Healthy human subjects have CD4+ T cells directed against H5N1 influenza virus[1]

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Abstract

It is commonly perceived that the human immune system is naïve to the newly emerged H5N1 virus. In contrast, most adults have been exposed to influenza A H1N1 and H3N2 viruses through vaccination or infection. Adults born before 1968 have likely been exposed to H2N2 viruses. We hypothesized that CD4+ T cells generated in response to H1N1, H3N2 and H2N2 influenza A viruses also recognize H5N1 epitopes. Tetramer Guided Epitope Mapping and antigen specific class II tetramers were used to identify H5N1 specific T cell epitopes and to detect H5N1 specific T cell responses. 15 out of 15 healthy subjects tested had robust CD4+ T cell responses against matrix protein, nucleoprotein and neuraminidase of the influenza A/Viet Nam/1203/2004 (H5N1) virus. These results are not surprising, since the matrix protein and nucleoprotein of influenza A viruses are conserved, while the neuraminidase of the H5N1 virus is of the same subtype as the circulating H1N1 influenza strain. However, H5N1 hemagglutinin reactive CD4+ T cells were also detected in 14 out of 14 subjects examined, despite the fact that hemagglutinin is less conserved. Most were cross-reactive to H1, H2 or H3 hemagglutinin epitopes. H5N1 reactive T cells were also detected *ex vivo*, exhibited a memory phenotype, and were capable of secreting IFN-γ, TNF-α, IL-5, and IL-13. These data demonstrate the presence of H5N1 cross-reactive T cells in healthy Caucasian subjects implying that exposure to influenza A H1N1, H3N2 or H2N2 viruses through either vaccination or infection may provide partial immunity to H5N1 virus.

Keywords

T cells; MHC; Epitopes; Viral; Vaccination

Introduction

In recent years, highly pathogenic influenza A H5N1 viruses have caused disease outbreaks in poultry and wild birds in Asia, Africa and Europe. Sporadic human cases have been reported in different countries, mostly in Asia (1–6). As of October 17, 2007, a total of 331 human cases have been confirmed, with a fatality rate of close to 60% (http://www.who.int/csr/disease/avian_influenza).

At present, most of the reported human cases have occurred in rural areas and direct contact with infected poultry was the probable route of transmission. However, the ability of

influenza A viruses to undergo genetic reassortment with gene segments from other influenza A strains and the high mutation rate of their RNA genomes have raised concerns that strains of H5N1 could adapt the ability to infect humans directly, eventually causing a human pandemic (7,8). Although the majority of the human population has not been exposed directly to H5N1 viruses, most adults in developed countries have been exposed to other influenza A viruses, including influenza A H1N1, and H3N2 viruses through either vaccination or infection. In addition, many adults born before 1968 have been exposed to H2N2 (9). Given the homology that exists between the viral proteins of different influenza A subtypes, we hypothesized that CD4+ T cells generated in response to other influenza A viruses could also recognize H5N1 viral proteins. In this study we examined whether healthy adults in the U.S. had measurable CD4+ T cell responses directed against the Matrix Protein 1 (M1), Nucleoprotein (NP), Neuraminidase (NA) and Hemagglutinin (HA) of the highly pathogenic A/Viet Nam/1203/2004 (H5N1) virus (10,11). All subjects examined had CD4+ T cells directed against M1, NP and NA of the H5N1 viruses. Moreover, H5N1 HA reactive CD4+ T cells were also detected. Thus the immune repertoire of most healthy subjects may afford some protection from the H5N1 virus.

Materials and Methods

Human Subjects

The study was approved by the Benaroya Research Institute Institutional Review Board. A total of 22 subjects were recruited with informed consent for these studies. All subjects were healthy volunteers of Caucasian descent. A two-step protocol was used to determine the class II HLA genotype for each subject. Low-resolution class II typing was carried out using an SSP UniTray typing kit (Invitrogen,). High-resolution class II typing was accomplished by DNA sequencing with allele specific primers based on low-resolution typing results. Subjects having DR0101, DR0404, DR0701 and DR1101 haplotypes were selected for detailed studies as reported in this manuscript. In addition, subjects with DR0301 and DR1501 were also examined. These alleles were selected based on their prevalence in the US Caucasian population and the availability of these soluble MHC molecules in our laboratory.

Production of class II tetramers

Soluble HLA class II molecules were used in this study. The production and purification of these recombinant class II molecules have been previously described (12). For production of class II tetramers, soluble class II molecules were labeled with biotin by using Biotin ligase according to the manufacturer's instruction (Avidity). Biotinylated class II molecules were dialyzed into phosphate storage buffer (pH 6.0) and loaded with pooled peptide mixtures or individual peptides by combining 0.5 mg/ml class II, 10 mg/ml of peptide (2 mg/ml of each individual peptide for pools), and 0.2% η-octyl-D-glucopyranoside. After incubating at 37°C for 48 hours, tetramers were cross-linked using PE-Streptavidin (Biosource) (13).

Peptides and recombinant H5 HA protein

Overlapping peptides that covered the entire A/Viet Nam/1203/2004 (H5N1) M1, NP, NA and HA proteins were purchased from Mimotopes. These peptides were 20 aa in length sharing a 12 aa overlap with the adjacent peptides. There were a total of 30 M1 peptides, a total of 61 NP peptides, 55 NA peptides and 70 HA peptides. In TGEM experiments, peptides spanning the sequence of each protein were divided up into different pools, with 5 consecutive peptides in each pool. There were a total of 6 pools for M1, 12 pools for NP (with 6 peptides in pool 12), 11 pools for NA, and 14 pools for HA. H1, H2, H3 and H5 HA peptides 12aa to 20aa in length were also purchased from Mimotopes. Recombinant H5 HA

protein derived from A/Viet Nam/1203/2004 (H5N1) was provided by Biodefense and Emerging Infections Research Resource Repository (BEI Resources).

TGEM

The TGEM procedure was as previously described (14,15). Briefly, CD4+ T cells were isolated from PBMC using the Miltenyi "no-touch" CD4+ T cell isolation kit. Non-CD4+ cells recovered from the magnetic column were incubated in 48 well plates (3×10^6 cells per well) for 1 hour and then washed, leaving adherent cells for use as APC. After adding 2 million CD4+ T cells per well, each well was stimulated with pooled influenza A peptides (10 μg/ml). Cells were fed with 20U/ml IL-2 in fresh medium starting at day 7, and were re-fed as needed. After 14 days incubation, 100 μl of resuspended cells were stained with PE-conjugated pooled peptide tetramers for 60 min at 37°C. Subsequently, cells were stained with CD4-PerCP, CD3-allophycocyanin and CD25-FITC mAbs (eBioscience) and analyzed by flow cytometry. Cells from pools that gave positive staining were analyzed again with individual peptide tetramers produced by loading empty class II molecules with each of the individual peptides from the positive peptide pool.

Other cell culture and tetramer staining assays

An approach that involved magnetic bead enrichment for tetramer positive cells, as previously described (16), was used to determine the frequencies of H5N1 reactive T cells. In brief, six million of PBMC in a volume of 100 μl was stained with 20 μg/ml tetramers at room temperature for 2 hrs. During the last 20 min, the cells were stained with FITC conjugated anti-CD45RA (eBioscience), allophycocyanin conjugated anti-CD4 (eBioscience), PerCP conjugated anti-CD14 (BD Pharmingen) and PerCP conjugated anti-CD19 (BD Pharmingen). Subsequently, cells were washed and then incubated with anti-PE magnetic bead (Miltenyi Biotec) at 4°C for another 20 min. The cells were washed again and a tenth of the fraction was saved for later analysis. The other fraction was passed through a Miltenyi magnetic column. The bound fraction was flushed out and collected. Cells in both the bound fraction and the pre-column fraction were stained with ViaProbe (BD Biosciences) for 10 min before flow cytometry. For analysis, cells were gated on

FCS/SSC, CD4+, CD14− and CD19− population. Dead cells were also gated out using Viaprobe. The frequency was calculated as the total number of tetramer positive cells in the bound fraction divided by 10 × the total number of CD4+ T cells in the pre-column fraction

For assays using fractionated memory T cells, CD4+ cells were isolated as described above. Cells were then stained with FITC-conjugated anti-CD45RA and PE-Cy5-conjugated anti-CD4 Abs, and the CD4+CD45RA+ and the CD4+CD45RA− fractions were sorted. 2.5×10^6 CD4+CD45RA− cells were stimulated with 10 μg/ml of HA peptide. Cells were fed with IL-2 starting at day 7, and were analyzed with tetramers on day 14.

