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Genome-wide screening of human T-cell epitopes in influenza A virus reveals a broad-spectrum of CD4⁺ T cell responses to internal proteins, hemagglutinins and neuraminidases

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Abstract

We performed a genome-wide screening for T cell epitopes using synthetic peptides that encompass all of the influenza A viral proteins, including subtype variants for hemagglutinin (HA) (H1, H3 and H5) and neuraminidase (NA) (human and avian N1 and N2) proteins, based on the sequence information of recently circulating strains. We found a total of 83 peptides, 54 of them novel, to which specific T cells were detectable in IFN- γ ELISPOT assays using peripheral blood mononuclear cells from four healthy adult donors. The surface glycoproteins, HA and NA, major components of vaccines, had many T cell epitopes. HA and matrix protein 1 had more T cell epitopes than other viral proteins, most of which were recognized by CD4⁺ T cells. We established several cytotoxic CD4⁺ T cell lines from these donors. We also analyzed H1 and H3 HA-specific T cell responses using the peripheral blood mononuclear cells of 30 hospital workers. 53% of donors gave a positive response to H3 HA peptides, while 17% gave a positive response to H1 HA peptides. Our genome-wide screening is useful in identifying T cell epitopes and complementary to the approach based on the predicted binding peptides to well-studied HLA-A, B and DR alleles.

Keywords

Human T cell epitopes; influenza A virus; CD4⁺ T cells; T cell epitope screening; hemagglutinin

INTRODUCTION

Human influenza is a contagious respiratory disease that results in substantial morbidity and mortality worldwide. With the recent cases of avian influenza infection in humans [1] and the heightened concern for an influenza pandemic [2], it is essential to understand host responses that would confer protective immunity to influenza. It is also important to generate influenza vaccines that can induce heterosubtypic immunity, which may in part be mediated by CD8⁺ and CD4⁺ T cells. A re-analysis of the archival records from the Cleveland Family Study,

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which was conducted before and during the 1957 pandemic when a shift from subtype H1N1 to H2N2 occurred, suggested an impact of accumulated heterosubtypic immunity in adults [3]. In addition, measures of the *ex vivo* cellular immune response to influenza in vaccinated older subjects correlated with protection against influenza while serum antibody responses had a limitation as a sole measure of vaccine efficacy [4]. Thus, understanding the roles of cell-mediated immune responses to influenza virus and evaluating the efficacy of influenza vaccines in humans require insight into the specificity and diversity of T cell epitopes elicited upon infection and upon vaccination.

In humans, the CD8⁺ and CD4⁺ T cell responses to influenza have not been fully elucidated. Earlier studies to identify T cell epitopes to influenza have found nucleoprotein (NP) and matrix protein 1 (M1) as the major antigens for CD8⁺ T cells [5-7] and the hemagglutinin (HA) for CD4⁺ T cells [7]. We [8,9] and others [10-13] showed that human memory cytotoxic T lymphocyte (CTL) responses to influenza A virus are broadly directed to epitopes on a wide variety of viral proteins. We previously established influenza A-specific CTL lines from healthy volunteers and identified epitopes using recombinant vaccinia viruses that expressed influenza A virus proteins. We then used synthetic peptides based on the amino acid sequences of the influenza viral proteins that were recognized by the CTL lines. Other groups synthesized peptides based on epitope prediction algorithms to several common human leukocyte antigen (HLA) molecules and screened them by peptide binding assays, and the peptides which bound with high enough affinity to a given HLA molecule were screened in HLA-transgenic mice splenocytes or with human peripheral blood mononuclear cells (PBMCs) [11-13]. Wang et al. [12] screened for influenza epitopes by focusing on well-conserved peptide sequences among influenza A H1N1 strains and, as a result, most of HA peptides were excluded. Utilization of epitope prediction algorithms may bias the screening since it precludes atypical but potentially important epitopes because of their poor binding scores. Additionally, we [8,9] and Gianfrani et al. [11] both utilized the sequences of the A/Puerto Rico/8/34 (H1N1) strain (A/PR/8), which was isolated more than 70 years ago and is not a circulating strain. Therefore, all of the above approaches were designed to identify highly conserved epitopes in viral internal proteins. As a result, we may have underestimated the CTL responses to the HA as well as the neuraminidase (NA), another surface glycoprotein which also undergoes antigenic drift.

In the present study, we performed an epitope screening approach using synthetic peptides that encompass all of the influenza A viral proteins based on sequence information from recently circulating influenza strains. Our peptide arrays include subtype variants for HA (H1, H3 and H5) and NA (human and avian N1 and N2) proteins. We screened these peptides using PBMCs from healthy adult volunteers without *in vitro* amplification of influenza A-specific T cells. Our genome-wide epitope screening confirmed a broad T cell response to influenza that was directed to several viral proteins. HA and M1 had more T cell epitopes than other viral proteins. We also detected cross-reactive T cell responses to H5 HA peptides in healthy humans who were unlikely to have been exposed to H5N1 viruses.

MATERIAL AND METHODS

Influenza A peptides and control peptides

17-mer peptides overlapping by 11-12 amino acids encompassing the entire sequence of all influenza viral proteins were obtained from the National Institutes of Health (NIH) Biodefense and Emerging Infections Research Resources Repository (BEI Resources) (Table 1). The length of the peptides was a debated compromise in an effort to detect most CD4⁺ and CD8⁺ T cell epitopes at a reasonable cost. The amino acid sequences of these peptides were based on the recent vaccine strains of influenza viruses A (H1N1) and (H3N2) (or an antigenically indistinguishable strain from the vaccine strain, when the amino acid sequences of the proteins were not available for the vaccine strain) and recent isolates of influenza virus A (H5N1).

Because of considerable difference between avian and human N1 NA amino acid sequences, peptides sets for both neuraminidases were synthesized. Polymerase B1-F2 peptides of both strains were also synthesized because of differences between H1N1 and H3N2 strains. Peptide arrays of control peptides for known major histocompatibility complex (MHC) Class I and II epitopes of Influenza A viruses were also provided by BEI Resources. Lyophilized peptides were reconstituted by dissolving in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml per peptide. The CEF peptide pool was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, the National Institute of Allergy and Infectious Diseases (NIAID), NIH [14] and was used as a positive control. It contains MHC class I-restricted human T cell epitope peptides of Cytomegalovirus, Epstein-Barr virus and Influenza A virus at 2 µg/ml per peptide. Other peptides described in this paper were synthesized by AnaSpec, Inc. (San Jose, CA).

Study subjects

Blood samples were obtained from four healthy adults and PBMC were purified by Ficoll-Hypaque density gradient centrifugation as previously described [15]. The donors were chosen based on the availability of PBMC for large scale screening and their reactivity to the influenza peptides in the CEF peptide pool in enzyme-linked immunosorbent spot (ELISPOT) assays that had been previously performed. Donors 1, 3 and 4 received influenza vaccine almost every year. Donor 2 never received any influenza vaccine. None of these four donors had a history of laboratory-confirmed influenza infection. The HLA alleles of donor 1 are A2, A24, B7, B62, Cw3, DP2, DQw5, DQw6, DRB1*0103, and DRB1*1501; Donor 2 has the HLA alleles A1, B8, B44, Cw5, DQw1, DQw2, DR2, DR3, and DRw52; Donor 3 has A1, A24, B35, Cw04, DRB1*11 and DRB1*13; Donor 4 has A30, A31, B13, B51, DRB1*07 and DRB1*13. Additional screening of the HA (H1 and H3) was done using PBMC from 30 healthy hospital workers, whose HLA-A, -B, and -DR typing were described previously [16]. HLA-typing for donors 1 and 2 was determined by the HLA Typing Laboratory at the University of Massachusetts Medical Center, and the HLA-typing for donor 3 and 4, and the 30 vaccinated donors was determined by the HLA Core Facility of the Center for Infectious Diseases and Vaccine Research at the University of Massachusetts Medical School.

