Title: Influenza A Virus Matrix Protein 1-Specific Human CD8+ T Cell Response

Induced in Trivalent Inactivated Vaccine Recipients

Running title: Flu M1-Specific CD8+ T Cell Response in TIV recipients

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Abstract

Among seventeen HLA-A2-positive healthy adults CD8+ T cell responses against an HLA-A2-restricted matrix protein 1 (M1) epitope increased after immunization with trivalent inactivated influenza vaccine (TIV) in two individuals. The presence of M1 in TIV was confirmed by Western blotting. T cell cytotoxicity assays showed that TIV is processed and the epitope is presented by antigen presenting cells to an M1 epitope-specific CD8+ T cell line for specific lysis. These data show that TIV, which is formulated to contain surface glycoproteins to induce serotype-specific antibody responses, also contains M1 capable of inducing subtype cross-reactive CD8+ T cell responses in some vaccinees.
Influenza A virus infections and complications are a major cause of human morbidity and mortality. Antibody responses to previous infection or vaccination are protective when the infecting strain is very similar to the vaccinating strain (1). However, the hemagglutinin (HA) and neuraminidase (NA) proteins undergo periodic antigenic shift when these HA and/or NA genes reassort with a virus of a different subtype, thus evading antibodies. HA and NA also undergo annual antigenic drift by accruing point mutations altering antibody binding sites (14).

Influenza virus-specific cytotoxic T lymphocytes (CTL) have been shown in murine studies to limit influenza A virus replication and to protect against lethal influenza A virus challenge (15, 16, 18, 26, 30, 31). In humans, McElhaney et al. reported that measures of the ex vivo cellular immune response to influenza in vaccinated older subjects correlated with protection against influenza while serum antibody responses had a limitation as a sole measure of vaccine efficacy (21). A recent re-analysis of the archival records from the Cleveland Family Study, which was conducted before and during the 1957 pandemic (when a shift from subtype H1N1 to H2N2 occurred), also suggested an impact of accumulated heterosubtypic immunity in adults, which may be mediated at least in part by subtype cross-reactive CD8+ and CD4+ T cells (5).

Licensed trivalent inactivated influenza vaccines (TIVs) are produced from the harvested allantoic fluids of infected embryonated hens’ eggs. The manufacturers process the fluids using zonal ultracentrifugation to concentrate and purify the
monovalent virus strains and then disrupt the virus particles to enhance recovery of the external major antigen, HA, and reduce the side effects of TIV. The monovalent vaccine preparations are later combined and each adult dose must contain at least 15µg of HA of each vaccine component (H1 and H3 HA of influenza A viruses and HA of influenza B virus) (14). Despite the information from mouse studies there has been a little interest in the potential of influenza vaccines to augment subtype cross-reactive T cell responses. It was reported in 1980 that HA NA subunit vaccine was not able to prime CTL responses in a mouse model (28). Live attenuated influenza vaccine (LAIV) is expected to induce CTL responses more efficiently. A larger proportion of elderly volunteers who received TIV intramuscularly and LAIV intranasally than of those who received TIV alone experienced a postvaccination rise in anti-influenza A virus CTL activity (9). He et al. reported that the mean percentages of influenza A virus-specific IFN-γ+ CD4+ and CD8+ T cells increased significantly after LAIV, but not after TIV immunization in children aged 5 to 9 years. No increase in the mean levels of influenza A virus-reactive IFN-γ+ T cells was observed in adults given LAIV or TIV. TIV induced a significant increase in influenza A virus-reactive T cells in 6-month- to 4-year-old children (LAIV was not evaluated in this age group) (11). We reported earlier that an influenza subunit vaccine which was presented with ISCOMATRIX™ significantly increased CTL activity after vaccination compared to non-adjuvanted vaccine, but we did not identify the viral epitopes inducing the CTL responses (4). Recently we reported that the number of interferon (IFN) -γ-producing cells responding in vitro to live influenza A viruses increased by more than two fold after TIV immunization in approximately 20% of healthy adult vaccinees (20% for H1N1 subtype and 17% for H3N2 subtype) (3).
Besides HA and NA, influenza subunit vaccines are known to have nucleoprotein (NP) (27), and one TIV (2000-2001 formulation by Aventis Pasteur) was reported to have 22 µg of NP per vial (20), and recently the presence of matrix protein 1 (M1) in TIV was reported by two groups (6, 7, 22). García-Cañas et al. identified it by two-dimensional high-performance liquid chromatography and mass spectrometry in one of three TIVs analyzed (6), and Rastogi et al. detected it by Western blotting using anti-M1 antibody (data were not shown in the article) (22). Rastogi et al. also showed that 40% of infants born from mothers who had received TIV vaccine in pregnancy had anti-M1 IgM antibodies and that 10% of them had M1_{58-66} epitope-specific CD8^+ T cells.