For experiments using H5 HA proteins (rather than peptides), CD4+ T cells and CD14+ cells at a 10 to 1 ratio were incubated with H5 HA protein at 100 μg/ml for 2 hr at 37°C in a conical tube. Cells were washed once, and then plated out at a density of 2.5×10^6 cells per well in a 48 well plate. Cells were analyzed with tetramers on day 14.

For cross-reactivity experiments, cells were plated into 4 different wells. Cells in each individual well were stimulated with H5 HA peptide, or with HA peptides from the corresponding region of H1, H2, H3 HA (all at 10 μg/ml). Cells were analyzed with tetramers on day 14.

Cytokine Assays

For *ex vivo* staining of PBMC for cytokines, 4 million PBMC were activated with a panel of tetramers (2.5 μg/ml each) and CD28/CD49d (1 μg/ml), Belfedin A (5 μg/ml) was added 2 hrs later. Cells were then incubated for another 16 hrs at 37°C, and permeabilized for intracellular cytokine staining (ICS) according to protocol as suggested by BD BioSciences. Briefly, cells were treated with 20 mM EDTA, washed, and then subsequently followed by washes with BD FACS Lyse buffer and BD FACS PERM II buffer. Cells were then stained with the appropriate allophycocyanin conjugated cytokine antibody, PerCP conjugated anti-CD4 and FITC conjugated anti-CD45RA. Cells were again washed and resuspended in FACS buffer with 1% paraformaldehyde and 1% FBS before analysis. Cells were gated on size scattering, and the CD4+ tetramer positive population was examined for cytokine staining.

For cytokine staining of *in vitro* stimulated PBMC, cells were stimulated with the peptide of interest as described above. On day 14, 200,000 cells in 100 μl were transferred to wells in a 96 well flat bottom plate which were precoated with 0.5 μg of the corresponding tetramers of interest. After an overnight culture, 25 μl of the supernatants were harvested and assayed for cytokines using the Meso Scale Discovery (MSD) Cytokine Multiplex kit according to the manufacturer's instruction. The MSD plate was read using the MSD SECTOR Imager 2400.

Results

Detection of CD4+ T cells that are specific for internal viral proteins of A/Viet Nam/1203/2004

In general, there is a perception that the human immune system is naive to the newly emerged H5N1 virus. However, the internal proteins of influenza A viruses, such as M1 and NP are highly conserved amongst different subtypes. As the general population has been exposed to various influenza A viruses, it seemed likely that T cells in the peripheral blood of human subjects would recognize the internal proteins of H5N1 viruses, such as A/Viet Nam/1203/2004.

To test this possibility, the Tetramer Guided Epitope Mapping (TGEM) approach (14,15) was used to identify CD4+ T cell epitopes within the M1 protein of A/Viet Nam/1203/2004. In one representative experiments, CD4+ T cells from a DRA1/DRB1*0404 (DR0404) subject were stimulated with the 6 different pooled overlapping peptides derived from A/Viet Nam/1203/2004 M1 peptides. Fourteen days later, cells were stained with the corresponding DR0404/M1 pooled peptide tetramers. M1 peptides that gave positive staining were observed within pools 2, 3, 4 and 6 (Figure 1A). Single peptide loaded tetramers for each individual peptide within the positive pools were used to stain the cells again. As shown in Figure 1B, tetramers loaded with individual peptides p9, p10, p13, p16, p26 and p27 gave clear positive staining for this DR0404 subject. Positive staining with a specific peptide identifies that peptide as a T cell epitope, and confirms the presence of CD4+ T cells specific for that particular epitope within the PBMC of the healthy subject studied. These experiments identified $M1_{65-84}$ (p9), $M1_{73-92}$ (p10), $M1_{97-116}$ (p13), $M1_{121-140}$ (p16), $M1_{201-220}$ (p26), and $M1_{209-228}$ (p27) as peptides containing DR0404 restricted H5N1 M1 epitopes for the subject studied. The sequences of these peptides are shown in Table I.

Similar experiments were also carried out to identify DR0404 restricted H5 NP specific T cells. A total of 61 NP peptides in 12 different pools were screened with DR0404/NP pooled peptide tetramers. Positive staining was observed with pools 9, 11 and 12. Subsequent staining identified NP peptides $NP_{321-340}$ (from pool 9), $NP_{401-420}$, $NP_{433-452}$ (from pool

11) and $NP_{441-460}$ (from pool 12) as DR0404 restricted NP epitopes. The sequences of these peptides are summarized in Table I. Experiments were repeated with other DR0404 subjects, and identical M1 and NP epitopes were identified. Thus the TGEM approach was successful in identifying DR0404 restricted T cells in multiple individuals with the DR0404 haplotype that recognize epitopes within the M1 and NP proteins of the A/Viet Nam/1203/2004 virus.

These results were not surprising, given the high degree of homology between the M1 and NP proteins of H5N1 and circulating strains of influenza A virus. Examination of the H5 M1 and NP amino acid (aa) sequences identified above and the corresponding M1 and NP regions in both the A/New Caledonia/20/99 (H1N1) and A/Panama/2007/99 (H3N2) viruses indicated that these sequences are either highly conserved or completely identical. The observed responses were not unique to subjects having the DR0404 allele, as CD4+ T cell responses to H5N1 M1 and NP proteins were also observed in 6 additional subjects with either DRA1/DRB1*0101(DR0101), DRA1/DRB1*0701(DR0701) or DRA1/DRB1*1101 (DR1101) alleles. These data are also summarized in Table I.

Both H5N1 M1 and NP reactive T cells were also detected *ex vivo* by tetramers. For these experiments, PBMC were stained with a panel of PE conjugated tetramers. For DR0404 subjects, the M1 tetramers used were $DR0404/M1_{73-92}$, $DR0404/M1_{121-140}$ and $DR0404/M1_{209-228}$; the NP tetramers used were $DR0404/NP_{321-340}$, $DR0404/NP_{401-420}$ and $DR0404/NP_{433-452}$. For DR0701 subjects, the M1 tetramers used were $DR0701/M1_{33-52}$, $DR0701/M1_{97-116}$ and $DR0701/M1_{177-196}$; the NP tetramers used were $DR0701/NP_{145-164}$, $DR0701/NP_{257-276}$ and $DR0701/NP_{321-340}$. For DR0101 subjects, the M1 tetramer used was $DR0101/M1_{17-36}$; and the NP tetramers used were $DR0101/NP_{73-92}$, $DR0101/NP_{297-316}$ and $DR0101/NP_{401-420}$. For DR1101 subjects, the M1 tetramers used were $DR1101/M1_{169-188}$ and $DR1101/M1_{201-220}$; the NP tetramers used were $DR1101/NP_{113-132}$ and $DR1101/NP_{185-204}$. Cells were treated with anti-PE magnetic beads and tetramer positive cells were enriched uisng a Miltenyi column. The cells were then co-stained with anti-CD4 and anti-CD45RA antibodies before flow cytometry analyses. Representative results of these experiments are shown in Figure 2. The results also indicated that these M1 and NP reactive T cells were CD45RA−, suggesting that these are memory CD4+ T cells. Amongst the 7 different subjects examined, the frequencies of M1 reactive T cells ranged from $4/10^6$ to $33/10^6$; frequencies of NP reactive T cells ranged from $5/10^6$ to $50/10^6$. The observed frequencies of these H5N1 T cells were similar to the frequencies of the H3N2 influenza T cells as reported in the literature (17). In summary, a total of 15 different subjects were examined by either the TGEM approach or the *ex vivo* tetramer staining approach, and all had measurable H5N1 M1 and NP T cell responses.

