

# Recognition of Homo- and Heterosubtypic Variants of Influenza A Viruses by Human CD8<sup>+</sup> T Lymphocytes<sup>1</sup>

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In the present study, the recognition of epitope variants of influenza A viruses by human CTL was investigated. To this end, human CD8<sup>+</sup> CTL clones, specific for natural variants of the HLA-B\*3501-restricted epitope in the nucleoprotein (NP<sub>418–426</sub>), were generated. As determined in <sup>51</sup>Cr release assays and by flow cytometry with HLA-B\*3501-peptide tetrameric complexes, CTL clones were found to be specific for epitopes within one subtype or cross-reactive with heterosubtypic variants of the epitope. Using eight natural variants of the epitope, positions in the 9-mer important for T cell recognition and involved in escape from CTL immunity were identified and visualized using multidimensional scaling. It was shown that positions 4 and 5 in the 9-mer epitope were important determinants of T cell specificity. The *in vivo* existence of CD8<sup>+</sup> cells cross-reactive with homo- and heterosubtypic variants of the epitope was further confirmed using polyclonal T cell populations obtained after stimulation of PBMC with different influenza A viruses. Based on the observed recognition patterns of the clonal and polyclonal T cell populations and serology, it is hypothesized that consecutive infections with influenza viruses containing different variants of the epitope select for cross-reactive T cells *in vivo*. *The Journal of Immunology*, 2004, 172: 2453–2460.

Cytotoxic T lymphocytes play an important role in the control of viral infections. In influenza A virus-infected mice, CTL were shown to contribute to protective immunity against viruses of various subtypes (1–5), while human CTL have been reported to reduce morbidity and virus titers in the lung (6, 7). Recently, it was demonstrated that CTL immunity can exert selective pressure on influenza A virus CTL epitopes, further implicating a role for CTL in the control of influenza virus replication in infected individuals (8, 9). Beside amino acid changes in the anchor residues of CTL epitopes (8), changes in nonanchor residues were observed in an HLA-B\*3501-restricted CTL epitope within the nucleoprotein (NP)<sup>3</sup> of influenza A virus (NP<sub>418–426</sub>) (9). Epitope variants of influenza A (H3N2) viruses (homosubtypic variants) were found to emerge in a chronological order (9). In addition, a variant of the epitope was identified in influenza A (H1N1) viruses, which differed from those found in A/H3N2 viruses (heterosubtypic variant). Although the majority of CTL epitopes are conserved between different subtypes (2, 10–13), both

human and murine CTL have been described that recognize a single subtype of influenza A virus (9, 10, 14–16). It has been shown that sequential infections with two influenza A viruses, containing different variants of the same epitope, induced CTL reactive against both epitopes, whereas infection with one influenza A virus only induced CTL reactive with the homologous peptide sequence (17, 18). These experiments were conducted in naive mice and are difficult to extrapolate to humans. Data on the cross-reactive nature of human influenza A virus-specific CTL are limited (19).

The observed variability in the HLA-B\*3501-restricted epitope NP<sub>418–426</sub> allows investigation of the cross-reactive nature of CTL obtained from humans which constitute a natural host for influenza viruses. In this study, the recognition of natural epitope variants by clonal and polyclonal T cell populations was studied. T cell recognition was studied in conventional <sup>51</sup>Cr release assays and also by direct visualization of T cells displaying monospecific or cross-reactive TCRs using HLA-B\*3501-peptide tetrameric complexes (20).

It was found that most CTL recognize one subtype of currently circulating influenza A viruses. However, some of the CTL exhibited a broader recognition pattern and recognized homosubtypic and even heterosubtypic variants of epitope NP<sub>418–426</sub>. Based on these findings obtained *in vitro*, the origin of these cross-reactive CTL is discussed.