In this report we analyzed influenza A virus epitope-specific CD8^+ T cell responses in seventeen HLA-A2-positive vaccinees, who received the licensed 2005-2006 TIV comprised of A/New Caledonia/20/99 (H1N1) and A/California/07/2004 (H3N2) strains manufactured by Sanofi Pasteur, because this is a common HLA allele and several HLA-A2-restricted epitopes have been defined on influenza A viruses (summarized in http://www.flu.lanl.gov/review/epitopes.html (17)). We performed IFN-γ-ELISPOT assays using prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs) (these vaccinees were described in the previous publication (3)). MHC class I genotyping was done by using Olerup SSP Combi-Kits (Qiagen Inc., Valencia, CA). Peptides tested are listed in Table 1. Among the 17 HLA-A2-positive vaccinees strong M1_{58-66} epitope-specific responses increased more than ten fold in two vaccinees (1010 and 1020) (Figure 1A), whose PBMC also showed more than two fold increase in the numbers of IFN-γ-producing cells after live influenza A virus stimulation (data not shown). This percentage of positive responses (12%) was similar to that
reported by Rastogi et al. (10%) (22). IFN-γ-producing cells specific to other epitopes were not detectable, at low frequency (most of them were less than 15 spots/10^6 PBMC) or did not show convincing increases including those specific to two CD4^+ T cell epitopes except for the response to PB1_{413-421} in donor 1020 (increase from 5 spots/10^6 PBMC prevaccination to 60 spots/10^6 PBMC). Neutralizing antibody titers were measured against the 2005-2006 vaccine strain (A/New Caledonia/20/99 (H1N1)) and a strain antigenically similar to the vaccine strain (A/Wisconsin/67/2005 (H3N2)) (3). Neither of these two donors had a four-fold or greater rise in neutralizing antibody titer (in donor 1010 neutralizing antibody titer increased by two fold against H1N1 and was unchanged against H3N2, and in donor 1020 neutralizing antibody titer increased by two fold against both subtypes). Average fold increases in neutralizing antibody titers in 30 vaccinees including the seventeen HLA-A2-positive vaccinees were 1.8 for both subtypes (3). These donors did not have any symptoms suggesting influenza infection during the observed period. Therefore, these increases in the number of IFN-γ-producing cells are likely to be due to vaccination. Although asymptomatic infection is impossible to rule out, the lack of convincing antibody responses makes that unlikely.

We then used cytotoxic CD8^+ and CD4^+ T cell lines known to recognize distinct epitopes located in the M1 protein (13) to see if the T cell lines recognized M1 epitopes on the target cells pulsed with TIV in cytotoxicity assays (12, 13). Since the TIV that these vaccinees had received were not available for the in vitro assays, we used TIV manufactured by Chiron Vaccines Limited for the 2006/07 season (Figure 2). We used B lymphoblastoid cell lines (BLCLs) as antigen-presenting cells (APCs) (Figure 1B). The CD8^+ T cell line, 1-7-K specific to the M1_{58-66} epitope, lysed target cells pulsed with the
TIV and ISCOMATRIX™, which can help APCs process and present viral proteins to specific CD8\(^+\) T cells (4, 20). When target cells were pulsed with the TIV alone, they were not lysed by the CD8\(^+\) T cell line (Figure 1B). The cytotoxic CD4\(^+\) T cell line, 1-3 specific to the peptide M1\(_{17-31}\) (containing the CD4\(^+\) T cell epitope M1\(_{18-29}\)), lysed target cells pulsed with the TIV alone (Figure 1C). We obtained similar results with the 2007/08 TIV formulation by Sanofi Pasteur (data not shown), which was used in the experiments shown in Table 2 and Figure 2.

