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Identification of HLA class II H5N1 hemagglutinin epitopes following subvirion influenza A (H5N1) vaccination

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ABSTRACT

Prophylactic immunization against influenza infection requires CD4+ T-helper cell activity for optimal humoral and cellular immunity. Currently there is one FDA approved H5N1 subvirion vaccine available, although stockpiles of this vaccine are insufficient for broad population coverage and the vaccine has only demonstrated modest immunogenicity. Specific activation of CD4+ T-helper cells using class II H5N1 HA peptide vaccines may be a useful component in immunization strategy and design. Identification of HLA class II HA epitopes was undertaken in this report by obtaining PBMCs from volunteers previously immunized with an H5N1 inactivated subvirion vaccine, followed by direct *ex vivo* stimulation of CD4+ T cells against different sources of potential HA class II epitopes. In the 1st round of analysis, 35 donors were tested via IFN-γ ELISPOT using pools of overlapping HA peptides derived from the H5N1 A/Thailand/4(SP-528)/2004 virus, recombinant H5N1 (rHA) and inactivated H5N1 subvirion vaccine. In addition, a series of algorithm-predicted epitopes coupled with the Ii-Key moiety of the MHC class II-associated invariant chain for enhanced MHC class II charging were also included. Specific responses were observed for all 20 peptide pools, with 6–26% of vaccinated individuals responding to any given pool (donor response frequency) and a magnitude of response ranging from 3- to >10-fold above background levels. Responses were similarly observed with the majority of algorithm-predicted epitopes, with a donor response frequency of up to 29% and a magnitude of response ranging from 3–10-fold (11/24 peptides) to >10-fold above background (7/24 peptides). PBMCs from vaccine recipients that had detectable responses to H5N1 rHA following 1st round analysis were used in a 2nd round of testing to confirm the identity of specific peptides based on the results of the 1st screening. Sixteen individual HA peptides identified from the library elicited CD4+ T cell responses between 3- and >10-fold above background, with two peptides being recognized in 21% of recipients tested. Eight of the putative MHC class II epitopes recognized were found in regions showing partial to significant sequence homology with New Caledonia H1N1 influenza HA, while eight were unique to H5N1 HA. This is the first study to identify H5N1 HA epitope-specific T cells in vaccine recipients and offers hope for the design of a synthetic peptide vaccine to prime CD4+ T-helper cells. Such a vaccine could be used to provide at least some minimal level of H5N1 protection on its own and/or prime for a subsequent dose of a more traditional but supply-limited vaccine.

1. Introduction

Vaccination against influenza H5N1 will likely be the only effective means of limiting morbidity and mortality in the event of a world-wide pandemic. Over the last several years, cases of direct avian-to-human transmission have been reported mainly in southern China and Southeast Asia [1,2]; however the more feared human-to-human transmission of virus has been limited to a few probable cases [3]. Should the virus re-assort its genetic material, allowing for direct human-to-human transmission, the potential exists for a world-wide pandemic. Traditional egg-based vaccines such as the tri-valent seasonal influenza vaccine, although highly effective against seasonal influenza subtypes, may not elicit sufficient cross-protection against H5N1 influenza. Early attempts to propagate H5N1 virus in embryonated chicken eggs for vaccine production were met with disappointment as the viral pathogenicity hindered high titer propagation resulting in relatively few vaccine doses. This limitation has been addressed by propagating subvirion vaccines, which includes strain-specific H5N1 HA and neuraminidase proteins combined with the internal viral proteins from the non-pathogenic A/PR/8/1934 (H1N1) strain. This approach, in addition to replacing the polybasic cleavage site

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between HA1 and HA2 has resulted in higher viral titers (increased vaccine supply) with minimal virulence in chicken eggs. Despite these improvements in manufacturing capability, clinical testing of these vaccines has induced only weak to modest immunogenicity [4–7], although a more recent clinical trial demonstrated that two 3.8 μg doses of a split-virion adjuvanted vaccine induced 77% seroconversion [8]. Stockpiling of such vaccines is of questionable utility due to the potential loss of potency over time and the emergence of mutant strains through antigenic drift, rendering such vaccines less effective.

Influenza infection has been most thoroughly investigated in murine model systems. Studies have shown that a lack of B cells in mice can lead to increased mortality following viral challenge [9,10], implicating the importance of having strong anti-viral humoral immunity, although the induction of CD8+ effector responses also contributes to viral clearance and recovery [11]. It has been demonstrated that activation of both arms of the immune system yields the most effective anti-viral response, and in most instances, relies heavily on the aid of CD4+ T cells. Activated CD4+ T cells provides indirect "help" for B cells and CD8+ T cells, as well as providing essential support for the induction of memory B and T cells [12,13]. Additional effector functions have been described for CD4+ T cells in the direct control of viral infections [14,15], including influenza-specific cytolytic activity [16,17]. Studies have also demonstrated that while CD4-depleted mice can clear the highly lethal PR8 murine influenza virus [18], the combination of CD4+, CD8+ and B cells greatly increases viral clearance and survival in mice [19,20], suggesting that a multi-pronged response is most efficient for protection. The contribution of each cell type in protecting humans against H5N1 infection is currently unknown and may depend in large part on the pathogenicity and overall virulence of the circulating strain. Taken together, H5N1 vaccines designed to induce multiple arms of the immune system and generate broad immunity will likely be most effective against an H5N1 outbreak.

The utilization of the Ii-Key technology to augment and drive stronger CD4+ T-helper cell activity may be advantageous to the development of an H5N1 vaccine. Specifically, modification of MHC class II epitopes with a fragment of the MHC class II-associated invariant chain termed Ii-Key has been shown to facilitate extracellular epitope loading of class II molecules, thereby bypassing the need for intracellular antigen processing. It has been suggested that extracellular peptide loading may activate naive T cells more quickly [21]. Such Ii-Key peptides have also been shown to enhance antigen-specific T cell responses *in vitro* [22] as well as T and B cell responses *in vivo* relative to non-Ii-Key-modified vaccine peptides [23–26]. Clinical testing of an Ii-Key class II Her2/neu peptide vaccine has shown it to induce a robust and specific immunological response in breast cancer patients [27].