Peptide screening

In our preliminary experiments, we detected IFN- γ -producing cells to the M1₅₈₋₆₆ in a pool with 15 non-overlapping peptides using donor 1 PBMC by ELISPOT assays. The number of IFN- γ -producing cells did not differ significantly if we used 5, 10 or 15 non-overlapping peptides in a pool, but decreased if we used peptides that overlapped with the peptide M1₅₈₋₆₆ (data not shown). Therefore, we made peptide pools that contained 15 non-overlapping peptides and made two sets of peptide pools to facilitate the peptide screening. The first set of peptide pools contains all peptides of the surface glycoproteins of H1, H3 and H5 HA, and avian N1 (aN1) and human N1 (hN1) and N2 NA. This set of peptide pools consists of 33 pools; Pool 1-6 contain H1 HA peptides, Pool 7-12 contain H3 HA peptides, Pool 13-18 contain H5 HA peptides, Pool 19-23 contain hN1 NA peptides, Pool 24-28 contain aN1 NA peptides, and Pool 29-33 contain N2 NA peptides. The second set of peptide pools consists of 38 pools and included all peptides of the internal viral proteins: NP and nonstructural protein 1 (NS1) (Pool 1-8), M1 and nonstructural protein 2 (NS2) (Pool 9-12), polymerase A (PA) and matrix protein 2 (M2) (Pool 13-21), and polymerase B1 (PB1) and polymerase B2 (PB2) (Pool 22-38). Three additional peptide pools were also made. These contained PB1-F2 peptides and H5 HA peptides representative of regions of amino acid sequence diversity among different strains of H5N1 viruses. Donor PBMC was tested against the influenza A peptide pools in ELISPOT. The cut-off value for a positive response in ELISPOT was 20 SFC per 10⁶ cells for donors 1, 2 and 4. This value was determined by the average spot forming cells (SFC) per 10⁶ cells greater than 3 standard deviations of the negative control wells. The PBMC of donor 3 had

higher media background than did the other PBMC and the cut-off value for a positive response in this donor was 37 SFC/10⁶. Peptide pools that had SFC values equal to or greater than the determined cut-off for each donor were then deconvoluted to identify the individual peptide/s eliciting the IFN- γ response. A final peptide concentration of 2 μ g/ml for each peptide was used in all ELISPOT assays.

IFN- γ ELISPOT assay

ELISPOT assays were done as previously described [8]. Briefly, cryopreserved PBMC (2-2.5 \times 10⁶ cells per well) were seeded onto polyvinylidene difluoride membrane 96-well plates (Millipore, Bedford, MA) precoated with 5 μ g/ml anti-IFN- γ mAb (clone D1K; Mabtech, Cincinnati, OH) in the presence or absence of peptide or peptide pools. Phytohemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO) (1:100), CEF peptide pool and/or virus were used as positive controls. After 18-24 hr incubation, cells were removed by washing with PBS plus 0.05% Tween 20. Secondary biotinylated anti-IFN- γ mAb (clone 7-B6-1; Mabtech) was added at 2 μ g/ml and the plates were incubated for 2 hours at room temperature. Plates were washed again and IFN- γ was detected with avidin-peroxidase (3420-2H, Mabtech) and substrate kit (NovaRed, Vector Laboratories, Burlingame, CA). The frequency of IFN- γ producing cells was determined by using the ImmunoSpot[®] S4 Pro Analyzer and the ImmunoSpot[®] Academic V.4 Software (Cellular Technologies Ltd.). Experiments were performed in triplicate wells.

CD4⁺ or CD8⁺ T cells depletion of PBMC

CD4 or CD8 expressing cell populations were depleted from PBMC by negative selection using anti-CD4 or anti-CD8 antibody-coated magnetic beads from the MACS purification system (Miltenyi Biotec, Bergisch Gladbach, Germany) and were processed according to the manufacturer's protocol. Depleted PBMC were used in ELISPOT to determine the cell population producing IFN- γ .

Preparation of antigen presenting cells (APCs) for ⁵¹Cr release assays and ICS

For virus-infected targets, autologous B lymphoblastoid cell lines (BLCL) were established by culturing with Epstein-Barr virus in 24 well plates as previously described previously [17]. BLCL target cells were infected with either of the following egg-adapted virus strains - A/New Caledonia/20/1999 IVR-66 or A/Wisconsin/67/2005X-161B. These virus strains were a gift from Dr. Michel DeWilde and Dr. Robert Ryall of Sanofi Pasteur. The optimal concentrations of the two strains were determined in preliminary experiments. Infected cells were incubated for 18 hours at 37°C. Virus-infected target cells were then radiolabeled for use in ⁵¹Cr release assays or used as APCs in ICS. Peptide-pulsed targets were prepared using autologous BLCLs that were either radiolabeled for one hour *in* CTL assays *or* used directly in ICS assays. Peptide is then added to the cells at a final concentration of 10 μ g/ml per peptide, unless indicated otherwise.

⁵¹Cr release assay

T cell lines or bulk culture effector cells were added to 1.5 \times 10³ ⁵¹Cr-labeled target cells at various effector to target (E:T) ratios [8]. After incubating for 4-6 hours at 37°C, supernatants were harvested (Skatron Instruments, Sterling, VA), and specific lysis was calculated as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] \times 100. All assays were performed in triplicates. Unpulsed target cells were used as negative control. Spontaneous lysis was <30% in all assays.

ICS and flow cytometry analysis

Bulk culture effector cells were washed and resuspended at 5 \times 10⁵ cells in RPMI-1640 medium supplemented with 10% FBS (RPMI-10). Autologous BLCLs were used as APCs at an E:T

ratio of 10 and were added to the effector cells, together with the peptide of interest at 10 µg/ml. These were incubated for 1 h at 37°C in a 5% CO₂ incubator, followed by an additional 5 h in the presence of Golgi Plug (BD Biosciences, San Jose, CA). The cells were then washed with FACS buffer (2% FBS and 0.1% sodium azide in PBS) and stained using the Live/Dead Aqua Fixable Dead Cell Stain Kit (Invitrogen, Eugene, OR) to identify live and dead cells. Cells were then stained for surface markers such as CD3-PerCPCy5.5, CD8-FITC, CD4-APC or -Pacific Blue (BD Biosciences) for 30 min at 4°C. After washing with FACS buffer, the cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained for the intracellular cytokine IFN-γ (PE-conjugate; BD Biosciences) for 30 min at 4°C. Cells were then washed with Permwash buffer (BD Biosciences) and resuspended in Cytofix (BD Biosciences) for flow cytometric analysis. Multiparameter flow cytometric analyses were performed using a FACSCalibur or ARIA flow cytometer. The number of events collected per donor varied from 150,000 to 300,000. List-mode data files were analyzed using FlowJo (Version 6.3, TreeStar 6 Inc., Ashland, CA). Graphs were plotted as dot plots of CD4⁺ vs. CD8⁺ T cells in the gated live, CD3⁺ IFN-γ⁺ cell population.

Generation of bulk culture T cell lines and clones

To generate peptide-specific bulk culture lines, PBMC (3 to 5 × 10⁶ cells) were washed and resuspended in 2 ml of AIM/V-10% FBS supplemented with 1:100 sodium pyruvate (Gibco) and 1:1000 2-Mercaptoethanol (Gibco). The corresponding influenza peptide was added at a final concentration of 10 µg/ml. Human rIL-7 (Peprotech, Inc., Rockyhill, NJ) was also added to the culture (5ng/ml) and incubated at 37°C. On Day 3, human rIL-2 (BD Discovery Labware, Bedford, MA) (25-50 U/ml) was applied, and the medium was replenished with AIM/V-10% FBS and rIL-2 every three to four days. Bulk culture ⁵¹Cr release assays were done between days 10 and 13 of culture. The cultures were restimulated once with autologous PBMC on day 14 to reduce non-specific background lysis and to generate enough cells for the assays.

To establish influenza A-specific T cell clones, a limiting dilution assay (LDA) was done as previously described [8]. Briefly, PBMC which had been stimulated in bulk culture for 14 days were plated at a concentration of 1, 3, 10, or 30 cells per well in 96-well round-bottom microtiter plates in 50 µl of AIM-V medium containing 10% FBS, 25 U of IL-2, a 1:1,000 dilution of anti-CD3 monoclonal antibody 12F6 (gift from Dr. Johnson Wong), and 1×10⁵ gamma-irradiated (3500 rads) allogeneic PBMC/well. On day 7, 50 µl of fresh AIM-V medium with FBS and IL-2 were added, and on day 14, fresh medium with 1×10⁵ gamma-irradiated allogeneic PBMC/well and a 1:1,000 dilution of the anti-CD3 12F6 were added. The cells were assayed for cytolytic activity using ⁵¹Cr assays between days 21-28. Cells from wells with influenza A peptide-specific cytolytic activity (specific killing of 15% and above at a peptide concentration of 10 µg/ml) were expanded to 48-well plates.

RESULTS

The PBMC of donor 1 had a broad IFN-γ response to peptide-epitopes on several influenza A proteins

We used ELISPOT assays to quantitate the number of IFN-γ-producing cells in donor PBMC that are specific for the influenza A viral proteins. This method of screening for T cell epitopes directed to viral proteins had been used with much success (reviewed in [18]), and in particular with vaccinia virus [19]. Donor 1 is HLA-A2-positive and was known to have T cells specific to M1₅₈₋₆₆, which is considered an immunodominant epitope [20], when responses to the CEF peptide pool were analyzed (data not shown).