To test if internal proteins other than M1 in the TIV were recognized by cytotoxic CD8\(^+\) T cells, we used CD8\(^+\) T cell lines specific to other proteins. For PB1 and PB2 we identified minimal epitopes which recognized by HLA-B27-restricted PB1-specific and PB2-specific CD8\(^+\) T cell lines, 1-2F8 and 10-1B7, respectively (13), using synthetic peptides (BEI Resources and Anaspec Inc (San Jose, CA)) by cytotoxicity assays. Line 1-2F8 recognized a peptide \(\text{RRAIA\_PGM}\)_\(238-246\), but, did not grow well and was not available for the assays we planned. Line10-1B7 recognized a peptide \(\text{SRTRE\_ILTK}\)_\(14-22\).

CD8\(^+\) T cell lines specific to the NP\(_{383-391}\) epitope restricted by HLA-B27 (1-1) (13) and NS1\(_{122-130}\) epitope (10-2C2) restricted by HLA-A2 (11, 25) were also tested. We observed \(\geq 20\%\) specific lysis by the NP\(_{383-391}\)-specific CD8\(^+\) T cell line (Table 2A). Specific lysis by an NS1\(_{122-130}\)-specific CD8\(^+\) T cell line and a PB2\(_{14-22}\)-specific CD8\(^+\) T cell line were much lower (Table 2B). There is probably no NS1 in inactivated vaccines.

We confirmed the presence of M1 and NP in the TIV by Western blotting (27). The left panel of Figure 2 shows a 28 kDa band detected by an antibody recognizing the N-terminus of M1 (vN-20, Santa Cruz Biotechnology, Santa Cruz, CA). The same band was also detected by an antibody recognizing the internal region of M1 (vF-20, Santa
Cruz Biotechnology) (data not shown). The right panel of the figure shows an approximately 55 kDa band detected by an anti-NP antibody (IMGENEX, San Diego, CA).

These results showed that the TIV contained M1 and NP and that the epitope peptides in the proteins were processed and presented by APCs to epitope-specific CD8+ and CD4+ T cells at least in the context of HLA-A2, HLA-B27 and HLA-DR1 (HLA-DRB1*0101), respectively. BLCLs required ISCOMATRIX™ to be added with the TIV in order to process the TIV and stimulate specific CD8+ T cells; whereas the TIV without ISCOMATRIX™ was processed and presented to specific CD4+ T cells. Dendritic cells have the capacity to cross-present antigens (23), and in vivo the M1 in the TIV may be cross-presented to the specific CD8+ T cells after vaccination.