In preliminary studies, mice primed with algorithm-predicted H5N1 HA MHC class II epitopes linked to Ii-Key demonstrated improved immunological response to a clinically tested rHA H5N1 subunit vaccine (unpublished observations). Specifically, priming with predicted class II H5N1 HA/Ii-Key epitopes derived from highly conserved regions of H5N1 HA increased the T-helper cell and antibody responses to a rHA boost. Unrelated studies also have demonstrated the utility of antigen-specific CD4+ priming prior to boosting with a recombinant vaccine, resulting in a more robust immunological response [28]. Therefore, it seems reasonable to pursue the use of Ii-Key-modified vaccine peptides as part of an overall H5N1 vaccine strategy with the goal of extending the limited supplies of more traditional H5N1 vaccines under development by using Ii-Key vaccines as a pre-emptive vaccine. As a "stand-alone" vaccine Ii-Key-modified H5N1 HA epitope(s) from conserved regions of H5N1 HA may provide some degree of protection against multiple H5N1 strains that may emerge in a pandemic.

Towards the development of such a vaccine, we have acquired PBMCs from subjects of an H5N1 subvirion vaccine trial to assess and identify specific CD4+ T cell epitope responses. Both algorithm-predicted class II HA peptides modified with Ii-Key as well as a library of overlapping peptides (peptide pool array) covering the entire H5N1 HA sequence were used as a source of potential MHC class II epitopes. The current study is the first to characterize CD4+ responses to an H5N1 subvirion vaccine and identify potential MHC class II epitopes suitable for H5N1 vaccine development.

2. Materials and methods

2.1. PBMC samples

The original double-blinded clinical trial involved 451 healthy adults who received two intramuscular doses of either 90, 45, 15 or 7.5 μg of an H5N1 subvirion influenza A vaccine (rgA/Vietnam/1203/2004), followed by safety, tolerability and hemagglutination inhibition analysis [4]. Six months following the second immunization, 337 study participants were given a third immunization, as a follow-up to the original study [5]. Of these participants, 35 study subjects (age 23–78) were recruited back to the University of Rochester site 20–29 months following study completion for collection of blood for PBMC isolation. PBMC samples were subsequently shipped to Antigen Express using a liquid nitrogen dry shipper and stored in liquid nitrogen until analysis.

2.2. Synthetic peptides, recombinant HA protein and H5N1 subvirion vaccine

For the identification of immunodominant class II HA epitopes, an influenza peptide array was utilized. This array, provided by BEI Resources (Manassas, VA), included 94 overlapping peptides (16–17 mers, overlapping by 11–12 amino acids) covering the entire A/Thailand/4(SP-528)/2004 HA protein and is >99% homologous to the HA of the Vietnam/1203/2004 strain used in the trial. Initial screening (1st round) of PBMCs to identify class II epitopes was performed by IFN-γ ELISPOT using a matrix-based approach. Briefly, the 94-peptide H5N1 HA array was divided amongst 20 different peptide pools, with 10 peptides represented in each pool (2 mg/ml), with the exception of Pools 5–10, which had 9 peptides each and Pool 20, which only included 4 peptides. Using a matrix-based strategy to more rapidly and efficiently identify potential new class II epitopes, similar to that described by Kaufmann et al. [29], each peptide was included in two different pools, such that a positive response in two different pools would permit identification of the individual peptide of interest. Individual peptides were subsequently retested in a 2nd round ELISPOT analysis to confirm reactivity.

In addition to screening a library of overlapping peptides, T cell responses were tested against twenty-four predicted Class II H5N1 epitopes. The SYFPEITHI algorithm (www.syfpeithi.de) was used in a manner to maximize the likelihood of identifying promiscuous, yet highly conserved HA epitopes from the H5N1 HA A/Duck/Anyang/AVL-1/2001 amino acid sequence (GenBank, accession #AF468837). Epitope peptides were selected based upon their cumulative binding affinity to six of the most common HLA-DRβ1 alleles (DRβ1*0101, DRβ1*0301, DRβ1*0401, DRβ1*0701, DRβ1*1101, and DRβ1*1501). It was later observed that all but one donor tested in these studies had at least one of these alleles (data not shown). Peptides were synthesized (NeoMPS, San Diego, CA) to include the Ii-Key motif (LRMK) for enhanced interaction with the class II molecule, which was covalently linked to the N-terminus of each epitope via a linker sequence (5-aminopentannoic acid, *ava*). Peptides were dissolved in 20% DMSO and frozen at −80 °C until use.

Measurement of T cell response was also tested against recombinant H5N1 HA (rHA) (A/Vietnam/1203/2004), H1N1 rHA (A/New Caledonia/20/99) (Protein Sciences, Meriden, CT), both at 5 μg/ml and H5N1 subvirion vaccine (rgA/Vietnam/1203/2004, BEI Resources) at 2.5 μg/ml.

2.3. PBMC preparation and CD8+ depletion

In preparation for ELISPOT analysis, donor PBMC samples were rapidly thawed in a 37 °C water bath and slowly added dropwise to prewarmed complete medium (X-Vivo 15, Cambrex, Walkersville, MD, 10% human AB, Gemini Bio Products, West Sacramento, CA). Since we did not have access to pre-vaccine samples, eight random naive donor PBMC samples (AllCells, Emeryville, CA) were utilized to assess potential cross-reactive T cell responses. Cells were subsequently centrifuged and supernatant decanted, followed by resuspension of PBMCs in complete media. Cell counts and viability were carried out by trypan blue exclusion, with viability generally >90%. PBMCs were depleted of CD8+ T cells using antibody-based magnetic separation columns (Miltenyi, Auburn, CA), followed by flow cytometric analysis to determine purity of cell populations. Residual CD8+ contamination was <1% in all samples.