The PBMC of donor 1 were broadly reactive to peptide pools from several influenza proteins as shown by the IFN-γ responses to peptide pools containing HA peptides (Fig. 1A; Pool 1, 7,

8, 10, 11, 15, 18), NA peptides (Fig. 1A; Pool 29, 30), NP peptides and NS1 peptides (Fig. 1C; Pool 2, 4, 5, 6, 8), M1 and NS2 peptides (Fig. 1C; Pool 9, 10, 11, 12), PA peptides (Fig. 1C; Pool 20) and PB1 and PB2 peptides (Fig. 1C; Pool 22). IFN- γ responses to individual peptides from positive pools containing H3 HA and M1 peptides are shown in Fig. 1B and 1D respectively. In addition, donor 1 had positive responses to two pools containing PB1-F2 and H5 HA variant peptides (data not shown). We also detected several IFN- γ responses to H3 but not to H1. For the internal proteins, the majority of responses were seen in pools containing M1, NP, NS1 and NS2 peptides. The pool that contained the peptide with the HLA-A2-restricted M1 epitope M1₅₈₋₆₆ (Fig. 1C; Pool 10) had the highest SFC value among all the peptide pools. The sum of the number of IFN- γ -producing cells responding to these positive peptides was 1108, which is 90.7 % of the number of IFN- γ -producing cells responding to live influenza virus stimulation (1221 SFC/10⁶ for H1N1 and 1219 SFC/10⁶ for H3N2) (Table 2). We also performed intracellular cytokine staining (ICS) to determine the cells producing IFN- γ in response to live influenza virus-stimulation for both H1N1 and H3N2 using donor 1 PBMC. In this donor's PBMC, ~96% of IFN- γ -producing cells responding to influenza virus were either CD4⁺ or CD8⁺ (with values ranging from 10-30 % CD4⁺ and 67-88 % CD8⁺ depending on the virus strain used to stimulate, data not shown).

IFN- γ responses to influenza A proteins in three other healthy adults

We selected three additional donors based on the availability of PBMC for large scale screening and their previous reactivity to the influenza peptides in the CEF peptide pool in ELISPOT assays (data not shown). All three of these donors responded to peptides from several influenza A viral proteins (Fig. 2). The PBMC of Donor 2 had less reactivity to influenza peptides with responses to only 4 peptides (Fig. 2, *black box*).

On average, the four donors' PBMC responded to 21 different peptides from 9 different viral proteins, including H3 and H5 HA. Responses to H3 and H5 HA were comparable, with SFC values ranging from 20 to 60 SFC per 10⁶ cells. Table 2 lists the sum of the number of IFN- γ -producing cells responding to these positive peptides for donors 2, 3 and 4 as well as the SFC values for live virus stimulation of these donors' PBMC. The sum of the responses after stimulation with a complete set of the viral peptides is comparable to that of live virus stimulation in ELISPOT assays. Results of our ELISPOT screening using PBMC from the four donors are summarized in Table 3. Most of these peptides do not contain known epitope sequence (underlined in Table 3) based on our search using the Immune Epitope Database (IEDB) (www.immuneepitope.org) [21,22]. We considered a peptide sequence to be a potential novel epitope if there was no record that the peptide sequence associated with positive T cell data in the IEDB. When we searched the IEDB, we considered both the amino acid sequence of the 17-mer peptide and the corresponding HLA allele of the donor's PBMC that responded. Some of the peptides we identified have a record of some positive T cell data in IEDB, but were not fully characterized as to their HLA restriction or minimal epitopes (indicated in Table 3). About 60% of these peptides gave "moderate" IFN- γ responses, compared to the number of IFN- γ -producing cells responding to the "immunodominant" HLA-A2-restricted M1₅₈₋₆₆ epitope (Fig. 3).

Determining phenotype of IFN- γ producing cells by depletion

To determine the T cell population that produced IFN- γ after peptide stimulation, we depleted CD4 or CD8 cells from the donors' PBMC. We then performed ELISPOT using the peptide of interest to stimulate the CD4 or CD8 depleted cells. Peptides that had SFC values greater than 60 in Table 3 were first analyzed to ensure that there are enough peptide-specific precursor cells. Majority of the IFN- γ producing cells that responded to the peptides in ELISPOT were CD4⁺ cells, given by a drastic decrease in SFC values when CD4-expressing cells are depleted from whole PBMC (Table 4). For the other candidate peptides, bulk culture cell lines using

donor PBMC were set-up and ICS was performed on days 12-14 to determine the phenotype of the IFN- γ producing cells. Figure 4 shows representative FACS plots determining the phenotype of IFN- γ producing cells from peptide-stimulated bulk culture using donor 3 PBMC. More than 80% of IFN- γ producing T cells expressed the CD4 surface marker upon cognate peptide stimulation in ICS (Figure 4). A complete list of candidate peptides tested by either ELISPOT or ICS is given in Table 3.

IFN- γ responses to HA proteins using additional source of PBMC

Since we detected HA-specific T cell responses in the first four donors and there was no well-characterized CD8⁺ T cells epitope, we decided to analyze HA-specific T cell responses using the PBMC of 30 hospital workers. The same ELISPOT strategy was used to screen these donors' PBMC. Only H1 HA and H3 HA peptides were screened because of the limited number of PBMC available from these donors (Table 5). Sixteen of 30 donors (53.3%) gave a positive response to H3 HA peptides, and five of 30 donors (16.7%) gave a positive response to H1 HA peptides.

Peptide-specific CD4⁺ T cell lines produce IFN- γ upon cognate peptide stimulation

We generated bulk culture lines by stimulating donor PBMC with peptides of interest to further characterize the peptide-specific responses we identified in our ELISPOT screening. We selected peptides that potentially contain novel epitopes by searching MHC class I and class II binding motifs within the peptides by two prediction algorithms, HLA Peptide Binding Predictions (http://www.bimas.cit.nih.gov/molbio/hla_bind/) [23] and SYFPEITHI (<http://www.syfpeithi.de>) [24]. After two weeks in culture, peptide-stimulated bulk culture lines that had specific CTL killing of $\geq 15\%$ in ⁵¹Cr release assays (E:T ratios of 10, 30 and 90 were tested) were used in a limiting dilution set-up to generate peptide-specific T cell lines. We generated T cell lines that are specific to peptides H3 HA₂₆₇₋₂₈₃, H3 HA₃₅₀₋₃₆₆, M1₂₀₅₋₂₂₁, and M1₉₁₋₁₀₇, as well as to known T-cell epitopes contained in peptides H3 HA₃₂₁₋₃₃₇ (PRYVKQNTLKLAT, HA₃₂₂₋₃₃₄) restricted by HLA-DR1 and M1₅₅₋₇₁ (GILGFVFTL, M1₅₈₋₆₆) restricted by HLA-A2. A ⁵¹Cr release assay was performed to determine the ability of the T cell lines to kill targets pulsed with decreasing doses of cognate peptide. All T cell lines were able to kill peptide-pulsed targets at a peptide concentration of 10 $\mu\text{g/ml}$ ($> 15\%$ specific lysis, Table 6). We also determined the surface expression of CD4 or CD8 of these T cell lines by flow cytometry. The T cell lines that were specific to H3 HA₂₆₇₋₂₈₃ (1-3E2), H3 HA₃₅₀₋₃₆₆ (2-10D8), and M1₂₀₅₋₂₂₁ (3-1C9) were CD4⁺ (Table 6, $>95\%$ purity), while the M1-10-specific T cell line was CD8⁺ (data not shown). We also performed ICS for IFN- γ by pulsing autologous BLCLs with the cognate peptide and adding the appropriate T cell line prior to Golgi plug application (Table 6). T cell lines were able to produce IFN- γ upon cognate peptide stimulation of autologous APCs, comparable to the virus-infected control, although only a low percentage of the 2-10D8 T cell line produced IFN- γ after stimulation with either live influenza A virus or the peptide.