When vaccine strains and circulating strains match, TIV should induce efficient neutralizing antibody and it may not make a great deal of difference whether the TIV contains these internal proteins which are able to induce T cell responses. On the other hand, when the vaccine strain and the circulating strain do not match well, T cell response might be important. For example this winter (2007 ~ 2008) 77% of influenza A (H3N2) and 98% of B viruses sent to Center for Disease Control and Prevention for further testing were not optimally matched to the 2007-08 influenza vaccine strains (2). Internal proteins in TIV may induce some cross-reactive T cell memory and provide a degree of protection, and influence the outcome of natural infection. More importantly there is a need to find safe and effective influenza vaccines that induce more CD8+ and CD4+ T cell responses to internal proteins to augment cross-reactive protection against influenza.
We thank Dr. Jeffrey S. Kennedy, Karen Longtine, Melissa O’Neill, and Jaclyn Longtine for their help in obtaining the human PBMC samples that were used in this study. We thank Christine Turcotte and Denise Marengo for the assistance with HLA typing, Kim West, Alan L. Rothman and James Evans for discussion, Dr David Burt and Pasteur-Merieux for providing ISOMATRIX™, Dr. Michel DeWilde and Dr. Robert Ryall of Sanofi Pasteur for the influenza virus strains used in this study. The following reagent was obtained through BEI Resources: Peptide Arrays, Control Peptides for MHC Class I & II Epitopes of Influenza Virus A & B Proteins, NR-2666; Influenza Virus A/New York/348/03 (H1N1) PB1 Protein, NR-2617; and Influenza Virus A/New York/348/03 (H1N1) PB2 Protein, NR-2616. This work was supported by the National Institutes of Health (NIH) / the National Institute of Allergy and Infectious Diseases (NIAID) grant U19 AI-057319. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH/NIAID.
References


Figure legends

**Figure 1.** Influenza M₁₅₈-₆₆ epitope-specific CD₈⁺ T cell response before and after vaccination quantitated by ELISPOT assays (A) and detection of M₁ protein in autologous BLCL pulsed with TIV (2006/07 formulation) by cytotoxic CD₈⁺ and CD₄⁺ T cell lines (B and C). (A) Cryopreserved PBMC were tested in IFN-γ ELISPOT assays as described previously (3). Cells were incubated at 37°C for 15 hours with peptides at a final concentration of 2µg/ml or live influenza A viruses (data not shown). Medium was used as a negative control. Assays were performed in triplicate. The frequency of peptide-specific IFN-γ-producing cells was calculated as (average number of spots in the virus wells – average number of spots in medium wells / number of cells/ well) and converted to the number of IFN-γ-producing cells per 10⁶ PBMC. (B) Detection of M₁ protein by the CD₈⁺ T cell line, 1-7-K specific to the M₁₅₈-₆₆ epitope. Effector/target ratio was 10. Peptide concentration was 25µg/ml for both M₁₅₈-₆₆ and M₁₁₇-₃₁ peptides. Autologous BLCLs as targets were pulsed with peptides, the TIV alone or the TIV and ISCOMATRIX™. (-): negative control (medium alone). Representative of the two experiments. (C) Detection of M₁ protein by the cytotoxic CD₄⁺ T cell line, 1-3 specific to the peptide M₁₁₇-₃₁ (containing epitope M₁₁₈-₃₂₉). Representative of the two experiments.

**Figure 2.** Detection of M₁ protein by Western blotting. 12.5 µl of the TIV (2007/08 formulation by Sanofi Pasteur) was separated in a sodium dodecyl sulfate polyacrylamide gel electrophoresis, and a goat polyclonal antibody recognizing the N-terminus of M₁ was used (left panel). For NP a rabbit polyclonal antibody specific to influenza A NP was used (fight panel). NC (H1N1) and W (H3N2) are A/New Caledonia/20/99 IVR-166
(H1N1) and A/Wisconsin/67/2005X-161B (H3N2) used as positive controls, and allantoic fluid was used as a negative control.
Table 1. HLA-A*0201-restricted CD8\(^+\) T cell epitopes and HLA-DR-restricted CD4\(^+\) T cell epitopes analyzed in this study.