CD4+ T cells which are specific for NA of A/Viet Nam/1203/2004

In contrast to the internal viral proteins, the viral envelope protein, NA, of influenza A viruses is less conserved, and is classified into 9 different subtypes (18,19). However, the NA gene of the A/New Caledonia/20/1999 virus (H1N1) (a current circulating influenza virus) and the NA gene of A/Viet Nam/1203/2004 (H5N1) virus belong to the same subtype and there is an 80% similarity in NA aa sequences between these two viruses. We used the TGEM approach to identify CD4+ T cell epitopes within the NA gene and to examine whether CD4+ T cells from healthy human subjects can recognize the NA protein of the A/Viet Nam/1203/2004 virus. A total of 55 NA peptides in 11 different peptide pools were screened by TGEM. Tetramer staining identified NA peptides $NA_{201-220}$ (from pool 6), $NA_{345-364}$ (from pool 9) and $NA_{409-428}$ (from pool 11), as DR0404 restricted NA epitopes. The sequences of these peptides are listed in Table II. In addition to these DR0404 restricted NA epitopes, DR0101, DR0701 and DR1101 restricted NA epitopes were also identified in subsequent experiments (Table II). In addition, NA reactive T cells were also detected *ex*

vivo by tetramer staining in 6 out of 7 individuals tested. Representative results are shown in Figure 3.

CD4+ T cells that are specific for HA of A/Viet Nam/1203/2004

Some degree of cross-recognition is to be expected between the M1, NP, and NA proteins given the homology of these proteins amongst the different influenza A subtypes. However, cross-recognition of hemagglutinin might be less likely given the fact that this protein is the least conserved among different subtypes (19–21). For example, amino acid sequence homology comparisons indicated there is only 63% homology between H5 and H1 HA proteins (comparison of the A/Viet Nam/1203/2004 strain and the A/New Caledonia/20/99 H1N1 strain), 74% homology between H5 and H2 (compared with the A/Singapore/1/57 H2N2 strain), and 41% homology between H5 and H3 (compared with the A/Panama/ 2007/99 H3N2 strain).

The general population has had exposure to H1N1 and H3N2 viruses, while the population born before 1968 has had exposure to H2N2 viruses (9). We reasoned that repeated exposure to influenza viruses in general would enhance HA T cell cross reactivity. Thus, to maximize our chance of detecting H5 HA reactive T cells, the subjects recruited for this portion of the study were all over 40 years old. To determine whether CD4+ T cells directed against the various influenza A viruses of H1, H2 and H3 subtypes can recognize H5 HA epitopes, the TGEM approach was used to identify H5 HA specific T cells from healthy human subjects. Results from an experiment with a DR0404 subject are shown in Figure 4. Positive tetramer staining was observed for peptide pools 5, 10, 11 and 12 and $HA_{169-188}$ (p22 from pool 5), $HA_{393-412}$ (p50 from pool 10), $HA_{401-420}$ (p51 from pool 11) and $HA_{441-460}$ (p56 from pool 12) were confirmed as DR0404 restricted H5 HA epitopes. Identical results were observed in another DR0404 subject. The results of the DR0404 restricted HA epitopes in these experiments are also summarized in Table III.

Similar experiments lead to identification of DR0101, DR0301, DR0701, DR1101 and DR1501 restricted H5 HA responses in 12 additional healthy subjects. These epitopes are shown in Table III. The epitopes identified in these subjects were confirmed in additional subjects with identical haplotypes. Also, by stimulating T cells with shorter peptides (in the corresponding regions) and staining with tetramers generated using these shorter peptides, H5 HA T cell epitopes that were 12 to 16 aa long were also identified. These data are also summarized in Table III.

Because the experiments described above were carried out using synthetic H5 HA peptides rather than protein, it is possible that some of the epitopes identified are not naturally processed. To investigate this possibility, PBMC from several subjects were stimulated with recombinant H5 HA protein and the resulting H5 HA specific T cell responses were evaluated using H5 HA tetramers generated with the 20 aa peptides as listed in Table III. Using this approach, all of the epitopes shown in Table III (with the exception of HA_{49-68} which not tested) were confirmed as naturally processed epitopes (data not shown).

In a separate series of experiments, CD4+ cells were fractionated into CD45RA+ and CD45RA− populations, and these two distinct populations were stimulated with H5 HA peptide. Two weeks after the initial culture, HA specific responses were assayed by tetramers staining. Responses to all the H5HA epitopes shown in Table III (with the exception of HA_{49-68} which was not tested) were detected in the CD45RA− population, suggesting that the observed responses originate primarily from cross-reactive memory T cells rather than naïve T cells. Representative results of these experiments are shown in Figure 5.

H5 HA reactive T cells were also detected by H5 HA specific tetramers *ex vivo* in 7 out of 12 subjects examined. The majority of these T cells also exhibited a memory T cell phenotype, with frequencies ranging from $4/10^6$ to $45/10^6$ in the 7 subjects with measurable *ex vivo* responses. Representative data for these experiments are shown in Figure 6.

Cross-reactivity of HA specific CD4+ T cells

To demonstrate more directly that HA responsive T cells can cross-recognize the HA protein of different influenza A subtypes, H5 HA epitopes and the corresponding HA peptide regions of H1 (A/New Caledonia/20/99), H2 (A/Singapore/1/57) and H3 (A/Panama/2007/99) were used to stimulate PBMC from healthy DR0701 and healthy DR0404 subjects. These cells were then analyzed by staining with H5 HA tetramers. A positive staining of an H1, H2, or H3 stimulated culture would indicate a response elicited by a circulating influenza strain that cross reacts with H5. Representative results are shown in Figure 7. For the DR0701 subject, these results indicated that cross-reactive H5 $HA_{253-265}$ specific T cells were elicited by HA from A/New Caledonia/20/99 viruses and that cross-reactive H5 $HA_{305-316}$ specific T cells were elicited by HA from both A/New Caledonia/20/99 and A/Singapore/1/57 viruses. For the DR0404 subject, cross-reactive H5 $HA_{408-420}$ specific T cells were elicited by HA from both A/New Caledonia/20/99 and A/Singapore/1/57 viruses (aa sequences were exactly identical between A/Viet Nam/1203/2004 (H5N1) and A/Singapore/1/57 (H2N2) in this region).

Similar experiments were also carried out with other H5 HA epitopes. Cross-reactive H5HA specific T cells that elicited by H1, H2 or H3 peptides were also observed for DR0101, and DR1101 CD4+ T cell epitopes. These results are summarized in Table IV. The cross-reactivity of the DR0301 and DR1501 HA reactive T cells was not determined.

Functional responses of M1, NP, NA and HA reactive T cells

To evaluate the functional responses of H5N1 reactive T cells, the cytokine profile of these T cells were examined by ICS of tetramer positive T cells *ex vivo*. For these assays, PBMC were activated with a panel of tetramers, and Belfedin A was added 2 hrs later. Cells were then incubated for another 16 hours, and permeabilized for ICS. Representative results of IFN-γ staining of M1, NP, NA and HA reactive T cells are shown in Figure 8. These results demonstrate the functional responses of H5N1 cross- reactive T cells upon challenge with H5N1 Ags.