2.4. ELISPOT analysis

ELISPOT analysis was performed using human anti-IFN-γ kits (BD Biosciences, San Jose, CA). In brief, PVDF plates were coated with 5 μg/ml anti-IFN-γ antibody diluted in sterile PBS (100 μl/well) and incubated overnight at 4 °C. Plates were blocked by washing 1× with 200 μl/well complete media followed by the addition of complete media (200 μl/well) and incubation at room temperature for 2 h. Complete media was decanted, followed by addition of $(1–4) \times 10^5$ CD8+ depleted PBMC/well depending on the donor sample tested. In the 1st round of T cell restimulation, peptide pools and algorithm-predicted H5N1 HA peptides modified to include the Ii-Key were added at 20 μg/ml (final concentration). Although this concentration of peptide is relatively high, given the length of time of last booster immunization (~2 years), detection of memory T cells generated from an already relatively weak vaccine was expected to be difficult. Therefore detecting all antigen-specific T cells, regardless of their affinity was preferred. Additional responses to H5N1 rHA (5 μg/ml) and subvirion inactivated H5N1 virus (2.5 μg/ml) were also tested. Positive control wells included ConA (10 μg/ml, Sigma, St. Louis, MO) and tetanus toxoid (1 μg/ml, Astarte Biologics, Redmond, WA) while negative controls consisted of each donor's PBMC tested in the absence of antigenic stimulation. ELISPOT plates were subsequently incubated at 37 °C for 24 h. Plates were washed 3× with PBS/0.5% Tween 20 (PBST) using a plate washer (Biotek Instruments, Winooski, VT), followed by the addition of biotinylated anti-human IFN-γ (2 μg/ml) diluted in PBS/10% FBS. After a 2 h incubation, plates were washed 3× with PBST, followed by the addition of streptavidin/HRP (1:100) diluted in PBS/10% FBS. Following 1 h incubation at room temperature, plates were washed 3× with PBST followed by two washes with PBS. Assay development was carried out by the addition of AEC substrate (BD Biosciences, San Diego, CA) until sufficient spot formation occurred (typically 1–2 min), followed by rinsing with ddH_2O and subsequent drying. Immunospots were counted using an AID ELISPOT reader (Autoimmun Diagnosticka, Strassberg, Germany). Data was calculated using the mean spot count of each antigen tested in triplicate. Responses were considered positive if there were >30 SFC/10^6 PBMC and at least 3× above unstimulated control wells. For some experiments ELISPOT data is presented as the fold increase of antigen stimulated samples relative to background unstimulated controls (3.0 considered baseline), while in others, the unstimulated background SFC was subtracted from antigen stimulated samples and reported as net SFC/10^6 PBMC.

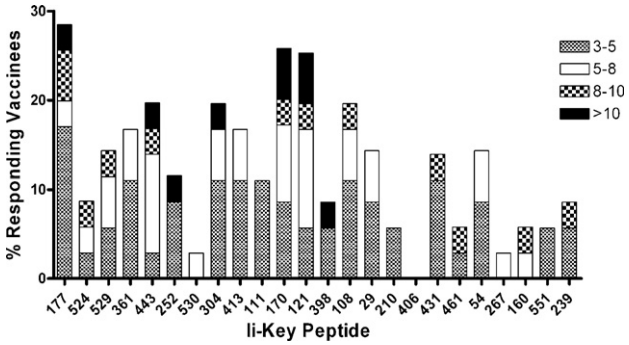

Fig. 1. CD4+ IFN-γ T cell frequency and magnitude following *in vitro* stimulation with algorithm-predicted class II HLA H5N1 HA peptides, modified to include Ii-Key moiety. Thirty-five donor PBMC samples were depleted of CD8+ T cells and incubated with 24 individual HA Ii-Key peptides. Following 24 h incubation, ELISPOT analysis was performed to measure the frequency and magnitude of the response to each peptide. The frequency of responding vaccinees to each peptide is shown. The overall magnitude of the response is arbitrarily segmented into 3–5-, 5–8-, 8–10- and >10-fold above background levels (3× above background, minimum of 30 SFC).

Second round T cell stimulation and ELISPOT analysis was performed using donors that were reactive to H5N1 rHA in 1st round stimulation, yielding 14 donors that were tested against individual library-derived peptides (20 μg/ml) predicted to be active based on their location in the matrix following 1st round screening. To examine the possibility of cross-reactivity between seasonal influenza HA and H5N1 HA, PBMC samples were tested against H1N1 A/New Caledonia/20/99 rHA (2.5 μg/ml).

3. Results

3.1. Ex vivo CD4+ T cell IFN-γ responses following stimulation with algorithm-predicted Ii-Key peptides and overlapping H5N1 HA peptides: 1st round PBMC analysis

In an effort to identify CD4+ immunodominant epitopes following H5N1 inactivated subvirion vaccination, we utilized CD8+ depleted PBMC samples from immunized volunteers and stimulated them for 24 h *ex vivo* against two different sources of potential class II epitopes: (1) algorithm-predicted HA class II epitopes linked to Ii-Key or (2) an overlapping HA peptide library. For predicted peptides, the SYFPEITHI algorithm was used in a manner to maximize the likelihood of identifying promiscuous yet conserved HA epitopes, such that a potential vaccine comprised of a few class II epitopes would have broad population coverage and elicit cross-strain protection. These peptides were then subsequently modified to incorporate the Ii-Key moiety. Studies have demonstrated that Ii-Key peptides induce greater *in vitro* T cell activity from human PBMCs relative to their native epitope counterparts [30]. The resulting 24 peptides tested span both the HA1 and HA2 regions. Algorithm-predicted peptides were able to elicit positive IFN-γ responses in up to 29% of the 35 vaccine recipients tested (Fig. 1). Responses were further characterized by arbitrarily assigning the response into four tiers: 3–5-, 5–8-, 8–10- and >10-fold above background. Ii-Key peptide-induced responses were primarily 3–10-fold above background level, while 7/24 peptides induced responses >10-fold above background. Of the 24 peptides tested, Ii-Key peptide nos. 177, 170 and 121 were most frequently recognized (10/35, 9/35 and 9/35 vaccine recipients respectively) and elicited the strongest *in vitro* IFN-γ responses.