<table>
<thead>
<tr>
<th>Protein</th>
<th>aa position</th>
<th>Peptide sequence</th>
<th>Restricting allele</th>
<th>reference</th>
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<tr>
<td>Neuraminidase N1 subtype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NA (N1))</td>
<td>75-84</td>
<td>SLCPIRGWAI</td>
<td>HLA-A*0201</td>
<td>(8)</td>
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<tr>
<td>NA (N1)</td>
<td>213-221</td>
<td>CVNGSCFTV</td>
<td>HLA-A*0201</td>
<td>(29)</td>
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<tr>
<td>Matrix protein 1 (M1)</td>
<td>58-66</td>
<td>GILGFVFTL</td>
<td>HLA-A*0201</td>
<td>(10)</td>
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<tr>
<td>Nonstructural protein 1 (NS1)</td>
<td>122-130</td>
<td>AIMDKNIIL</td>
<td>HLA-A*0201</td>
<td>(19)</td>
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<tr>
<td>Polymerase A (PA)</td>
<td>46-54</td>
<td>FMYSDFHFI</td>
<td>HLA-A*0201</td>
<td>(8)</td>
</tr>
<tr>
<td>PA</td>
<td>225-233</td>
<td>SLENFRAYV</td>
<td>HLA-A*0201</td>
<td>(8)</td>
</tr>
<tr>
<td>Polymerase B1 (PB1)</td>
<td>413-421</td>
<td>NMLSTVLGV</td>
<td>HLA-A*0201</td>
<td>(8)</td>
</tr>
<tr>
<td>M1</td>
<td>18-29</td>
<td>GPLKAEIARLE</td>
<td>HLA-DRB1*0101</td>
<td>(24)</td>
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<tr>
<td>NS1</td>
<td>34-42</td>
<td>DRLRRDQKS</td>
<td>HLA-DR3</td>
<td>(13)</td>
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Table 2. Cytotoxic T cell assays using epitope-specific CD8+ T cell lines.

<table>
<thead>
<tr>
<th>T cell line</th>
<th>% specific lysis vaccine + ISCOMATRIX</th>
<th>(-)</th>
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<tbody>
<tr>
<td>1-1 (NP-specific)a</td>
<td>23.4%</td>
<td>0.0%</td>
</tr>
<tr>
<td>min/max</td>
<td>29.4%</td>
<td>19.9%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T cell line</th>
<th>% specific lysis vaccine + ISCOMATRIX</th>
<th>% specific lysis vaccine + M1 58-66 10µg/ml</th>
<th>NS1 122-130 10µg/ml</th>
<th>(-)</th>
</tr>
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<tr>
<td>1-7-K (M1-specific)</td>
<td>31.4%</td>
<td>77.3%</td>
<td>75.2%</td>
<td>0.3%</td>
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<tr>
<td>10-2C2 (NS1-specific)</td>
<td>15.3%</td>
<td>22.4%</td>
<td>23.3%</td>
<td>2.1%</td>
</tr>
<tr>
<td>10-1B7 (PB2-specific)b</td>
<td>7.3%</td>
<td>22.4%</td>
<td>23.3%</td>
<td>2.0%</td>
</tr>
<tr>
<td>min/max</td>
<td>38.0%</td>
<td>22.4%</td>
<td>23.3%</td>
<td>21.2%</td>
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</table>

Vaccine used in these experiments was the 2007/2008 TIV formulation by Sanofi Pasteur. Effector/target ratio was 10. (-) was medium alone control. The percent specific ⁵¹Cr release was calculated as \[\frac{(cpm \text{ experimental release} - cpm \text{ spontaneous release})}{(cpm \text{ maximum release} - cpm \text{ spontaneous release})} \times 100. \] “Min/max” was calculated as \[\frac{cpm \text{ spontaneous release}}{cpm \text{ maximum release}} \times 100. \] Maximum release was measured by lysing target cells with detergent Renex (Uniqema, New Castle, DE). All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells. A is a representative of two experiments.

a: In a separate experiment line 1-1 specifically lysed target cells infected with influenza A virus (A/New Caledonia/20/99 (H1N1)) or recombinant vaccinia virus expressing NP (25) or target cells pulsed with the NP383-391 epitope peptide (specific lyses were 40.6%, 79.8% and 73.9%, respectively).
b: In a separate experiment line 10-1B7 specifically lysed target cells infected with influenza A virus (A/New Caledonia/20/99 (H1N1)) or recombinant vaccinia virus expressing PB2 (25) (specific lyses were 74.7% and 75.0%, respectively).