Although similar data were also obtained for *ex vivo* TNF-α staining (Figure 8), it was not possible to detect IL-5 and IL-13 using this approach. To establish whether H5N1 reactive T cells are capable of producing other cytokines such as IL-5 and IL-13, an *in vitro* stimulation assay was developed. For these assays, CD4+ T cells were stimulated with individual NA or HA peptides. At day 14, cells were transferred to new wells which were pre-coated with the corresponding peptide specific tetramers. Supernatants were harvested after an overnight culture and assayed for the presence of IFN-γ, IL-5 and IL-13. As shown in Figure 9A and 9B, T cells generated by H5N1 NA and H5N1 HA stimulation secreted IFN-γ, IL-5 and IL-13 during the overnight culture. As shown in Figure 9C, T cells generated by H1 HA stimulation produced similar levels of IFN-γ, IL-5 and IL-13 during the overnight culture regardless of whether these cells were restimulated with H1 HA peptide or with the corresponding H5 HA peptides. Apparently, CD4+ T cells which have been previously activated by H1 HA (*in vivo* or *in vitro*) can give a functional response when rechallenged with H5 HA. Together these results suggest that cross-reactive T cells can provide help for cellular and humoral responses to diverse strains of influenza A.

Discussion

In this study, we report the detection of CD4+ T cells that recognize H5N1 influenza viral antigens in the peripheral blood of healthy Caucasian subjects. Although the results being shown focused on subjects with DR0101, DR0404, DR0701 and DR1101 haplotypes, we have also examined subjects that have DR0301 and DR1501 haplotypes. In total, we examined 22 healthy subjects, and T cell responses directed against H5N1 viral proteins were observed in every subject. These findings demonstrated the presence of H5N1 cross-reactive T cells in the Caucasian populations. These A/Viet Nam/1203/2004 (H5N1) responsive CD4+ T cells recognized peptides derived from the well conserved M1, NP, and NA proteins and even the more weakly conserved HA protein.

In concept, our results are in agreement with published reports describing cross-reactivity between different influenza strains. Jameson et al demonstrated that CD4+ and CD8+ human cytotoxic T cell lines generated against A/PR/8/34 (H1N1) could recognize B-LCL infected with various strains of swine and avian influenza A viruses (22). Most of the T cell lines they characterized recognized internal viral proteins, such as M1and NP. There were also lines that recognized NS1, PB1 and PB2. Jameson et al also observed a CD4+ A/PR/ 8/34 NA specific line that could lyse B-LCL infected with other avian H1N1 viruses. However, cross-reactivity with H5N1 infected cells was not observed. Working with a CD8+ CTL line specific for NP, Boon et al. observed that these CTL recognized NP from other heterosubtypic variants (23). Collectively, these data suggest that most healthy adults in the US have T cells that are directed against the internal proteins of different influenza A subtypes, including the H5N1 subtype which is completely naïve to the US population.

In this current study, we used a more extensive approach to demonstrate that T cells directed against the NA of H5N1 were present in non-exposed Caucasian subjects. This can be accounted for by the fact that most healthy subjects have been exposed to the H1N1 viruses through natural infection or through vaccination. H1N1 and H3N2 viruses are the major influenza A viruses that have circulated the globe since 1968. As the NA protein of H1N1 and H5N1 is of the same NA subtype, it is not unexpected that T cells that are directed against the NA protein of the H1N1 virus would be cross-reactive to the NA protein of the H5N1 virus.

In addition to demonstrating the presence of H5N1 M1, NP, and NA reactive T cells, this study also detected H5N1 HA specific T cells after *in vitro* expansion in 14 out of 14 healthy Caucasian subjects examined. This finding was less expected because HA is the least conserved protein among different influenza subtypes. The H5 HA epitopes detected were located in both the HA1 and HA2 domains of HA and exhibited a memory T cell phenotype. Since it is unlikely that the subjects studied had been exposed to H5N1 viruses, these observations support the hypothesis that the H5 HA responsive T cells are derived from cross-reactive T cells. Indeed, of the eleven H5 HA T cell epitopes examined (Table IV), five showed cross-reactivity to H1 and H2 HA, one had cross reactivity to H1 HA only and one had cross-reactivity to H2 and H3 HA. The higher degree of cross-reactivity with H5 HA observed for H1 and H2 HA as compared with H3 HA is probably due to the higher degree of sequence homology between H5 HA and H1/H2 HA.

Interestingly, none of the H5 HA epitopes identified were uniquely cross-reactive to H2 HA. Thus, exposure to H2N2 viruses is probably not essential for cross-reactivity to H5 HA. We also noted that cross-reactivity occurred between antigenic peptides that have two or more mismatched amino acids. This illustrates the flexibility of the TcR-MHC/peptide interaction which leads to the cross-reactivity observed. Four of the H5N1 HA T cell epitopes that were detected did not show cross-reactivity to any of the corresponding H1, H2 or H3 HA region

under the experimental conditions tested. As these cross-reactivity experiments were carried out with HA peptides selected from only one representative strain of each subtype, it is possible that the H5 HA responsive T cells can recognize the corresponding region of other strains of the H1, H2 or H3 subtypes. Alternatively, it is possible that these H5 HA reactive T cells recognize other unrelated proteins.

Though H5 HA responses were detected after *in vitro* expansion in all 14 subjects examined, it is notable the HA responses could not be detected *ex vivo* by tetramers in 5 out of the 12 subjects examined. In contrast, M1 and NP reactive T cells were detected *ex vivo* in 7 out of 7 subjects examined, and NA reactive T cells were detected in 6 out of 7 subjects examined. These observations suggested that H5 HA reactive T cells may be present at lower frequencies in some portion of the population, while H5 M1, NP and NA reactive T cells are more prevalent.

In general, these experimental results verify a recent literature survey by Bui et al (24). In this comprehensive database search of influenza T cell epitopes, Bui et al identified T cell epitopes that are highly conserved amongst different influenza A subtypes as analyzed by amino acid sequence comparisons. Specifically, they observed that nearly 45% of the published influenza A T cell epitopes (mostly from A/PR/8/34 and A/X-31 strains) have greater than 80% aa sequence homology to A/Viet Nam/1194/2004 or A/Hong Kong/156/97 (H5N1). Because influenza A internal proteins such as M1 and NP are so well conserved amongst different subtypes, it is likely that memory CD4+ T cells elicited by previous exposure to influenza A viruses of the H1, H2 or H3 subtypes can respond to the M1 and NP of H5N1. It is also likely that T cells recognizing other internal viral proteins such as Acidic Polymerase (PA) and Basic Polymerases 1 and 2 (PB1 and PB2) are present, since these internal viral proteins are also well conserved.

Our results also demonstrated that H5N1 cross-reactive T cells yield a productive functional response, secreting cytokines such as TNF-α, IFN-γ, IL-5 and IL-13. It is of particular interest that H5 HA cross-reactive T cells that had been generated by stimulation with the H1 HA peptides produced significant amounts of IFN-γ, IL-5 and IL-13 when rechallenged with H5 HA peptides. These data strongly suggest that vaccination with the current influenza vaccine, which has an H1N1 component and an H3N2 component, can activate NA and HA specific T cells that are cross-reactive to other strains such as the H5N1 virus, thus increasing the pool size of H5N1 NA and HA reactive T cells. In addition, vaccination with live attenuated vaccine can be expected to modulate a more polyclonal pool of influenza A specific T cells compared to subunit vaccines. Subunit influenza vaccines are deprived of internal viral proteins, while live attenuated vaccines include internal viral proteins, which are well conserved. Thus the attenuated vaccines should increase the pool size of T cells that are directed against multiple internal viral proteins of multiple influenza strains including H5N1 viruses.

