21. Miotto O, Heiny A, Tan TW, August JT, Brusic V (2008) Identification of human-to-human transmissibility factors in PB2 proteins of influenza A of by large-scale mutational information analysis. BMC Bioinformatics 9 Suppl 1:S8.
22. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, et al. (1997) HLA-B*0702 transgenic, H-2KbDb double-knockout mice: phenotypical and functional characterization in response to influenza virus. Int Immunol 15: 765-772.
23. Gianfrani C, Oerhoff C, Sidney J, Chesnut RW, Sette A (2000) Human memory CTL response specific for influenza A virus is broad and multispecific. Hum Immunol 61: 438-452.
24. Heiny AT, Miotto O, Srinivasan KN, Khan AM, Zhang GL, et al. (2007) Evolutionarily conserved protein sequences of influenza A viruses, avian and human, as vaccine targets. PLoS ONE 2: e1190.
25. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, et al. (1997) HLA-A2 restricted education and cytotoxic activity of CD8+ T lymphocytes from beta2 microglobulin (beta2m) HLA-A2 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 185: 2043-2051.
26. Kreijtz JH, de Mutsert G, van Baalen CF, Fouchier RA, Osterhaus AD, et al. (2000) Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 82: 5161-5166.
27. Kreijtz JH, de Mutsert G, van Baalen CF, Fouchier RA, Osterhaus AD, et al. (2000) Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol 82: 5161-5166.
36. Sloan-Lancaster J, Allen PM (1996) Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. Annu Rev Immunol 14: 1–27.

37. Tsitoura DC, Holter W, Cerwenka A, Gelder CM, Lamb JR (1996) Induction of anergy in human T helper 0 cells by stimulation with altered T cell antigen receptor ligands. J Immunol 156: 2801–2808.

38. Frahm N, Yusim K, Suscovich TJ, Adams S, Sidney J, et al. (2007) Extensive HLA class I allele promiscuity among viral CTL epitopes. Eur J Immunol 37: 2419–2433.

39. Richards KA, Chaves FA, Sant AJ (2009) Infection of HLA-DR1 transgenic mice with a human isolate of influenza a virus (H1N1) primes a diverse CD4 T-cell repertoire that includes CD4 T cells with heterosubtypic cross-reactivity to avian (H5N1) influenza virus. J Virol 83: 6566–6577.

40. Lee LY, Ha do LA, Simmons C, de Jong MD, Chau NV, et al. (2008) Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J Clin Invest 118: 3478–3490.

41. Doytchinova IA, Flower DR (2005) In silico identification of supertypes for class II MHCs. J Immunol 174: 7085–7095.

42. Niedermann G (2002) Immunological functions of the proteasome. Curr Top Microbiol Immunol 268: 91–136.

43. Le Gall S, Stamegna P, Walker BD (2007) Portable flanking sequences modulate CTL epitope processing. J Clin Invest 117: 3563–3575.

44. Alexander-Miller MA, Leggatt GR, Berzofsky JA (1996) Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci U S A 93: 4102–4107.

45. Derby M, Alexander-Miller M, Tse R, Berzofsky J (2001) High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. J Immunol 166: 1690–1697.

46. Seflik C, Dadaglio G, Saron MF, Deriaud E, Rojas M, et al. (2000) In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity. J Virol 74: 5769–5773.

47. Gonzalez S, Zurcher T, Ortin J (1996) Identification of two separate domains in the influenza virus PB1 protein involved in the interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Res 24: 4456–4463.

48. Poole E, Elton D, Medcalf L, Digard P (2004) Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology 321: 120–131.

49. Rimmelzwaan GF, Kreijtz JH, Bodewes R, Fouchier RA, Osterhaus AD (2009) Influenza virus CTL epitopes, remarkably conserved and remarkably variable. Vaccine 27: 6363–6365.

50. Berkholz IG, de Wit E, Geelhuis-Mieras MM, Boon AC, Symons J, et al. (2006) Fitness costs limit escape from cytotoxic T lymphocytes by influenza A viruses. Vaccine 24: 6594–6596.