To identify additional class II H5N1 HA epitopes for vaccine development, we undertook a more traditional brute force approach by screening vaccine recipient PBMCs against a library

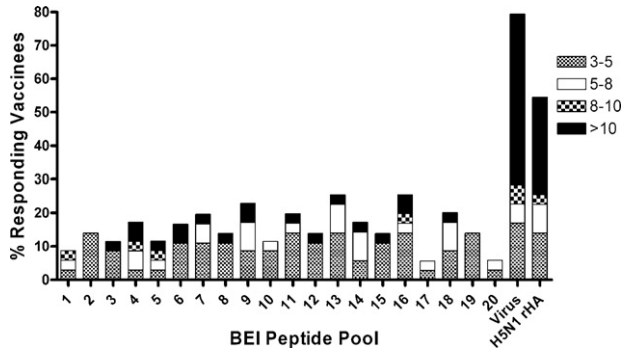

Fig. 2. CD4+ IFN-γ peptide pool response and frequency of recognition following *in vitro* stimulation with overlapping A/Thailand/4(SP-528)/2004 HA peptide pools. Thirty-five donor PBMC samples were depleted of CD8+ T cells and incubated with 20 individual peptide pools covering the entire H5N1 HA sequence. Following 24 h incubation, ELISPOT analysis was performed to measure the frequency and magnitude of the response to each peptide pool. The frequency of responding vaccinees to each peptide pool, subvirion vaccine (Virus) and H5N1 rHA are shown. The overall magnitude of the response is arbitrarily segmented into 3–5-, 5–8-, 8–10- and >10-fold above background levels (3× above background, minimum of 30 SFC).

of overlapping class II peptides covering the entire HA sequence. This was accomplished by using a matrix approach, whereby 20 individual peptide pools, each containing up to ten overlapping 16–17-mers spanning the entire A/Thailand/4(SP-528)/2004 HA protein sequence and having 99% homology to the vaccinating strain, were used to stimulate CD4+ T cells. Such an approach made it more feasible to screen many peptides at one time and permitted identification of individual peptides that were later retested. Following a 24 h incubation with each peptide pool, donor PBMCs were assayed via ELISPOT for the induction of IFN-γ. After normalizing the number of spot forming cells (SFC) for each donor tested, the frequency of peptide pool responses was determined. The frequency of responding vaccine recipients were between 6 and 26%, depending upon the specific peptide pool (Fig. 2). The magnitude of the T cell response was generally 3–5-fold above background with several pools yielding responses 5–8-fold above background, followed by a few eliciting responses 8–10- and >10-fold above background. CD8+ depleted samples were also tested against the clinical trial subvirion vaccine, which was expected to induce the highest frequency and strongest response relative to peptides. Indeed, 80% of individuals tested had measurable T cell responses against subvirion vaccine tested *in vitro*, with a range of 3.2–494-fold above background. Since the subvirion vaccine is a whole virus inactivated preparation carrying other viral proteins (NA, NP, M2) some having high homology with seasonal influenza strains, it is likely that part of the response against the H5N1 vaccine was driven by T cell cross-reactivity. To partially account for this, PBMCs were also restimulated with purified H5N1 rHA as a means of assessing the "HA-only" response. Despite the high degree of HA homology (>95%) among H5N1 strains, there is only ∼63% homology between the HA of A/Vietnam/1203/2004 and the more recently circulating seasonal influenza strain A/New Caledonia/20/99, increasing the likelihood that the H5N1 rHA responses were vaccine induced. We found that 55% of the volunteers had detectable responses to rHA, with 29% of those donors having >10-fold response (Fig. 2). A 2nd round of T cell screening to identify specific peptides present in the peptide pools active in stimulating T cells was performed using only PBMC from those donors having a response against rHA. Using the criteria of rHA-positivity, and being constrained by limited donor PBMC material, resulted in 2nd round testing of 14 donor PBMC samples of the original 35 (see below).

3.2. Measured IFN-γ responses to algorithm predicted Ii-Key hybrid peptides and overlapping peptides are primarily vaccine induced

It was anticipated that donor PBMC samples responsive to rHA in 1st round analysis would show more frequent responses to HA peptide pools and algorithm-predicted peptides, suggesting that the observed responses were vaccine induced and not the result of cross-reactivity. To test this, we compared the peptide pool response frequency of rHA-responsive versus rHA-non-responsive vaccinated donors and eight naive individuals (non-vaccinated). Because "pre-vaccine" PBMC samples from vaccinated volunteers were not available to assess baseline T cell activity for each donor, it was difficult to establish whether PBMC responses to class II epitopes identified from this study were vaccine induced or represented cross-reactive preexisting immunity. It was expected that vaccinated donors having a rHA response would have the most frequently recognized peptide pool responses. Of the 19 rHA positive vaccine recipients tested in 1st round screening, 14/19 had responses to both rHA (denoted by "X") and to 18/20 peptide pools (denoted by closed circles, Fig. 3, panel A). Interestingly, five donors in this panel had responses to rHA but not to any peptide pool. Although peptide pool responses in these individuals were detectable, they did not meet the minimum threshold for being considered a positive response. This may be related to the low concentration of each peptide in a given peptide pool. In examining PBMC samples from rHA non-responsive vaccinated donors, only 3/16 (Donor Nos. 1004, 25, 33) had measurable responses to several peptide pools (Fig. 3, panel B). rHA responsiveness also correlated with the frequency of responsiveness to Ii-Key-modified predicted peptides (data not shown). Finally the same analysis was performed using naive (non-vaccinated) donor PBMCs. Although the overall frequency of 'hits' per peptide pool was clearly lower than the frequency of 'hits' in the PBMC from vaccinated individuals, T cell responses were observed in 3/8 donor samples to several peptide pools and rHA, with magnitudes comparable to vaccinated individuals (Fig. 3, panel C). This is consistent with a recent finding demonstrating that healthy human subjects have detectable CD4+ T cell responses to H5N1 HA class II epitopes [31] most likely the result of cross-reactivity to seasonal influenza viruses. Collectively, these data clearly show a positive correlation between the frequency of rHA responders and frequency of both peptide pool and Ii-Key peptide responders and supports our expectation that these responses were primarily vaccine induced.