Humoral immunity is well established as the primary protective mechanism against influenza infection. However, multiple experiments in different animal models have demonstrated the role of T cell mediated protection in influenza infection (25–27). Experiments with murine models also demonstrated that CD4+ T cells can mediate protection through B cell dependant and B cell independent mechanisms, leading to improved viral clearance and a more rapid and effective antibody response (28–30). While we are cautious to directly extrapolate these findings to humans, a few observations clearly support the possibility of cell mediated cross-protection in human influenza infection. For example, Epstein demonstrated in a retrospective study that prior infections of H1N1 protected subjects from subsequent H2N2 infection in the adult population (31). Similar cross-protection was not observed in children. The author suggested that repeated exposures

to the influenza A virus were essential to generate cross-protective immunity through cross-reactive T cells or cross-reactive antibodies. In addition, it is interesting to note that the fatality rate for H5N1 infections is 68% for those that are 39 years of age or younger and only 41% for those that are 40 or older. This is in contrast to the usual pattern seen for other influenza infections, in which fatality rates are highest amongst older age groups. The incidence of disease is also lower for those over 40 years old (32,33). Our data indicates that previous exposure to H1N1, H2N2 or H3N2 influenza viruses can generate CD4+ T cells that are cross-reactive toward H5N1 epitopes. It is likely that older adults have a larger or more varied influenza reactive CD4+ and CD8+ T cell pool (as a consequence of repeated exposures to influenza) that is more cross-reactive to H5N1 viruses. It seems plausible that these preexisting cross-reactive T cells could lead to improved viral clearance directly or by providing help for the generation of H5 reactive antibodies. Thus, it is tempting to conclude that the immune repertoire of healthy subjects, especially those with repeated exposures to other influenza A viruses, have partial immunity to the H5N1 virus. This speculation is supported by a recent finding that healthy subjects do have detectable NA antibodies that recognize H5 NA (34). In any case, our results indicate that healthy subjects exhibit memory CD4+ T cell responses directed against multiple H5N1 viral proteins. These findings raise the prospect that widespread administration of the currently available influenza vaccines could reduce the severity of a human H5N1 influenza pandemic.

Non-standard Abbreviations

HA   Hemagglutinin

M1   Matrix Protein 1

NP   Nucleoprotein

NA   Neuraminidase

PB   Basic Polymerase

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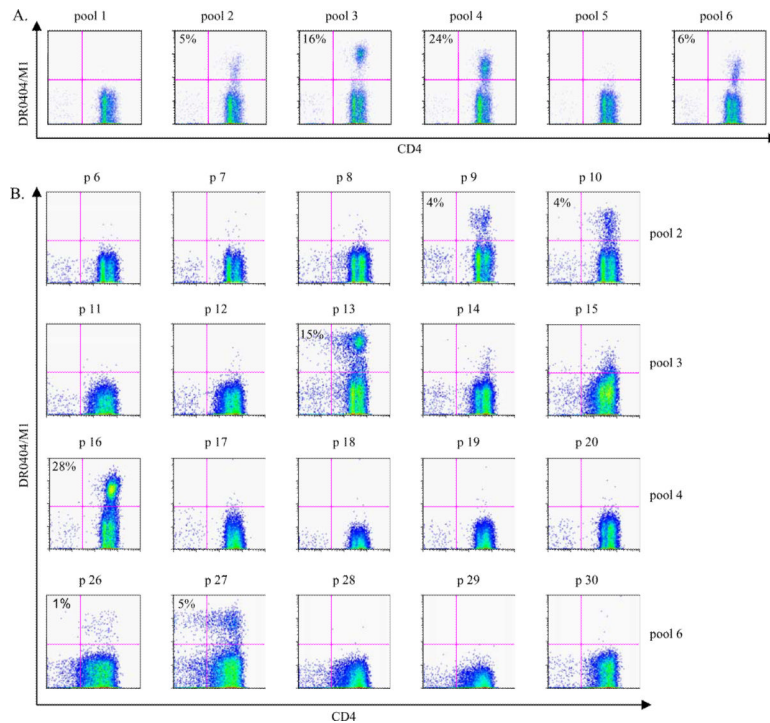
Figure 1.
Identification of DR0404 restricted M1 epitopes. CD4+ T cells from a DR0404 subject were stimulated with 6 peptide pools derived from the M1 protein of A/Viet Nam/1203/2004. A. Cells were stained with the corresponding DR0404 pooled M1 peptide tetramers at day 14. Pools 2, 3, 4 and 6 gave positive staining. B. Cells stimulated with peptide pools that gave positive staining were stained with DR0404 individual peptide tetramers at day 17. The percentage of tetramer positive cells is indicated. Background staining in these experiments was 0.3% or lower. Peptides p9, p10, p13, p16, p26 and p27, which correspond to $M1_{65-84}$, $M1_{73-92}$, $M1_{97-116}$, $M1_{121-140}$, $M1_{201-220}$ and $M1_{209-228}$ respectively, gave staining significantly above background and were identified as H5 M1 epitopes.