DISCUSSION

Characterization of epitope-specific T cell responses is critical in the understanding of immune responses to infection or vaccination. In this study, we performed a less biased and comprehensive screening of peptides covering all influenza A virus proteins. We confirmed previous reports by us [8,9] and others [10-13] that the T cell responses to influenza are broadly directed to several viral proteins. We found that surface glycoproteins, HA and NA, which are major components of inactivated vaccines, had many T cell epitopes. Recently we [16,25] and others [26] reported that trivalent inactivated influenza vaccine can induce T cell responses and part of these T cell responses may be targeting epitopes on HA, NA and M1. Overall, HA and M1 had more T cell epitopes than other viral proteins, most of which were recognized by

CD4⁺ T cells. One limitation of this study is that for some of the peptides identified in our screening, we were not able to determine whether T cells responding to a given peptide were CD4⁺ or CD8⁺ T cells. We did not use *in vitro* amplification of influenza A-specific T cells to avoid the skewing the relative frequency of the T cells specific to each epitope. As a result, the frequency of most peptide-specific T cells in PBMC was not high enough to determine the phenotype by performing ICS or depletion experiments. Based on the ICS of PBMC stimulated with live influenza A viruses it is estimated that approximately 80% of T cells responding to the viruses are CD8⁺ T cells (data not shown). However, for peptides that we were able to do further studies, they were all recognized by CD4⁺ T cells, with the exception of the CD8⁺ T cell epitope, M1₅₈₋₆₆. The peptides we used for the screening are 17mers, which may have stimulated CD4⁺ T cells better than CD8⁺ T cells and as a result the sensitivity of detecting CD4⁺ T cells may have been relatively higher. Ideally we should have included influenza-naïve donors as a negative control to show that T cells responding to influenza peptides have been generated against influenza virus. However, it is practically not possible to find influenza-naïve adults (we cannot obtain large volume of blood for genome-wide screening experiments from very young influenza-naïve children).

Assarsson et al. recently found that PB1 was the major target for both CD4⁺ and CD8⁺ T cell responses [13]. In our screening, HA (especially H3) and M1 were major targets of T cell responses. The peptides they screened were predicted to have high-affinity binding to HLA class I or class II molecules and to be highly conserved. Our peptides were 17mers overlapping by 11 amino acids covering all influenza A viral proteins. Using minimal epitope peptides in the assays is likely to increase the sensitivity of detecting responding T cells, especially in the case of CD8⁺ T cells. Using longer peptides covering all viral proteins is probably less sensitive in detecting specific CD8⁺ T cells, but can detect T cells recognizing atypical T cell epitopes or epitopes restricted by MHC class I or class II molecules whose binding motif predictions are not available (for example HLA-DP and DQ alleles and rare HLA-A, B and C alleles). These differences in the peptide sets may explain why the two screenings by Assarsson et al. and the present study produced different results. Because HLA-restriction of the epitope candidate peptides identified in this screening has not been determined, we were not able to test if these epitopes could be identified by the computer predictions. We think that our results are complementary to those of Assarsson et al. and that both approaches may be used, if possible, to identify T cell epitopes on a virus.

The abundance of T-cell responses against HA was confirmed by analyzing the PBMC of 30 more donors. In screening using the PBMC of 30 hospital workers, more responses to H3 HA were seen than to H1 HA (53.3% to H3 and 16.7% to H1). These may reflect the epidemiology of currently circulating influenza A strains, prior infections with H3N2 versus H1N1 viruses, or higher virulence of H3N2 strain than H1N1 strain. Additionally, only 17% of the hospital workers had responses to H1, thus it is probably not surprising that none of the four healthy donors we previously screened had responses to H1. The amino acid sequence identity between the HA and NA of A/New Caledonia/20/99 (H1N1) and A/Wisconsin/67/2005 (H3N2), is 40% for the HA (AAP34324 and ABW80978) and 42% for the NA (CAD57252 and ABP52004) respectively. This suggests a low probability of identifying subtype cross-reactive T cell epitopes in these proteins. However, in two of the four healthy adult donors whose PBMC were screened, we detected T cells responding to the peptides encoded by the H5 HA gene in IFN- γ ELISPOT assays. Although the frequencies of these T cells were not high, they were comparable to those responding to the peptides encoded by H3 HA gene. Recently Roti and colleagues [27] reported the presence of CD4⁺ T cells recognizing epitopes encoded by H5 HA gene in healthy individuals, who were unlikely to have been exposed to the H5N1 virus, although *in vitro* amplification of specific T cells were needed to detect them, suggesting a low frequency of these H5 HA cross-reactive T cells. They found that none of the H5 HA epitopes identified were uniquely cross-reactive to H2 HA, which is the closest subtype to H5 and

suggested that exposure to H2N2 viruses is not essential for cross-reactivity to H5 HA. Except for two peptides (H5₁₅₁₋₁₆₇ and H5₂₄₃₋₂₅₉ in Table 3) which have a four- to eight-amino acid overlap with the H5 HA epitopes identified by them, the H5 HA peptides that our donors' PBMCs responded to are different. We did not observe responses to the H1 and H3 HA peptides corresponding to these eight H5 HA peptides in these donors. Other groups have also identified cross-reactive memory T cell responses to avian H5N1 proteins in healthy individuals who were previously infected or exposed to seasonal influenza [28,29], as well as in individuals who were recently vaccinated for influenza [30]. Most of the cross-reactive responses were towards the internal proteins M1 and NP [28,29], which is expected since the internal proteins are highly conserved even among the different subtypes. They were also able to identify cross-reactive responses to the HA [28] and NA [30] proteins.

Many of the novel H3 HA T cell epitopes we detected and those previously defined are situated at conserved segments of the H3 protein sequence, with the majority of them clustering at the C-terminus. This confirms a previous study that correlated the H3 three-dimensional structure and the epitopes that had been identified in mice and humans and found that dominant epitopes to HA are primarily located in conformationally stable segments of the C-terminal region [31]. In addition, a recent study using HLA-DR1 transgenic mice [32] identified a diverse HA-specific, HLA-DR1-restricted CD4⁺ T cell response, with the majority of epitopes located in conserved HA regions. Our data also suggest that the T cell responses are directed to influenza HA regions that are structurally and functionally conserved. Repeated infection or immunization by different virus strains may selectively stimulate T cells specific to the epitopes located in conserved regions.

CD4⁺ T cell effector and memory responses to influenza infection have been studied to some extent in mice (reviewed in [33,34]), however, our knowledge of CD4⁺ T cell responses to influenza in humans is quite limited. A few HA CD4⁺ T cell epitopes have been identified in humans [35-38], and from recent study done using HLA-DR1 transgenic mice [32]. These influenza-specific CD4⁺ T cell responses may have important roles during influenza infection. An earlier study done in athymic mice reported a differential ability of influenza T helper clones to afford help to B-cells, depending on whether the B cells presented either viral surface proteins or internal viral components on their cell surface [39]. In Balb/c mice, CD4⁺ effector T cells mediated protection against a lethal influenza infection by perforin-mediated cytotoxicity [40]. Moreover, higher neutralizing antibody titers were attributed to CD4⁺ T cell help [40]. In another study, murine CD4⁺ T cells were able to traffic to the lungs during influenza infection, but needed antigen to be present to proliferate [41]. By stimulating PBMC with peptides of interest, we established CD4⁺ T cell lines specific to three peptides. These three lines were able to lyse target cells pulsed with the peptides. One CD4⁺ T cell line, 2-10D8, produced little IFN- γ after stimulation with autologous BLCL pulsed with the peptide or infected with influenza A virus, although the peptide recognized by the line was initially identified in IFN- γ ELISPOT assays, suggesting that measuring IFN- γ production alone is likely to underestimate the T cell responses to influenza A virus.

In summary, our screening data showed the strength and breadth of T cell responses against influenza A virus at baseline levels of healthy adults. These T cell responses target both subtype specific (most of the epitopes localized with HA and NA peptides) and subtype cross-reactive (internal protein peptides) peptides. Genome-wide screening using overlapping peptides covering all viral proteins is useful for identifying T cell epitopes and complementary to the approach based on the predicted binding peptides to well-studied HLA-A, B and DR alleles. It is important to analyze the effect of influenza vaccination on T cell responses, and the information reported here will be useful for that purpose.