3.3. Identification and confirmation of matrix-derived HA peptides: 2nd round PBMC analysis

To identify specific H5N1 HA class II peptides from the overlapping peptide library, peptides derived from the matrix in the 1st round of T cell screening were individually retested in a 2nd round assay to confirm activity. Only PBMC from donors that demonstrated a positive response to H5N1 rHA in the 1st round were used for 2nd round screening. This selection criteria eliminated those donor samples that likely did not have vaccine-induced T cell specific responses towards the HA of the subvirion vaccine. Of the 94 overlapping peptides from H5N1 HA, there was a wide distribution of peptide "hits" throughout the HA sequence for each of the 14 donors. Since the identification of false positives using this method is possible following 1st round screening, each donor was tested against all of their respective matrix-derived peptide "hits" individually, resulting in up to 28 peptides tested for each donor. Testing of these matrix-derived peptides revealed a smaller number of genuinely active peptides. Some donors only responded to one of the matrix-derived peptides, while others (e.g., Donor No. 1008), responded up to seven peptides (Table 1). In total, 16/94 array pep-

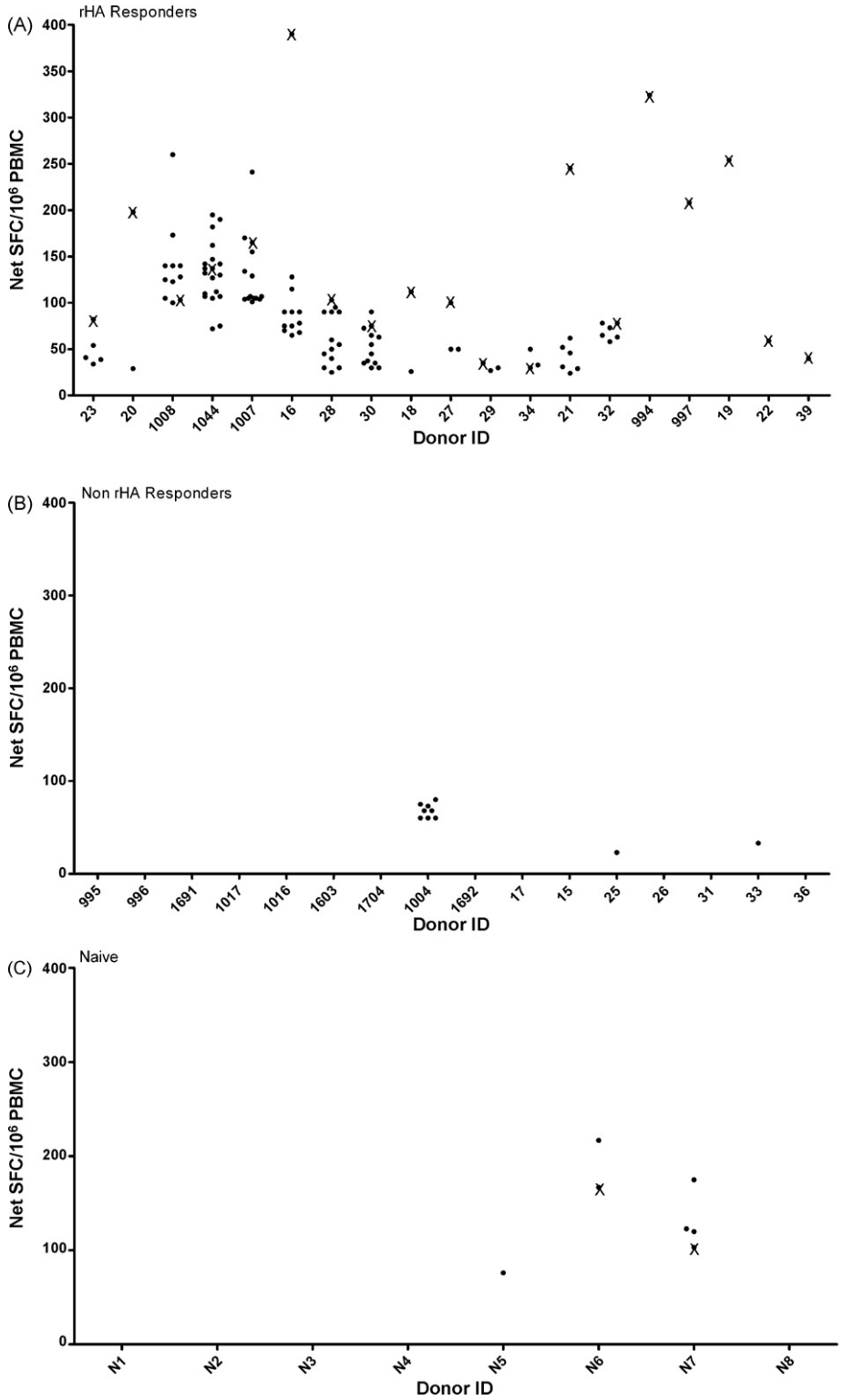

Fig. 3. CD4+ IFN-γ peptide pool response frequency and magnitude between rHA-responsive, rHA-non-responsive and naive (non-vaccinated) donor PBMCs. Background responses (unstimulated PBMC) were subtracted from each peptide pool or rHA response with the net SFC response to peptide pools (closed circles) or rHA ("×") plotted. Data represents the mean SFC response to peptide pools or rHA, assayed in triplicate. Panel A illustrates 19 rHA-responsive donors and their respective frequency of peptide pool "hits", while Panel B illustrates the peptide pool frequency of 16 non-rHA-responsive donors. Naive (non-vaccinated) donors are designated N1–N8 with peptide pool and rHA response depicted in panel C.