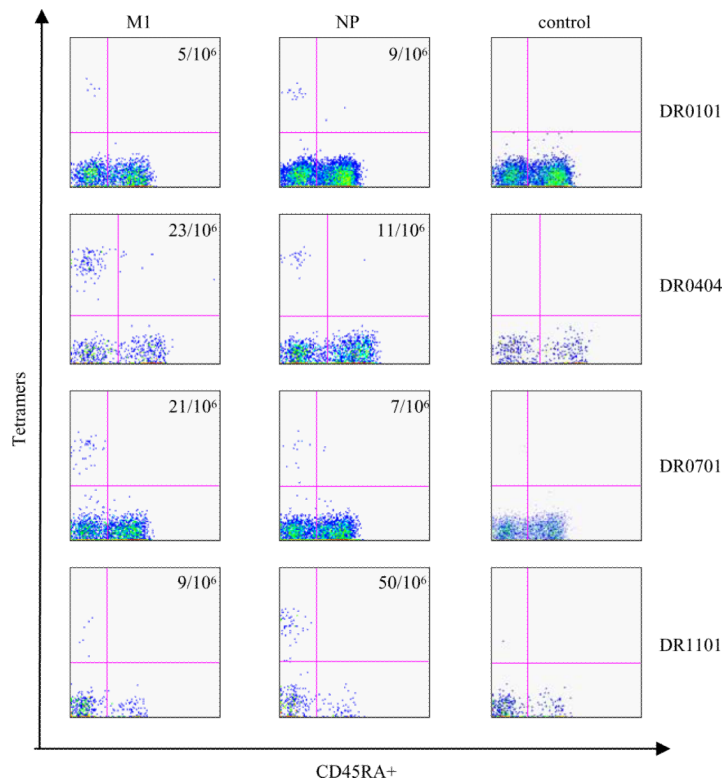
Figure 2.
Ex vivo staining of H5 M1 and H5 NP reactive T cells. PBMC from DR0101, DR0404 DR0701 and DR1101 subjects were stained with a panel of HLA- matched DR0101/M1, DR0101/NP, DR0404/M1, DR0404/NP, DR0701/M1, DR0701/NP, DR1101/M1, DR1101/ NP tetramers or the appropriate control tetramers. The panel of tetramers being used and the *ex vivo* staining protocol were as stated in the text. The DR0101 control tetramers was DR0101/GAD65$_{555-567}$, the DR0404 control tetramers was DR0404/GAD65$_{555-567}$, and both the DR0701 and DR1101 control tetramers were empty tetramers. The frequency of antigen specific T cell per million CD4+ T cells is as indicated.
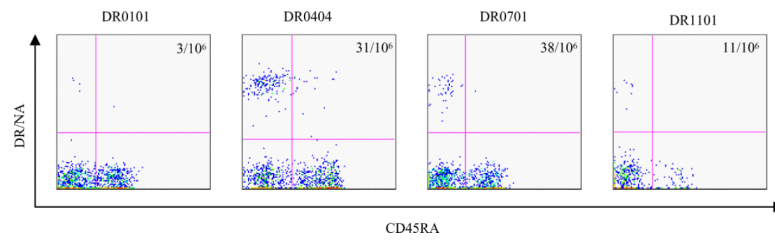
Figure 3.
Ex vivo staining of NA reactive T cells. PBMC from DR0101, DR0404, DR0701 and DR1101 subjects were stained with a panel of HLA- matched NA tetramers. For the DR0101 subject, the tetramer was $DR0101/NA_{105-124}$. For the DR0404 subject, the tetramers were $DR0404/NA_{345-364}$ and $DR0404/NA_{409-428}$. For the DR0701 subject, the tetramers were $DR0701/NA_{73-92}$ and $DR0701/NA_{105-124}$. For the DR1101 subject, the tetramers were $DR1101/NA_{225-244}$ and $DR1101/NA_{393-412}$. The frequency of antigen specific T cell per million CD4+ T cells is as indicated.
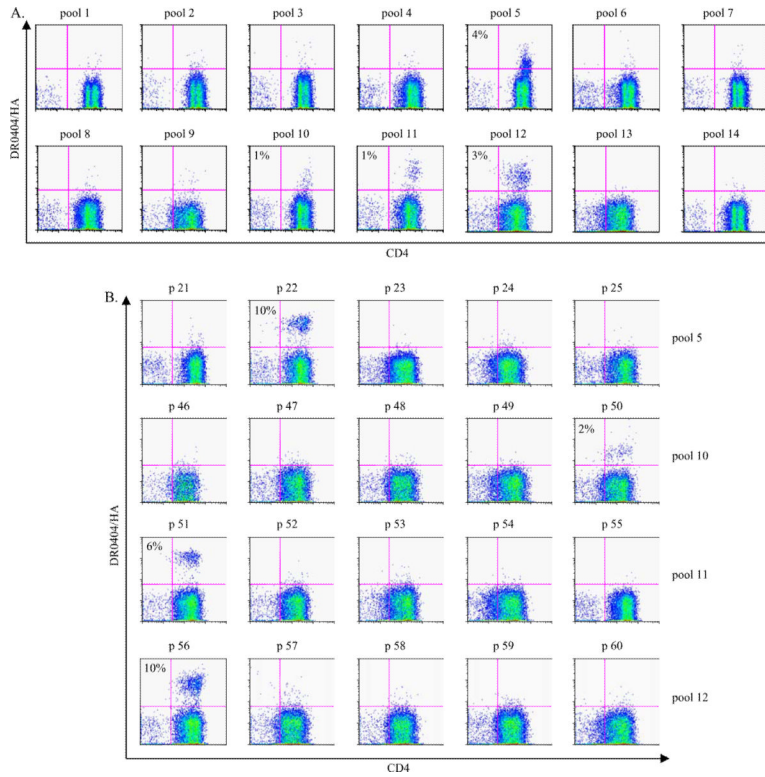
Figure 4.
Identification of DR0404 restricted HA epitopes. CD4+ T cells from a DR0404 subject were stimulated with 14 peptide pools derived from the HA protein of A/Viet Nam/1203/2004. A. Cells were stained with the corresponding DR0404 pooled HA peptide tetramers at day 14. Pools 5, 10, 11 and 12 were identified as positive pools. B. Cells stimulated with peptide pools that gave positive staining were stained with DR0404 individual peptide tetramers at day 17. The percentage of tetramer positive cells is indicated. Background staining in these experiments was 0.3% or lower. Peptides p22, p50, p51 and p56, which correspond to $HA_{169-188}$, $HA_{393-412}$, $HA_{401-420}$ and $HA_{441-460}$ respectively, were identified as T cell epitopes.
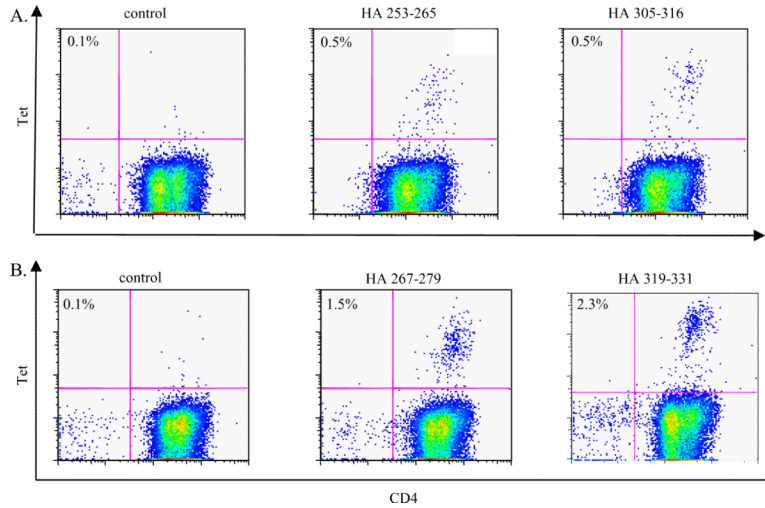
Figure 5.
H5N1 HA CD4+ T cells are memory CD4+ T cells. CD4+ T cells were sorted into CD45RA + and CD45RA− fractions using a FACSVantage. The CD4+CD45RA− fraction was stimulated with H5 HA peptides. A. Cells from the CD45RA− fraction of a DR0701 subject were stimulated with $HA_{253-265}$ (middle panel) or $HA_{305-316}$ peptide (right panel). At day 14, these cells were stained with the corresponding DR0701 tetramers. The negative control was cells which have been stimulated with a $M1_{33-52}$ peptide and stained with DR0701/ $HA_{253-265}$ tetramers. B. Cells from the CD45RA− fraction of a DR1101 subject were stimulated with $HA_{267-279}$ (middle panel) or $HA_{319-331}$ peptide (right panel). At day 14, these cells were stained with the corresponding DR1101 tetramers. The negative control was cells that have been stimulated with $M1_{97-116}$, and stained with DR1101/ $HA_{267-279}$ tetramers.