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Abbreviations used

NP, nucleoprotein
 M1, matrix protein 1
 HA, hemagglutinin
 CTL, cytotoxic T lymphocyte
 HLA, human leukocyte antigen
 A/PR/8, A/Puerto Rico/8/34 (H1N1) strain
 NA, neuraminidase
 PBMCs, peripheral blood mononuclear cells
 NIH, National Institutes of Health
 BEI Resources, Biodefense and Emerging Infections Research Resources Repository
 MHC, major histocompatibility complex
 DMSO, dimethyl sulfoxide
 NIAID, the National Institute of Allergy and Infectious Diseases
 ELISPOT, enzyme-linked immunosorbent spot
 IFN, interferon
 aN1, avian N1
 hN1, human N1
 M2, matrix protein 2
 NS1, nonstructural protein 1
 NS2, nonstructural protein 2
 PB1, polymerase B1
 PB2, polymerase B2
 PA, polymerase A
 SFC, spot forming cells
 PHA, Phytohemagglutinin
 APCs, antigen presenting cells
 ICS, intracellular cytokine staining
 BLCL, B lymphoblastoid cell lines
 FBS, fetal bovine serum
 PBS, phosphate buffered saline
 rIL, recombinant interleukin
 IEDB, Immune Epitope Database

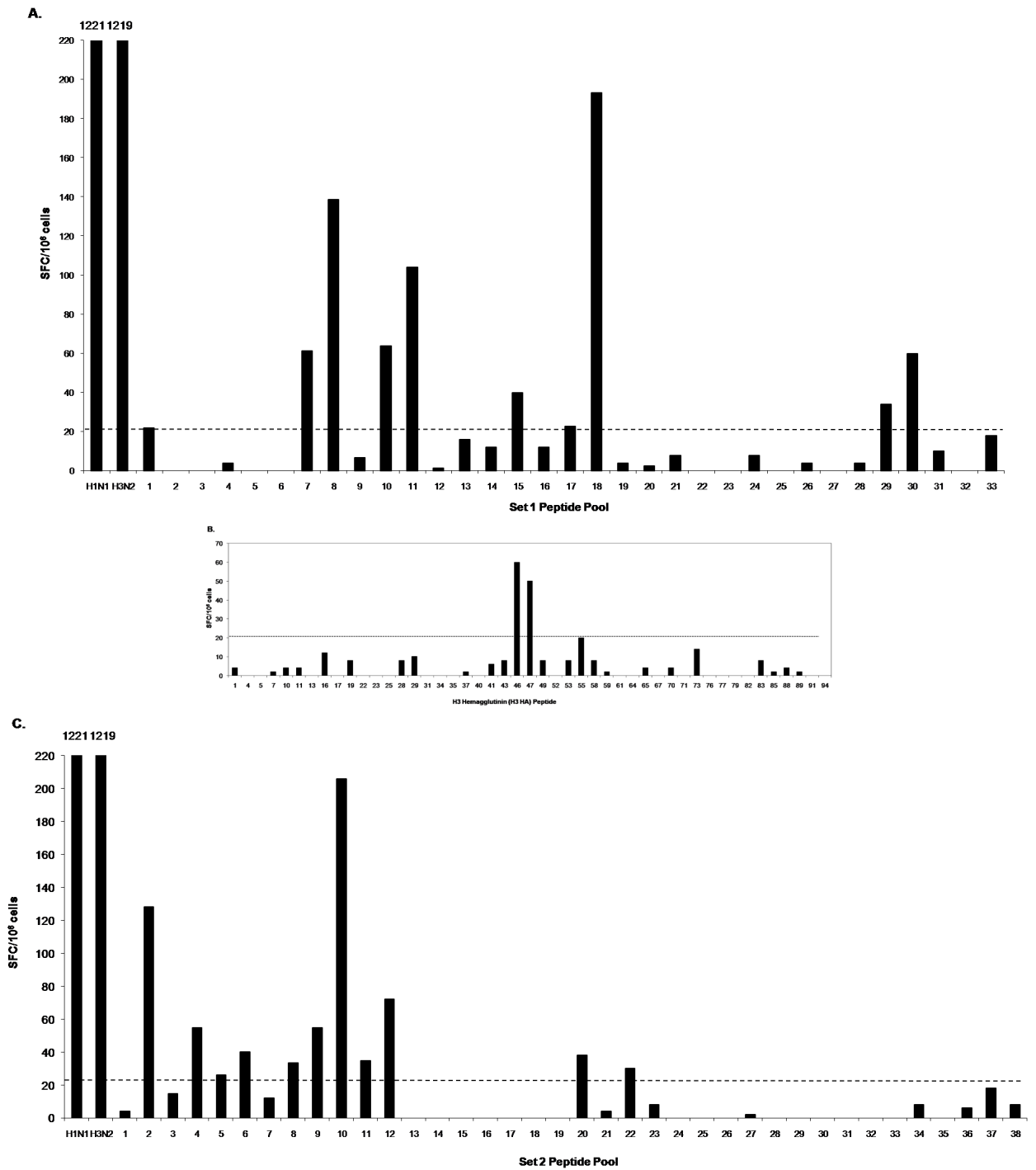
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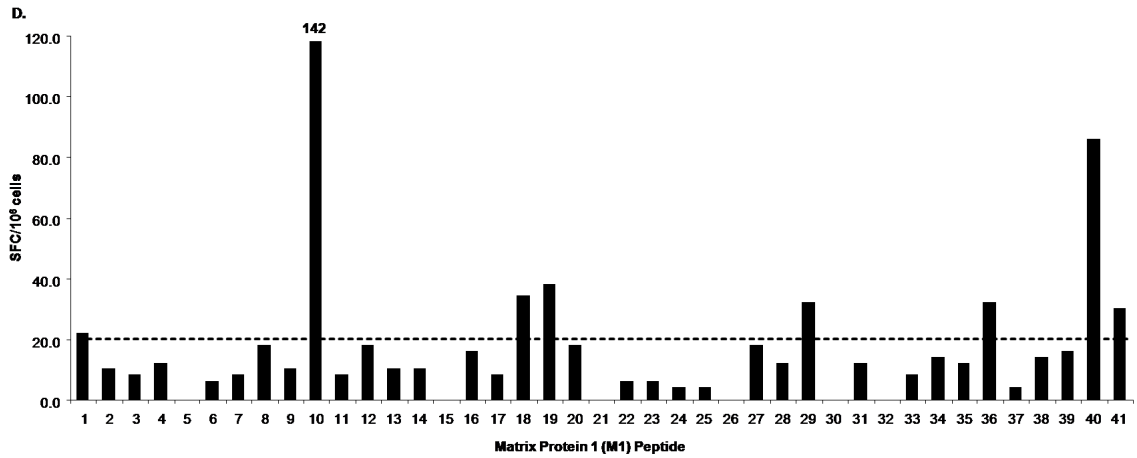


Figure 1.

IFN- γ response of donor 1 PBMC to peptides from all influenza proteins. PBMC was first tested in ELISPOT against Set 1 peptide pools (1-33), which include the hemagglutinin (H1 HA, H3 HA and H5 HA) and neuraminidase (aN1 NA, hN1 NA and N2 NA) peptides (A) and Set 2 peptide pools (1-38), which include peptides spanning all the viral internal proteins (C). Positive pools were deconvoluted to test individual peptides. An example of such screening is shown in B and D. ELISPOT assays were performed using individual peptides in Pools 7, 10, and 11 consisting of hemagglutinin H3 HA peptides (B); and individual peptides in Pools 9, 10, 11, and 12 consisting of matrix protein 1 (M1) peptides (D). A/Wisconsin/67/2005X-161B H3N2 virus and A/New Caledonia/20/1999 IVR-66 H1N1 virus were used as positive controls for live influenza A virus infection. Peptide pools and individual peptides were tested in three replicate wells with 200,000 to 250,000 cells per well. The final concentration of peptide used in all experiments was 2 μ g/ml per each peptide. The dotted line indicates the cut-off SFC value for this donor (20 SFC/10⁶).

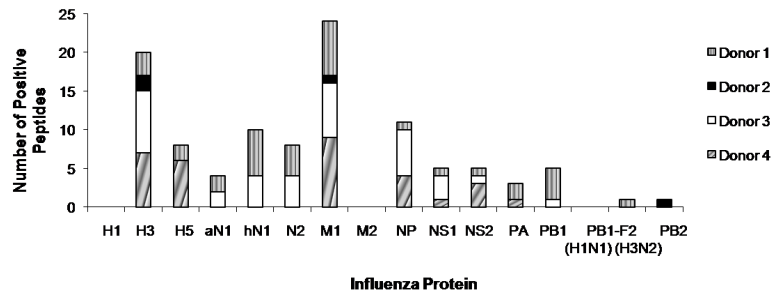


Figure 2. Broad T cell responses to influenza viral proteins in PBMC from healthy adults. PBMC from four healthy adults were screened for IFN- γ responses to all influenza proteins in ELISPOT assays.