tides were detected (75% located in HA1), with eight (BEI 12, 39, 54, 57, 59, 73, 74, 78) having partial to almost complete homology to the New Caledonia rHA sequence and eight being unique to H5N1 HA (BEI 7, 8, 22, 27, 28, 29, 36, 38). The latter would thus appear to be vaccine specific. A few matrix-derived peptides were more frequently recognized than others, such as BEI 36 and 59 (21% recognition); while the magnitude of the observed responses was widely variable (3.3–57-fold above background). It is important to note that the results in Table 1 depict only those peptides that scored positive following 2nd round testing. For instance, Donor

Table 1
In vitro CD4 responses against matrix-derived H5N1 HA peptides*.

Matrix derived peptide[a]		7	8	12	22	27	28	29	36	38	39	54	57	59	73	74	78	H5N1 rHA	H1N1 rHA
Donor ID																			
23									39		3.8							3.6	3.1
20																		74	35
1008			3.4	3.3	4.6		3.7	3.6				3.8	4.1						
1044		12				12				13				28		20		39	37
1007												3.9							
16																		6.6	
28									5.3									3.9	
30															15				46
18																			43
27									3.9								3.3		
29																			
34														39				21	46
21														57		49		55	240
32																		5.3	7.3

*Boxed numbers indicate fold above background response for each peptide.
[a]Peptide numbers denote which peptide out of the 94 peptide array panel were tested and correspond to the following regions within the A/Thailand/4(SP-528)/2004 HA sequence: BEI 7 (aa 36–52), BEI 8 (aa 42–57), BEI 12 (aa 65–81), BEI 22 (aa 125–141), BEI 27 (aa 153–169), BEI 28 (aa 159–175), BEI 29 (aa 165–181), BEI 36 (aa 207–223), BEI 38 (aa 219–235), BEI 39 (aa 225–241), BEI 54 (aa 315–331), BEI 57 (aa 333–349), BEI 59 (aa 345–361), BEI 73 (aa 429–445), BEI 74 (aa 435–451), BEI 78 (aa 459–475).

No. 27 yielded 20 matrix-derived peptide hits, but upon subsequent testing of those peptides individually, only two were actually confirmed to be active (BEI 36, 78). Likewise, Donor No. 32 scored six matrix-derived hits following 1st round analysis, but when tested individually, none were above background. This leads to a potential limitation of using a matrix strategy for screening a peptide library, in that the PBMC response to a pool of 10 peptides (1st round) may respond differently (e.g., peptide competition) *in vitro* compared to the same peptides tested individually. Nonetheless, screening individual peptides within the library (94 H5N1 HA peptides) for all 14 donors would not have been feasible given the number of cells available.

While the T cell responses to H5N1 rHA during 2nd round screening were in general comparable to 1st round analysis, only 8/14 donors responded to H5N1 rHA unlike 1st round analysis whereby all 14 donors were responsive. Three donors (Nos. 1008, 1007, 27) had weak 1st round rHA responses and were not detectable after 2nd round testing. The lack of detection in 2nd round stimulation may be attributed to interassay variability of the ELISPOT assay, which has been reported to vary by up to 11% [32,33] or suboptimal T cell activation.

Given that responses to MHC class II epitopes derived from H5N1 HA have been observed in non-H5N1-exposed individuals [31], it is possible that some of the responses we observed were the result of cross-reactive seasonal influenza T cells. To address this possibility, donor PBMCs were tested against New Caledonia (H1N1) rHA. Although other subtypes could induce cross-reactivity, we chose to test the responses to the New Caledonia strain, as this provided a good comparator for examining the potential cross-reactive T cell responses due to its high prevalence in the population either due to natural infection or vaccination. Indeed, 8/14 donors tested during 2nd round stimulation had detectable levels of IFN-γ to this antigen (Table 1) with a magnitude response of 3.1–240-fold above background. As an example, Donor Nos. 23, 20, 1044, 34, 21, and 32 all had measurable responses to H5N1 rHA and H1N1 rHA, making it difficult to conclude the subvirion vaccine was wholly responsible for inducing H5N1 rHA antigen-specific responses. Even with only ~63% HA sequence homology, it is possible that preexisting immunity to New Caledonia (or other seasonal subtypes), may have elicited cross-reactive H5N1 HA T cell immunity. Further, there was evidence of this for Donor Nos. 1044, 34 and 21, all of whom had strong reactivity to BEI 59 (28-, 39-, 57-fold respectively), corresponding to aa 345–361 of the A/Thailand/4(SP-528)/2004 strain. With the exception of the first and last amino acid in the 17-mer, there is complete sequence homology with the HA of A/New Caledonia/20/99. Likewise for Donor Nos. 1044 and 21, there was strong reactivity to BEI 74 (20- and 49-fold respectively), corresponding to aa 435–451, which shares 88% sequence homology to New Caledonia HA. Cross-reactivity to this region has been previously demonstrated in examining the human CD4+ T cell repertoire to influenza HA [31,34] and in HLA-DR transgenic mice infected with A/New Caledonia/20/99 [35]. Finally, Donor No. 30, having modest T cell responses against BEI 73 (15-fold), corresponding to aa 429–445, did not have detectable responses to H5N1 rHA, implying the peptide response was likely driven by a preexisting seasonal influenza memory T cell response. Similarly, some algorithm-predicted epitopes modified with Ii-Key also were active in donor samples that were non-responsive to H5N1 rHA.