Figure 6.
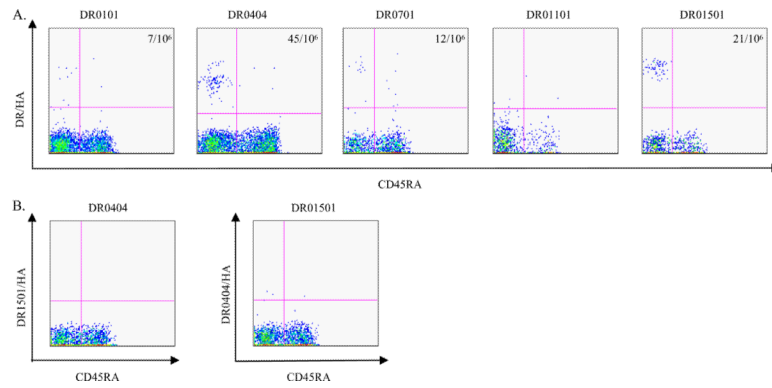*Ex vivo* staining of H5 HA reactive T cells. A. PBMC from DR0101, DR0404, DR0701, DR1101 and DR1501 subjects were stained with a panel of HLA-matched DR0101/HA, DR0404/HA, DR0701/HA, DR1101/HA and DR1501/HA tetramers. The tetramers used were as followed, DR0101/ HA_{91-104}, DR0101/ $HA_{157-169}$ and DR0101/ $HA_{443-458}$ for DR0101 subjects, DR0404/ $HA_{174-188}$, DR0404/ $HA_{400-412}$ and DR0404/ $HA_{443-458}$ for DR0404 subjects, DR0701/ HA_{49-68}, DR0701/ $HA_{253-265}$ and DR0701/ $HA_{305-316}$ for DR0701 subjects, D1101/ $HA_{267-279}$, DR1101/ HA319–331 and DR1101/ $HA_{408-420}$ for DR1101 subjects, and DR1501/ $HA_{433-452}$ for DR1501 subjects. B. As a negative control for the tetramer staining, PBMC from a DR0404 subject was stained with the same DR1501/ HA tetramers used in A, and PBMC from a DR1501 subject was stained with the set of DR0404/HA tetramers described as in A. The frequency of antigen specific T cell per million CD4+ T cells is as indicated. No significant staining was observed in the DR1101 subject in A. and the two negative controls in B.
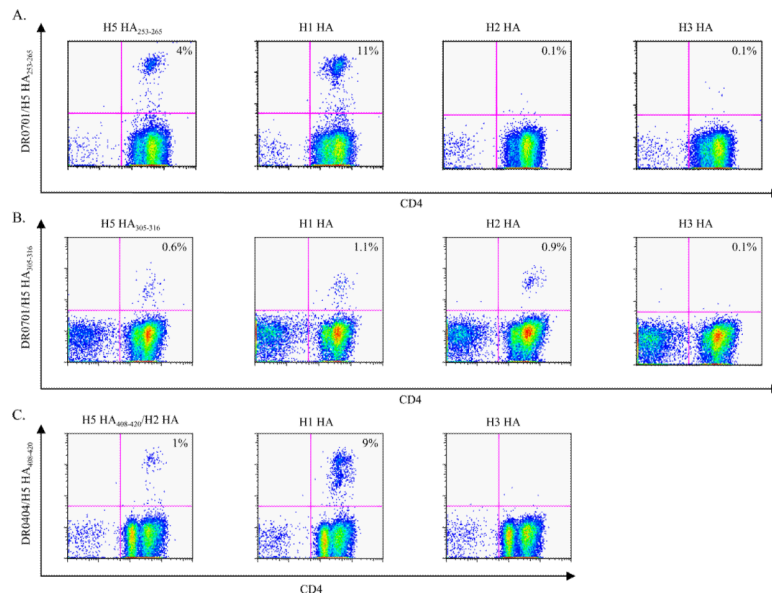
Figure 7.
Cross-reactivity of H5 HA$_{253-265}$ and HA$_{305-316}$ reactive T cells. A. CD4+ T cells from a DR0701 subject were stimulated with H5 HA$_{253-265}$, or with peptides in the corresponding region of H1N1, H2N2 or H3N2 HA (sequences are as indicated in Table IV). Fourteen days post-stimulation, cells were stained with DR0701/ H5 HA$_{253-265}$ tetramers. B. CD4+ T cells from a DR0701 subject were stimulated with H5 HA$_{305-316}$, or with peptides in the corresponding region of H1N1, H2N2 or H3N2 HA (sequences are as indicated in Table IV). Fourteen days post-stimulation, cells were stained with DR0701/ H5 HA$_{305-316}$ tetramers. C. CD4+ T cells from a DR0404 subject were stimulated with H5 HA$_{408-420}$, or with peptides in the corresponding regions of H1N1 or H3N2 HA (see Table IV). Fourteen days post-stimulation, cells were stained with DR0404/ H5 HA$_{408-420}$ tetramers. The aa sequences of H5 HA$_{408-420}$ and the corresponding region of H2 HA are exactly identical.
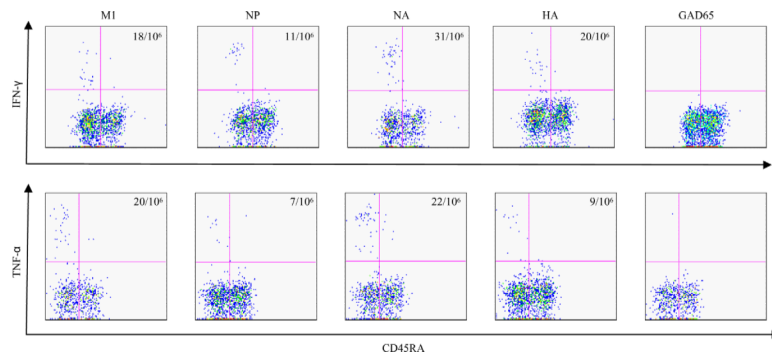
Figure 8.
Ex vivo cytokine analysis of H5N1 reactive T cells. DR0404 restricted H5N1 M1, NP, NA, and HA T cells were analysis for secretion of IFN-γ and TNF-α after an overnight culture of the PBMC from a DR0404 subject with a panel of M1, NP, NA or HA specific tetramers. The DR0404 M1, NP, NA and HA tetramers used were as previously described. The DR0404/GAD65$_{555-567}$ tetramers were used as a negative control. The frequencies of IFN-γ and TNF-α secreting cells per million CD4+ T cells are as indicated.
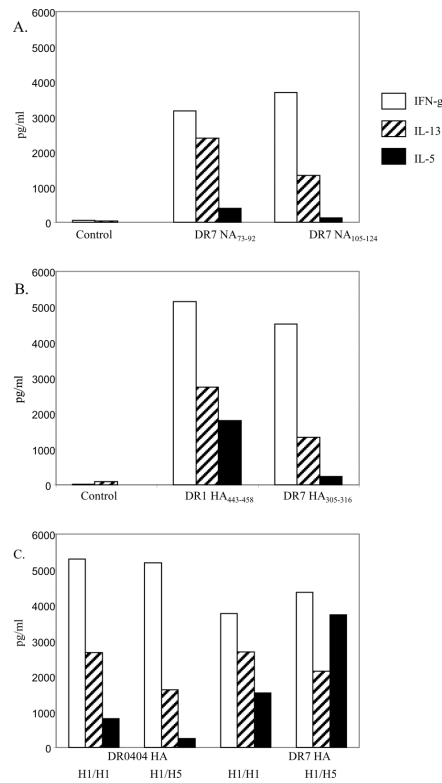
Figure 9.
Cytokine profiles of NA and HA reactive T cells. A. Cytokine profiles of NA reactive T cells. PBMC from a DR0701 subject were stimulated with the NA_{73-92} (middle panel) or the $NA_{105-124}$ (right panel) peptide. On day 14, cells were transferred to a well that were precoated with the corresponding tetramers, and supernatants were harvested after an overnight culture. Cytokines were assayed by the Meso Scale multiplex kit. Cells had been stimulated with the $NA_{105-124}$ peptide were also reactivated with an irrelevant tetramer as a negative control (panel on the left). B. Cytokine profiles of HA reactive T cells. PBMC from a DR0101 subject (middle panel) and a DR0701 subject (right panel) were stimulated with the H5 $HA_{443-458}$ peptide and the H5 $HA_{305-316}$ peptide respectively. Cytokines were assayed by the Meso Scale multiplex kit as described above. Cells from the DR0101 subjects were also restimulated with an irrelevant tetramer as a negative control (left panel). C. Cross-reactivity of H1 and H5 HA reactive T cells. PBMC from a DR0404 subject were stimulated with the H1 HA peptide SVIEKMNTQFTAV (Table IV). Cells were reactivated with either the H1 HA peptide or the H5 $HA_{400-412}$ peptide and then assayed for cytokines (first set and second set of data respectively). PBMC from a DR0701 subject were stimulated with the H1 HA peptide DTIIFEANGNLIA (Table IV). Cells were reactivated with either the H1 HA peptide or the H5 $HA_{253-265}$ peptide and then assayed for cytokines (third and fourth set of data respectively).