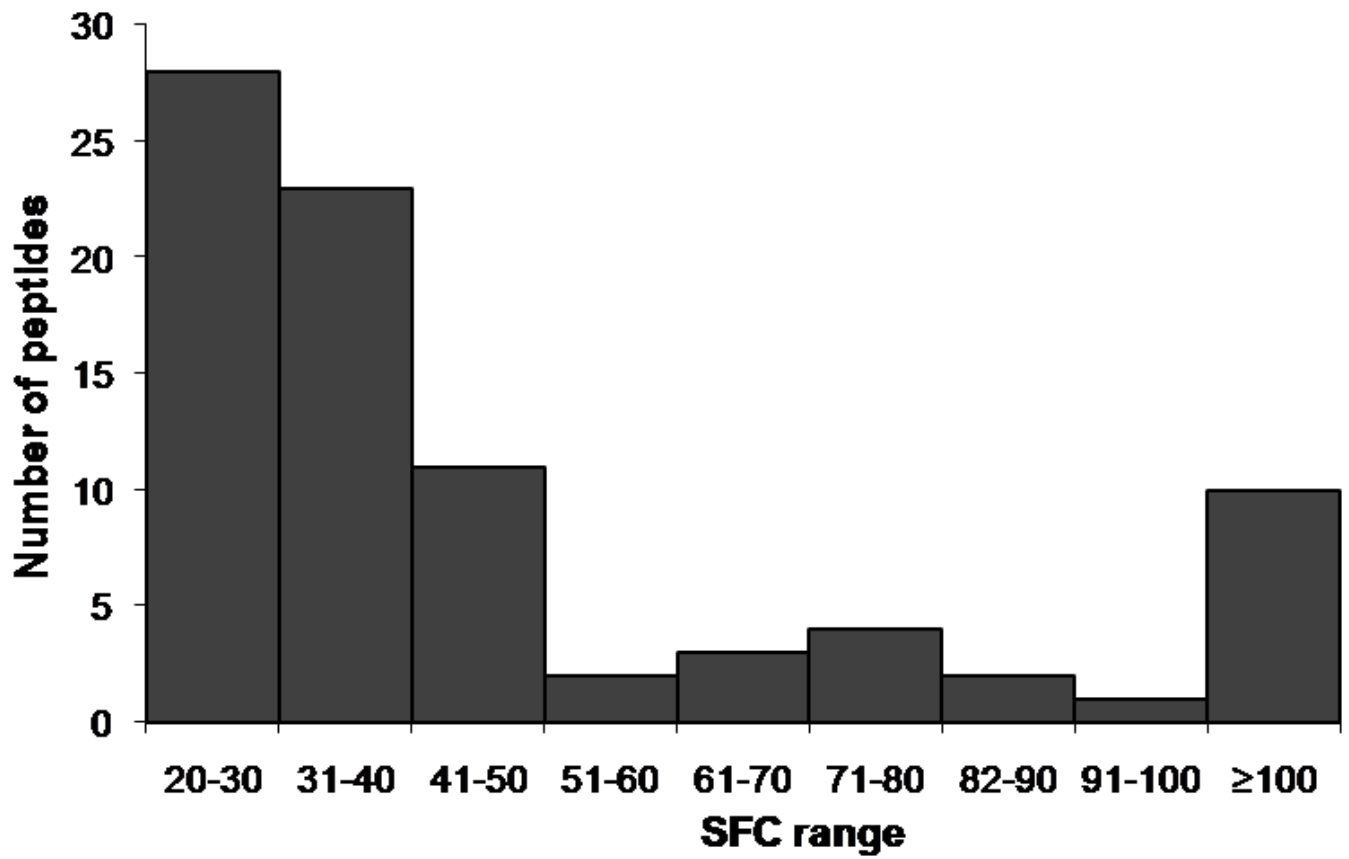


Figure 3. Distribution of SFC values of positive peptides in ELISPOT assays. The SFC values for each peptide that gave a positive IFN- γ response were plotted in a histogram chart to determine the frequency of IFN- γ -producing cells among PBMC of the four donors.

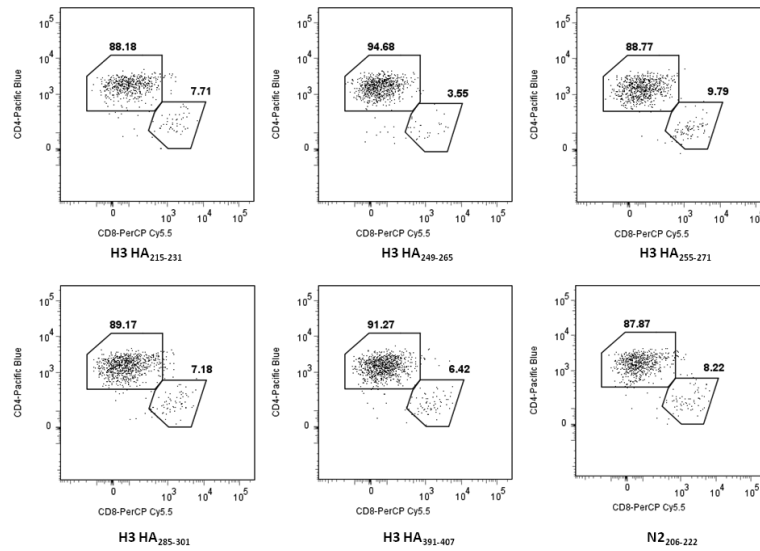


Figure 4.

Flow cytometry to determine the phenotypes of IFN- γ producing cells in donor 3 PBMC. Peptide-stimulated bulk culture cells using donor 3 PBMC were used as effectors in an ICS. The peptides shown here were used to initially stimulate whole PBMC and establish the bulk culture. The final peptide concentration was 10 μ g/ml in all experiments. Events were gated for the live, CD3⁺ IFN- γ ⁺ cells.

Table 1

Influenza A peptides used in this paper

Influenza Protein	Abbreviation	Strain	No. of Peptides	BEI Catalog No.
Hemagglutinin	H1 HA	A/New Caledonia/20/1999 H1N1	94	NR-2602
Hemagglutinin	H3 HA	A/New York/384/2005 H3N2	94	NR-2603
Hemagglutinin	H5 HA	A/Thailand/4(SP-528)/2004 H5N1	94	NR-2604
Neuraminidase	aN1 NA	A/Thailand/4(SP-528)/2004 H5N1	74	NR-2607
Neuraminidase	hN1 NA	A/New Caledonia/20/1999 H1N1	78	NR-2606
Neuraminidase	N2 NA	A/New York/384/2005 H3N2	78	NR-2608
Matrix Protein 1	M1	A/New York/348/2003 H1N1	41	NR-2613
Matrix Protein 2	M2	A/New York/348/2003 H1N1	15	NR-2614
Nucleoprotein	NP	A/New York/348/2003 H1N1	82	NR-2611
Nonstructural Protein 1	NS1	A/New York/444/2001 H1N1	37	NR-2612
Nonstructural Protein 2	NS2	A/New York/348/2003 H1N1	19	NR-2615
Polymerase A	PA	A/New York/348/2003 H1N1	119	NR-2618
Polymerase B1	PB1	A/New York/348/2003 H1N1	126	NR-2617
Polymerase B1-F2	PB1-F2 (H1N1)	A/New York/348/2003 H1N1	8	NR-2685
Polymerase B1-F2	PB1-F2 (H3N2)	A/New York/504/1998 H3N2	16	NR-2685
Polymerase B2	PB2	A/New York/348/2003 H1N1	126	NR-2616

Table 2

Total SFC values. The sum of the number of IFN- γ producing cells that responded to influenza peptide stimulation in ELISPOT was determined for each donor and compared to live virus infection

	Total SFC values ^a					
	H1 and hN1 Peptides	H3 and N2 Peptides	H5 and aN1 Peptides	Internal Protein Peptides	H1N1 Virus Infection	H3N2 Virus Infection
Donor 1	202	264	180	620	1221	1219
Donor 2	0	52.5	0	52.5	N.D. ^b	420
Donor 3	0	985	0	1825	1182	1478
Donor 4	0	273	193	714	1033	1135

^aTotal SFC values are representative of one ELISPOT experiment, with triplicate wells per peptide or virus stimulation.