Table 2
HLA-DR typing results.

Donor ID[a]	MHC II
23	DRB1*1101, 14, DRB3*02
20	DRB1*0701, 1101, DRB3*02, DRB4*01
1008	DRB1*0301, 0701, DRB3*01, DRB4*01
1044	DRB1*1104, 1501, DRB3*02, DRB5*01
1007	DRB1*0301, 1501, DRB3*01, DRB5*01
16	DRB1*0701, 0802, DRB4*01
28	DRB1*14, 1601, DRB3*02, DRB5*0202
30	DRB1*15, 16, DRB5*01, 02
18	DRB1*0301, 12, DRB3*01, 02
27	DRB1*0301, 0701, DRB3*01, DRB4*01
29	DRB1*03, DRB3*01
34	DRB1*09, 1303, DRB3*01, DRB4*01
21	DRB1*0301, 12, DRB3*01, 02
32	DRB1*1001, 1103, DRB3*02

[a] HLA typing performed on donors screened following 2nd round T cell stimulation.

While in agreement with others in demonstrating cross-reactive T cell responses to H5N1 HA-derived peptides in individuals not exposed to H5N1, the data presented here strongly suggests that at least half of the peptides identified in this 2nd round analysis are the result of prior H5N1 subvirion immunization.

3.4. Allelic restriction of H5N1 HA class II epitopes identified from library peptides

While Ii-Key/H5 hybrid peptides were selected using algorithm prediction to identify promiscuous MHC class II epitopes for providing greater population coverage, one concern was that the matrix-derived peptides would be strongly restricted to specific class II alleles. To address this, all 14 donors were HLA typed following 2nd round analysis of matrix-derived peptides. As shown in Table 2, DRβ1*03 was most prevalent (6/14), while DRβ1*07 and DRβ1*11 were both expressed by 4/14 donors, while other DRβ1 alleles were less representative (DRβ1*8, 12, 13, 14, 15, 16). DRβ3*01 and 02 were expressed in 7/14 donors, while DRβ4*01 and DRβ5 were expressed in 5/14 and 4/14 donors respectively. Investigating those peptides that were most frequently recognized and/or elicited the strongest T cell responses (BEI 7, 27, 36, 38, 59, 73 and 74), it did not appear as though these peptides were restricted to one individual HLA type. As an example, BEI 36 was able to elicit CD4+ T cell responses in three donors expressing different HLA-DR genotypes, suggesting that the peptide is not restricted to one DR type. Likewise, BEI 59, which induced strong T cell activity, also appeared not to be strongly restricted. Although the number of donors typed was small, there did not appear to be strong restriction of any of the MHC class II epitopes identified from the library peptides for given HLA-DR genotypes.

3.5. Increased vaccine dose does not correlate with increased T cell responses

An additional goal of this study was to establish if volunteers receiving a higher dose of vaccine exhibited a stronger T cell response to the antigens employed for this study (subvirion vaccine, H5N1 HA, overlapping peptide pools, active peptides from the pools, and Ii-Key peptides). It was anticipated that those individuals who were vaccinated with three doses of 90 μg subvirion vaccine would demonstrate the strongest recall T cell responses, as measured via ELISPOT assay. However, the majority of donors in this group had no response or only weak responses to overlapping peptide pools or predicted class II epitopes modified with Ii-Key (data not shown). This is in contrast to two donors receiving three doses of 45 μg that had moderate to strong responses to several peptide pools and to predicted epitopes. Similarly, PBMC from donors receiving higher doses of vaccine did not show increased T cell responses either to the vaccine itself or to H5N1 rHA. In short, while strong T cell responses were observed selectively in PBMC from vaccinated individuals, neither the frequency nor the magnitude of the response correlated with the dose of vaccine.

4. Discussion

With the possibility of a world-wide H5N1 pandemic, continued efforts are being made to better prepare for such an outbreak. Vaccination is likely the most effective means of curtailing a pandemic that could claim the lives of millions. Although there is one FDA approved subvirion vaccine currently being stockpiled in the US and another available in Europe, both vaccines, as tested in clinical trials are not optimally immunogenic and require multiple doses to induce protective hemagglutinin inhibition titers.

Previous studies have demonstrated that priming CD4+ T cells using antigen-specific peptides can enhance the production of viral neutralizing antibodies and promote viral clearance [36,37]. It is anticipated that the use of conserved class II epitopes, modified to include a fragment of the invariant chain, offers a unique approach to improve the immunogenicity of H5N1 vaccines already approved or under development as well as providing heterosubtypic immunity. To our knowledge, this is the first report that has identified class II H5N1 HA epitopes for the purposes of vaccine design. Although others have investigated the human CD4+ T cell repertoire to HA following seasonal influenza infection [34,35] or vaccination [38–40], we undertook this analysis to determine what epitopes are most frequently recognized following vaccination with an H5N1 inactivated subvirion vaccine.

To identify CD4+ immunodominant epitopes following H5N1 inactivated subvirion vaccination, we utilized CD8+ depleted PBMC samples and stimulated them directly *ex vivo* initially using a set of 24 algorithm-predicted peptides, modified to include the Ii-Key moiety. Screening these Ii-Key hybrid peptides revealed several that induced a high frequency of IFN-γ response among donor PBMC. Although we did not directly compare these Ii-Key peptides to the matched-control native peptides, previous *in vitro* and *in vivo* studies have demonstrated greater T cell activity and immunological responses with class II epitopes modified with the Ii-Key moiety [22,23,26,30,41]. Some active Ii-Key peptides partially overlapped library-derived peptides identified as active in 2nd round T cell analysis, others contained sequences not identified by library screening. In particular, Ii-Key peptides 121, 170, 431 and 443 showed good activity and overlapped with library-derived BEI peptides 22, 29, 73 and 74 (respectively) which also showed activity. This provides corroborating evidence that these regions indeed contain active MHC class II epitopes. Not surprisingly, however, there were sequences identified through peptide library screening that were not represented in our set of algorithm-predicted peptides and vice versa. Therefore, the combined use of these two methods for epitope identification gave a better representation of H5N1 HA epitopes than either one alone.