Table I. HLA-DR restricted A/Viet Nam/1203/2004 M1 and NP epitopes

DR0101 restricted H5N1 M1 epitopes		**DR0101 restricted H5N1 NP epitopes**	
$M1_{17-36}$	SGPLKAEIAQKLEDVFAGKN	NP_{65-84}	RMVLSAFDERRNRYLEEHPS
		NP_{73-92}	ERRNRYLEEHPSAGKDPKKT
		$NP_{161-180}$	PRMCSLMQGSTLPRRSGAAG
		$NP_{185-204}$	GVGTMVMELIRMIKRGINDR
		$NP_{297-316}$	SLVGIDPFRLLQNSQVFSLI
		$NP_{401-420}$	ASAGQISVQPTFSVQRNLPF
		$NP_{441-460}$	RTEIIRMMESARPEDVSFQG
DR0404 restricted H5N1 M1 epitopes		**DR0404 restricted H5N1 NP epitopes**	
$M1_{65-84}$	TLTVPSERGLQRRRFVQNAL	$NP_{321-340}$	NPAHKSQLVWMACHSAAFED
$M1_{73-92}$	GLQRRRFVQNALNGNGDPNN	$NP_{401-420}$	ASAGQISVQPTFSVQRNLPF
$M1_{97-116}$	VKLYKKLKREITFHGAKEVA	$NP_{433-452}$	TEGRTSDMRTEIIRMMESAR
$M1_{121-140}$	TGALASCMGLIYNRMGTVTT	$NP_{441-460}$	RTEIIRMMESARPEDVSFQG
$M1_{201-220}$	EAMEIANQARQMVQAMRTIG		
$M1_{209-228}$	ARQMVQAMRTIGTHPNSSAG		
DR0701 restricted H5N1 M1 epitopes		**DR0701 restricted H5N1 NP epitopes**	
$M1_{9-28}$	TYVLSIIPSGPLKAEIAQKL	$NP_{137-156}$	MIWHSNLNDATYQRTRALVR
$M1_{33-52}$	AGKNTDLEALMEWLKTRPIL	$NP_{145-164}$	DATYQRTRALVRTGMDPRMC
$M1_{41-60}$	ALMEWLKTRPILSPLTKGIL	$NP_{257-276}$	IFLARSALILRGSVAHKSCL
$M1_{97-116}$	VKLYKKLKREITFHGAKEVA	$NP_{265-284}$	ILRGSVAHKSCLPACVYGLA
$M1_{161-180}$	SHRQMATITNPLIRHENRMV	$NP_{321-340}$	NPAHKSQLVWMACHSAAFED
$M1_{169-188}$	TNPLIRHENRMVLASTTAKA		
$M1_{177-196}$	NRMVLASTTAKAMEQMAGSS		
DR1101 restricted H5N1 M1 epitopes		**DR1101 restricted NP H5N1 epitopes**	
$M1_{97-116}$	VKLYKKLKREITFHGAKEVA	$NP_{105-124}$	VRELILYDKEEIRRIWRQAN
$M1_{169-188}$	TNPLIRHENRMVLASTTAKA	$NP_{113-132}$	KEEIRRIWRQANNGEDATAG
$M1_{201-220}$	EAMEIANQARQMVQAMRTIG	$NP_{185-204}$	GVGTMVMELIRMIKRGINDR
$M1_{209-228}$	ARQMVQAMRTIGTHPNSSAG	$NP_{193-212}$	LIRMIKRGINDRNFWRGENG
		$NP_{377-396}$	NTLELRSRYWAIRTRSGGNT
		$NP_{385-404}$	YWAIRTRSGGNTNQQRASAG

Table II. HLA-DR restricted H5N1 NA CD4+ T cell epitopes

DR0101 restricted H5N1 NA epitopes		DR0701 restricted H5N1 NA epitopes	
$NA_{105-124}$	SHLECRTFFLTQGALLNDKH	NA_{73-92}	PINGWAVYSKDNSIRIGSKG
		$NA_{105-124}$	SHLECRTFFLTQGALLNDKH
		$NA_{113-132}$	FLTQGALLNDKHSNGTVKDR
DR0404 restricted H5N1 NA epitopes		**DR1101 restricted H5N1 NA epitopes**	
$NA_{201-220}$	NNILRTQESECACVNGSCFT	$NA_{225-244}$	GPSNGQASHKIFKMEKGKVV
$NA_{345-364}$	TNSRSGFEMIWDPNGWTETD	$NA_{233-252}$	HKIFKMEKGKVVKSVELDAP
$NA_{409-428}$	GRPKESTIWTSGSSISFCGV	$NA_{393-412}$	TGLDCIRPCFWVELIRGRPK
		$NA_{401-420}$	CFWVELIRGRPKESTIWTSG

Table III. HLA-DR restricted A/Viet Nam/1203/2004 HA T cell epitopes

Epitope identified by TGEM		Minimal epitope		HLA restriction
HA$_{49-68}$	LEKKHNGKLCDLDGVKPLIL		ND	DR0701
HA$_{89-108}$	VPEWSYIVEKANPVNDLCYP	HA$_{91-104}$	EWSYIVEKANPVND	DR0101
HA$_{153-172}$	YQGKSSFFRNVVWLIKKNST	HA$_{157-169}$	SSFFRNVVWLIKK	DR0101
HA$_{169-188}$	KNSTYPTIKRSYNNTNQEDL	HA$_{174-188}$	PTIKRSYNNTNQEDL	DR0404
HA$_{249-268}$	LKPNDAINFESNGNFIAPEY	HA$_{253-265}$	DAINFESNGNFIA	DR0701
HA$_{265-284}$	APEYAYKIVKKGDSTIMKSE	HA$_{267-279}$	EYAYKIVKKGDST	DR1101
HA$_{297-316}$	PMGAINSSMPFHNIHPLTIG	HA$_{305-316}$	MPFHNIHPLTIG	DR0701
HA$_{305-324}$	MPFHNIHPLTIGECPKYVKS	HA$_{305-316}$	MPFHNIHPLTIG	DR0701
HA$_{313-332}$	LTIGECPKYVKSNRLVLATG	HA$_{319-331}$	PKYVKSNRLVLAT	DR1101
HA$_{393-412}$	GVTNKVNSIIDKMNTQFEAV		ND	DR0301
HA$_{393-412}$	GVTNKVNSIIDKMNTQFEAV	HA$_{400-412}$	SIIDKMNTQFEAV	DR0404
HA$_{401-420}$	IIDKMNTQFEAVGREFNNLE	HA$_{400-412}$	SIIDKMNTQFEAV	DR0404
HA$_{401-420}$	IIDKMNTQFEAVGREFNNLE	HA$_{408-420}$	QFEAVGREFNNLE	DR1101
HA$_{433-452}$	GFLDVWTYNAELLVLMENER		ND	DR1501
HA$_{441-460}$	NAELLVLMENERTLDFHDSN	HA$_{443-458}$	ELLVLMENERTLDFHD	DR0101/DR0404

Table IV. Cross recognition of H5 HA reactive T cells

			Cross-reactivity			Cross-reactivity
DR0701 restricted cross-reactivity						
H5	HA$_{253-265}$	DAINFESNGNFIA		HA$_{305-316}$	MPFHNIHPLTIG	
H1		*T*I**A***L**	+		L**Q*V**V***	+
H2		*T*****T**L**	–		L****V******	+
H3		*ILLIN*T**L**	–		K**Q*VNRI*Y*	–
DR0101 restricted cross-reactivity						
H5	HA$_{91-104}$	EWSYIVEKANPVND		HA$_{157-169}$	SSFFRNVVWLIKK	
H1		S******TP**E*G	–		***Y**LL**TG*	–
H2		E****M**E**RDG	–		*F*RNM*WLTE*G	–
H3		**DLF**RSKAYSN	–		K***SRLN**HQL	–
H5	HA$_{443-458}$	ELLVLMENERTLDFHD				
H1		*****L**********	+			
H2		****************	+			
H3		****AL**QH*I*LT*	–			
DR0404 restricted cross-reactivity						
H5	HA$_{174-188}$	PTIKRSYNNTNQEDL		HA$_{400-412}$	SIIDKMNTQFEAV	
H1		*NLSK**V*NKEKEV	–		*V*E******T**	+
H2		*VA*G*****SG*QM	–		*V*E*********	+
H3		*ALNVTMP*NEKF*K	–		RL*E*T*EK*HQI	ND
H5	HA$_{443-458}$	ELLVLMENERTLDFHD				
H1		*****L**********	+			
H2		****************	+			
H3		****AL**QH*I*LT*	–			
DR1101 restricted cross-reactivity						
H5	HA$_{267-279}$	EYAYKIVKKGDST		HA$_{319-331}$	PKYVKSNRLVLAT	
H1		W**FALSRGFG*G	–		****R*AK*RMV*	–
H2		**GF**S*R*S*G	–		******EK*****	+
H3		RGYF**RSGKS*I	–		*****Q*T*K***	+
H5	HA$_{408-420}$	QFEAVGREFNNLE				
H1		**T***K***K**	+			
H2		******K**S***	+			
H3		K*HQIEK**S***	–			

* amino acid is identical to H5 HA