^bN.D., not determined

Influenza peptides that gave a positive IFN- γ response in our ELISPOT screening using PBMC from donors 1, 2, 3 and 4

Table 3

Surface Glycoproteins						
Protein	AA Position	Peptide Sequence ^d	Donor	SFC/10 ^{6b}	Comments ^c	CD4/CD8 ^d
H3 HA	71-87	PHQILDGENCTLIDALL	3	37.5	novel	ND
H3 HA	179-195	ALNVTMPNNEKFKLYI	4	22.5	novel	ND
H3 HA	209-225	SLYAQAASGRITVSTKRS	4	42.5	novel	ND
H3 HA	215-231	SGRITVSTKRSQOIVIP	3	195	novel	CD4
H3 HA ^e	243-259	PSRISYWTIVKPGDIL	2, 4	27.5, 80	novel	CD4
H3 HA ^e	249-265	YWTIVKPGDILLINSTG	3, 4	127.5, 47.5		CD4
H3 HA	255-271	PGDILLINSTGNLAPR	3	67.5	CD4 T cell epitope restriction not known	CD4
H3 HA	267-283	LIAPRGYFKIRSGKSSI	1	60	CD4 T cell epitope restriction not known	CD4
H3 HA	273-289	YFKIRSGKSSIMRSDAP	1	50	DRB1*1001; DQA1*0102; DQB1*0602	CD4
H3 HA	279-295	GKSSIMRSDAPIGKCS	4	20	novel	ND
H3 HA	285-301	RSDAPIGKCSSECTPN	3	75	novel	ND
H3 HA	321-337	CPRYVKONTLKLATGMR	1, 3	20, 52.5	DRB1*0101; DRB1*0401; DRB5	CD4
H3 HA	350-366	AIAGFIENGWEGMVDGW	2	25	novel	CD4
H3 HA	391-407	NQINGKLNRLJGKTNEK	3, 4	75, 20	novel	CD4
H3 HA	505-521	DVYRDEALNRRFOIKGV	3	47.5	CD4 T cell epitope restriction not known	CD4
H3 HA	528-543	KDWILWISFAISCELL	4	40	CD4 T cell epitope restriction not known	CD4
H5 HA	30-46	EQVDTIMEKNVTVTHAQ	4	35	novel	ND
H5 HA	136-152	SSWSSHEASLGVSSACP	4	22.5	novel	ND
H5 HA	151-167	SSACPYQRKSSFFRNVV	1	80	novel	ND
H5 HA	207-223	YQNPITYISVGTSTLNQ	4	55	novel	CD4
H5 HA	243-259	EFFWTLKPNDAINFES	4	20	novel	ND
H5 HA	351-367	AIAGFIEFFWQGMVDGW	4	20	novel	ND
H5 HA	387-403	TQKAIDGVTKVNSIID	4	32.5	novel	ND
H5 HA	470-486	LQLKDNAKELGNGCFEF	1	34	novel	ND
hN1 NA	13-29	TSIAIGHLSMLQIGNI	1	44	A*0201	CD8
hN1 NA	42-58	SONHTGVCNQRIITYEN	1	38	novel	ND
hN1 NA	309-325	NLDYQIGYICSGVFGDN	1	30	novel	ND
hN1 NA	434-450	NITWTSGSISFCGVN	1	40	novel	ND
hN1 NA ^e	452-468	DTANWSWPDGAELPFTI	1	32	novel	ND
hN1 NA ^e	458-470	WPDGAELPFTIDK	1	28	novel	ND
aN1 NA	1-17	MNPKKIHTIGSICMVT	1	38	novel	ND
aN1 NA	24-40	LQIGNLISIWVSHSHI	1	42	novel	ND
N2 NA	13-29	VSLTITICFFMQIAL	1	44	novel	ND
N2 NA ^e	176-192	IAWSSSCHDGGKAWLHV	1, 3	50, 120	novel	CD4
N2 NA ^e	188-204	AWLHVCVTGDDKNATAS	1	22	novel	ND
N2 NA	206-222	IYNGRLVDSIVSWKEI	3	87.5	novel	ND
N2 NA	236-252	TCTVVMITDGSASGKADT	3	47.5	novel	ND
N2 NA	266-282	STLSGSAQHVEECSCYP	1, 3	24, 52.5	novel	ND

Internal Proteins

Protein	AA Position	Peptide Sequence ^d	Donor	SFC/10 ^{6b}	Comments ^c	CD4/CD8 ^d
M1 ^e	1-17	MSLLTEVETYYVLSIVPS	1, 4	34, 45	novel	ND
M1 ^e	7-23	VETYYVLSIVPSGPKAE	1	22	A*1101	CD8
M1 ^e	13-29	SIVPSGPKAEIAQRLE	1	26	DRB1*0101; DRB1*1501	CD4

Internal Proteins						
Protein	AA Position	Peptide Sequence ^d	Donor	SFC/10 ^{6b}	Comments ^c	CD4/CD8 ^d
M1	37-53	TDLEALMEWLKTRPILS	1	50	CD4 T cell epitope restriction not known	CD4
M1	43-59	MEWLKTRPILSPLTKGI	4	47.5	CD4 T cell epitope restriction not known;	CD4
M1	55-71	LTKGILGFVETLTPVSE	1	142	A*0201	CD8
M1 ^e	91-107	NNMDRAVKLYRKLKREI	3, 4	157.5, 22.5	DRB1*0103	CD4
M1 ^e	97-113	VKLYRKLKREITFHGAK	3	212.5	DRB1*0103	CD4
M1	121-137	AGALASCMGLIYNRMGA	3	42.5	B*3501	CD8
M1	139-155	TTESAFGLICATCEQIA	4	45	novel	ND
M1	163-179	RQMVTTTNPLRHENRM	4	20	novel	CD4
M1	169-185	TNPLIRHENRMVLA ^{STT}	1	42	DRB1*0103	CD4
M1 ^e	187-203	KAMEQ ^{MA} GSSEQA ^{AEAM}	3, 4	47.5, 37.5	DRB1*0103; DRB1*1501; DRB5	CD4
M1 ^e	193-209	AGSSEQAAEAMEV ^{ASQA}	4	37.5	novel	ND
M1 ^e	205-221	VASQARQMVQAMRAIGT	3	210	novel	CD4
M1 ^e	210-226	RQMVQAMRAIGTHPSSS	3, 4	142.5, 65	novel	CD4
M1 ^e	216-232	MRAIGTHPSSSTGLKND	3	115	novel	ND
M1	228-244	GLKNDLLENLQAYAKRM	2	22.5	DRB5	CD4
M1	234-250	LENLQAYQKRMGVOMOR	1	40	DRB1*0103; DQw3, DQw1	CD4
M1	240-252	YQKRMGVQMQREFK	4	35	DQw1	CD4
NS1	13-29	CFLWHVRKQVADQDKGD	4	25	novel	ND
NS1	120-136	DOAIMDKNILKANFSV	1	24	A*0201	CD8
NS2 ^e	13-29	LMRMSKMLGSSSGDLN	4	35	novel	ND
NS2 ^e	19-35	MQLGSSGDLNGMITQF	4	30	novel	ND
NS2	37-53	SLKLYRDSLGEAVMRLG	1, 4	50, 35	novel	ND
NS2	85-101	HKLKTTENSFEQITMQ	4	37.5	novel	ND
NP	55-71	RLIQNSLTIERMVLSAF	4	47.5	novel	ND
NP ^e	109-125	VLYDK ^{EE} IRRIW ^{RQ} ANN	3	165	novel	CD4
NP ^e	115-131	EIRRIW ^{RQ} ANN ^{GD} DATA	3	70	Novel	CD4
NP ^e	175-191	RS ^{GA} AA ^{AA} V ^K GV ^{GT} MVL	3	95	B*2705 ^f	CD4
NP ^e	187-203	GT ^{MV} LE ^{LR} MI ^{KR} GI ND	3	75	A*1101 ^f	CD4
NP ^e	193-209	LIRMIKRGINDRNFWRG	3	102	novel	CD4
NP ^e	246-262	RNPGNAEIEDLTLFARS	4	22.5	novel	ND
NP	258--274	FLARSAJLRGSAVHKS	1	40	A3	CD8
NP	294-310	EGYSLVGVDPFKLLOTS	4	22	novel	ND
PA	220-236	PPNFSCIENERAYVDGF	1	22	A*0201	CD8
PA	310-326	CMRTFFGWKEPTVVKPH	4	32	novel	ND
PA	516-532	DVVNFVSMFESLTDPRL	1	20	novel	ND
PA	576-592	KWGMEMRRCLLSLQQL	4	36	novel	ND
PB1	406-422	GMMGMFMMLSTVLGVS	1	26	novel	CD8
PB1	447-463	FALIVNAPNYAGIQAGV	1	44	A*0201	ND
PB1 ^e	537-553	NDLGPATAQMAALQLFIK	1	32	novel	CD8
PB1 ^e	548-564	LQLFIKDYRYTYRCHRG	1	36	B7	ND
PB1-F2 (H3N2)	65-81	KNPTQGS ^{LR} THAL ^K QW ^K	1	48	novel	ND
PB2	203-219	VAYMLERELVRKTRFLP	2	30	novel	ND

^aUnderlined amino acid sequences are previously published T cell epitopes (also see comments). Amino acid/s highlighted in bold reflects an amino acid change in our peptide sequence compared to the previously published epitope.