To extend our findings, we took on a more brute force approach to epitope identification and acquired a peptide array set that consisted of overlapping HA peptides. Preliminary analysis entailed testing these overlapping peptide pools using 35 vaccine recipients in conjunction with a matrix-derived approach, followed by retesting individual peptides, making it more practical to screen many peptides at one time. Using this approach in 1st round T cell analysis permitted for rapid assessment of subvirion vaccine and H5N1 rHA responses *in vitro* in addition to identifying class II epitopes. Interestingly, nearly half of the vaccinated donors did not show reactivity to rHA. One possible explanation may be that samples obtained for

this study were collected approximately 2 years after vaccination; therefore memory T cells may have been undetectable. A second possibility is that the vaccine was ineffective at inducing a *de novo* immune response. The latter explanation is partially supported by lack of HA-specific antibody responses in some individuals from the original and extended clinical trial following subvirion vaccination [4,5]. It was notable that some donors receiving the highest vaccine dose (90 $\mu g \times 3$) did not have detectable T cell responses to either peptide pools or rHA while some receiving the lower vaccine doses did respond (data not shown), therefore there was no strict correlation between vaccine dose and T cell response. Furthermore, we did not observe a correlation between H5N1 specific hemagglutinin titers from the original trial and the magnitude of the T cell response to peptide pools in PBMC samples tested within this study (data not shown). Not surprisingly, there was an overwhelming preponderance of reactivity to both peptide pools and Ii-Key-modified predicted epitopes in donor samples responding to rHA, while non-responsive rHA donors and naive (non-vaccinated) donors elicited little to no response to peptide pools (Fig. 3).

Due to yearly seasonal influenza virus infection and/or immunization in the population, a concern of the current study was to distinguish between vaccine-induced *de novo* CD4+ T cell responses and cross-reactivity to prior seasonal virus infection or immunization. While we did observe some minimal responses to algorithm-predicted peptides and to pools containing overlapping H5N1 HA peptides in naive (non-vaccinated) or rHA non-responsive donors, there was clearly a much more frequent and stronger response in H5N1 vaccinated donors that were H5N1 rHA responsive. The detection of H5N1 HA-specific T cell responses in naive (non-vaccinated) individuals is in line with the findings of Roti et al. who demonstrated that healthy individuals with no prior exposure to H5N1 had detectable CD4+ T cell responses against H5N1 HA, NA, matrix and nucleoprotein epitopes. The analysis of our data was further complicated by not having access to "pre-vaccine" control PBMCs, which could have been used to establish background levels of T cell responses to antigens tested, although this was partially remedied by screening several H5N1 naive (non-vaccinated) donor PBMCs. Peptide pools generally induced weak to moderate IFN-γ activity for vaccine recipients (3–5-fold above background), although several pools elicited stronger activity depending on the donor. Given the length of time between the last booster vaccine dose and when PBMCs were collected (∼24 months), it is not surprising that many of the peptide pool responses were weak.

To maximize the possibility of identifying H5N1 HA-specific epitopes from the H5N1 peptide library, only those donors that were reactive against H5N1 rHA following 1st round testing were utilized for 2nd round screening of specific peptides. While active peptides were identified in this group, some of the donors in which positive responses were observed were also reactive to H1N1 rHA. Therefore, we cannot exclude the possibility that some of the peptides active in 2nd round screening were the result of cross-reactivity. Sixteen individual epitopes were confirmed to be active after 2nd round T cell stimulation. Of these, eight were found to have partial to almost complete sequence homology with a common seasonal influenza H1N1 strain, while the remaining eight peptides were unique to H5N1 HA, in that there was little to no homology with New Caledonia HA. Following 2nd round T cell stimulation, it was determined that BEI 59, 73 and 74 evoked strong (20–57-fold above background) *in vitro* recall responses in Donor Nos. 1044, 34 and 21 and likely represent cross-reactivity based on sequence alignment of A/Vietnam/1203/04 and A/New Caledonia/20/99 HA.

The importance of antigen-specific CD4+ T cells in generating or contributing to protective immunity to influenza viral infection has been clearly demonstrated in a variety of different studies [10,14,42]. However, there have been only a limited number of studies examining the human CD4+ T cell repertoire to seasonal influenza, and none to our knowledge investigating such responses to H5N1 HA following vaccination. For the purposes of vaccine design, specifically utilizing a peptide-based approach, an important first step is to identify immunodominant viral epitopes, followed by identification of conserved and promiscuous epitopes that could yield cross-strain H5N1 protection and broader population coverage. The current study has identified highly conserved MHC class II epitope peptides with a high likelihood of specificity towards H5N1 HA as well as cross-reactive seasonal influenza virus HA epitopes. Which combination of epitopes might serve as the most useful and effective vaccine is unclear at this point, though further experimentation is warranted. Immunization with highly conserved MHC class II epitope peptides for the generation of CD4+ T cells reactive to H5N1 HA can reasonably be expected to provide some level of partial immunity that alone could reduce fatalities in the event of an H5N1 pandemic, while also potentially increasing heterosubtypic immunity. In addition, use of MHC class II epitope peptides may be used as a pre-emptive immunization strategy to allow for antigen dose sparing of more traditional but supply-limited vaccines to achieve greater population coverage.

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