^bSFC values are representative of one ELISPOT experiment with three replicate wells per donor.

^cThe HLA class I and class II alleles described in the comments are the restricting MHC allele of the underlined amino acid sequence.

^dT-cell phenotype was determined by either IFN- γ ELISPOT using CD4 or CD8 depleted donor PBMC or IFN- γ ICS using peptide-stimulated bulk culture cells as effectors. ND (not determined).

^eThis peptide contains amino acid residues that overlaps with another peptide and is identified by the same donor.

^fThis peptide contains a known CD8 T-cell epitope, but the peptide-stimulated bulk culture we generated has a CD4 T-cell phenotype.

Table 4Phenotype of IFN- γ -producing cells after CD4⁺ or CD8⁺ cell depletion

Peptide	SFC/10 ⁶		
	Whole PBMC	CD4-depleted PBMC	CD8-depleted PBMC
DONOR 1			
^a M1 ₅₈₋₆₆	401.7	281.7	0
^b H3 HA ₃₂₂₋₃₃₄	8.3	6.7	25
H3 HA ₂₇₃₋₂₈₉	46.7	8.3	65
M1 ₂₃₄₋₂₅₀	53.3	1.3	57.3
N2 ₁₇₆₋₁₉₂	34.7	0	68
DONOR 3			
M1 ₉₁₋₁₀₇	90.7	0.0	164.2
M1 ₉₇₋₁₁₃	124	27.5	152.5
M1 ₂₀₅₋₂₂₁	68.0	7.5	174.2
M1 ₂₁₀₋₂₂₆	97.3	7.5	235
M1 ₂₁₆₋₂₃₂	22.7	0.8	50
N2 ₁₇₆₋₁₉₂	44	0.8	135
NP ₁₀₉₋₁₂₅	74.7	7.5	95
NP ₁₁₅₋₁₃₁	30	0.8	45
NP ₁₇₅₋₁₉₁	22	0.8	20
NP ₁₈₇₋₂₀₃	37.3	2.5	42.5
NP ₁₉₃₋₂₀₉	52	4.2	102.5
H3 HA ₂₄₉₋₂₆₅	25	2.5	25

^aM1₅₈₋₆₆ is an HLA-A2-restricted immunodominant epitope. It is used here as a control peptide for positive CD8 T cell responses in HLA-A2 donors.

^bH3 HA₃₂₂₋₃₃₄ is a HLA-DR1-restricted epitope. It is used here as a control peptide for positive CD4 T cell responses in HLA-DR1 donors.

Table 5

H1 and H3 HA peptides that gave a positive IFN- γ response in ELISPOT screening using PBMC from an additional 30 donors

Protein	AA Position	Sequence ^a	No. of Positive Donors	SFC/10 ^{6b}
H3 HA	13-29	LVFAQKLPNGDNSTATL	1	62.5
H3 HA	37-53	PNGTIVKTTINDGIEVT	2	50, 107.5
H3 HA	43-59	KTITNDQIEVTNATELV	2	30, 37.5
H3 HA	59-75	VQSSSTGGICDSPHQIL	1	57.5
H3 HA	83-99	IDALLGDPQCDFQNKK	1	52.5
H3 HA	107-123	SKAYSNCYPYDVPDYAS	2	40, 50
H3 HA	119-135	PDYASLRSLVASSGTLE	1	22.5
H3 HA	131-147	SGTLEFNNEFNWTVGT	1	37.5
H3 HA	155-171	CKRRSNNSFFSRLNWL	1	20
H3 HA ^c	209-225	SLYAQASGRITVSTKRS	4	20, 25, 330, 167.5
H3 HA ^c	215-231	SGRITVSTKRSQQTVIP	2	37.5
H3 HA ^c	243-259	PSRISIYWTIVKPGDIL	5	33.3, 37.5, 40, 42.5, 75
H3 HA ^c	249-265	YWTIVKPGDILLINSTG	4	27.5, 27.5, 32.5, 32.5
H3 HA ^c	255-271	PGDILLINSTGNLIAPR	4	30, 22.5, 35, 50
H3 HA	267-283	LIAPRGYFKIRSGKSSI	1	20
H3 HA	291-307	GKCNSCITPNGSIPND	1	22.5
H3 HA	321-337	CPRYVKQNTLKLATGMR	3	50, 35, 67.5
H3 HA	344-360	TRGIFGAAGFIENGWE	1	50
H3 HA	368-384	GFRHQNSEGIGQAADLK	1	27.5
H3 HA	386-402	TQAAINQINGKLNRLIG	2	27.5, 32.5
H3 HA	397-413	LNRLIGKTNEKFHQIEK	1	22.5
H3 HA	403-419	KTNEKFHQIEKFSEVE	1	57.5
H3 HA	409-425	HQIEKEFSEVGRIQDL	1	57.5
H3 HA	421-437	RIQDLEKYVEDTKIDLW	1	90
H3 HA	433-449	KIDLWSYNAELLVALEN	3	35, 27.5, 57.5
H3 HA	439-455	YNAELLVALENQHTIDL	2	22.5, 35
H3 HA	457-473	DSEMKNLFERTKKQLRE	1	55
H3 HA	463-479	LFERTKKQLRENAEDMG	1	20
H3 HA	481-497	GCFKIYHKCDNACIGSI	3	62.5, 22.5, 80
H3 HA	505-521	DVYRDEALNNRFQIKGV	1	85
H3 HA	528-543	KDWLWISFAISCFLL	1	50
H3 HA	550-566	FIMWACQKGNIRCNICI	1	47.5
H1 HA	37-53	LEKNVTVTHSVNLLLED	1	27.5
H1 HA	156-172	GKSSFYRNLLWLTGKNG	1	35
H1 HA	262-278	GNLIAPWYAFALSRGFG	1	60
H1 HA	416-432	LERRMENLNKKVDDGFL	1	40
H1 HA	434-450	IWTYNAELLVLENERT	1	25
H1 HA	458-474	VKNLYEKVKSQKNNNAK	1	37.5
H1 HA	464-479	KVKSQLKNNNAKEIGNG	2	65, 75
H1 HA	480-496	CFEFYHKCNNECMESVK	1	45
H1 HA	510-526	KLNREKIDGVKLESMGV	1	35
H1 HA	527-543	YQILAIYSTVASSLVLL	1	20
H1 HA	545-560	SLGAISFWMCSNGSLQ	1	42.5
H1 HA	550-565	SFWMCSNGSLQCRICI	1	20

^aUnderlined sequences are known influenza HA epitopes. Amino acids highlighted in bold reflect an amino acid change in our peptide sequence compared to the previously published epitope.

^bSFC values are representative of one ELISPOT experiment with three replicate wells for each donor positive for the peptide.

^cThis peptide contains amino acid residues that overlaps with another peptide and tested positive for IFN- γ response using PBMC from the same donor.

Recognition of influenza virus peptides by CTL lines generated from donor PBMC

Cell Line ^b	Protein	Epitope (AA position)	CD4/CD8 ^c	% CD4 T-cells producing IFN- γ ^d	HLA restriction ^e	% Specific ⁵¹ Cr release ^d			
						10 μ g/ml peptide	1 μ g/ml peptide	0.1 μ g/ml peptide	0.001 μ g/ml peptide
1-3E2	H3 HA	267-283	CD4	84.5	DRB1*01	59.2	38.0	31.7	31.5
2-10D8	H3 HA	350-366	CD4	0.73 ^f	N.D.	30.2	8.5	12.9	8.4
3-1C9	M1	205-221	CD4	27.6	N.D.	55.7	35.5	37.9	7.4

^aE:T ratio was 10:1; values are representative of one experiment. Specific lysis was determined by subtracting the percentage of lysis of unpulsed targets by effector cells from that of peptide-pulsed targets. Lysis of unpulsed targets were <5%.

^bCell lines were tested in CTL assay at least twice in triplicate wells.

^cT cell phenotype was determined by flow cytometry. Purity of T cell population is >98%.

^dIFN- γ producing cells were identified by ICS. Cell lines were added to either virus or peptide-stimulated autologous BLCLs. PMA/ionomycin was used as a positive control.

^eHLA restriction was determined by CTL assay against various BLCLs expressing different combinations of MHC class II molecules and by pre-treating target cells with anti-HLA-DR, -DQ, and -DP antibodies.

^fFor this T-cell line, 62.4% of CD4 T-cells produced IFN- γ upon PMA/ionomycin stimulation, but only 0.22% of CD4 T-cells produced IFN- γ upon H3N2 virus infection.