## Materials and Methods

### Cells

PBMC from normal healthy nonvaccinated HLA-B35<sup>+</sup> blood donors (Table I), between 30 and 48 years of age, were isolated between 1999 and 2001 using Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and cryopreserved at –135°C. HLA sero- and genotyping was performed in the laboratory for Histocompatibility and Immunogenetics at the Sanquin Bloodbank Rotterdam (The Netherlands) using a commercial typing system (GenoVision, Vienna, Austria).

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<sup>3</sup> Abbreviations used in this paper: NP, nucleoprotein; HAI, hemagglutinin inhibition; EC<sub>50</sub>, epitope concentration; MDS, multidimensional scaling.

Table I. HLA-A and -B serotype and age of blood donors

| Donor | HLA-A and -B Serotype | Age (years) |
|-------|-----------------------|-------------|
| 1     | A1,2; B57,35          | 44          |
| 2     | A3,-; B27,35          | 46          |
| 3     | A1,2; B7,35           | 41          |
| 4     | A3,26; B27,35         | 30          |
| 5     | A1,2; B8,35           | 37          |
| 6     | A1,2; B8,35           | 48          |
| 7     | A1,3; B8,35           | 38          |

### Serology

Plasma samples were stored at  $-20^{\circ}\text{C}$  and tested for the presence of influenza A virus (H3N2 and H1N1)-specific Abs in a hemagglutination inhibition (HAI) assay according to standard methods (21, 22) using turkey erythrocytes and four hemagglutinating units of virus. The sera were tested for Abs against 11 vaccine strains of subtype H3N2 and 6 vaccine strains of the H1N1 subtype used since 1968. Ferret sera raised against the test Ags were used as positive controls. A HAI titer of  $>20$  against a vaccine strain used in a particular season was considered indicative of an infection with an epidemic influenza A virus of that year.

### Influenza A viruses and peptides

Sucrose gradient purified influenza A viruses were used for the infection of PBMC and cells of B lymphoblastoid cell lines (BLCL). Resvir-9 is a reassortant virus between A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2) containing the NP, hemagglutinin, and neuraminidase of A/Nanchang/933/95 with the LPFEKSTVM variant of the NP<sub>418-426</sub> epitope (designated NP<sub>ESV</sub>), which was first identified in 1980 (Table II). A second virus, A/Victoria/3/75 (H3N2) containing the LPFDKSTIM variant of the NP<sub>418-426</sub> epitope (designated NP<sub>DSI</sub>), introduced in 1972, was also used. As prototype of currently circulating H1N1 viruses in The Netherlands, we used A/Netherlands/306/00. This virus contains the LPFDKTTIM variant of the NP<sub>418-426</sub> epitope (designated NP<sub>DTI</sub>, Table II). The infectious virus titers of the virus preparations were determined in cell culture using Madin-Darby canine kidney cells as indicator cells, as described previously (23). All peptide analogues of epitope NP<sub>418-426</sub> found in human influenza A viruses were manufactured, HPLC purified, and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). Influenza A virus NP sequence information was obtained from the Influenza Sequence Database (Los Alamos National Laboratories, Los Alamos, NM) (24). Peptides were dissolved in DMSO at 5.0 mg/ml, diluted to 100  $\mu\text{M}$  in RPMI 1640 (Invitrogen, Breda, The Netherlands), and stored at  $-20^{\circ}\text{C}$  until use.

### In vitro stimulation of PBMC with influenza A virus

Stimulation of PBMC with influenza A virus was performed as previously described (25). The cells were resuspended at  $10^6$  cells/ml in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 100 IU/ml penicillin (R10F). Following a 1-h infection at  $37^{\circ}\text{C}$  with Resvir-9, A/Victoria/3/75 or A/Netherlands/306/00 at a multiplicity of infection of three, the cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin, 100 IU/ml penicillin, and 20  $\mu\text{M}$  2-ME (R10H) and added to uninfected PBMC at a ratio of 1:1 in a 25-cm<sup>2</sup> culture flask. The PBMC were incubated for 2 days before rIL-2 (final concentration 50 U/ml; Chiron, Amsterdam, The Netherlands) was added. Subsequently, the cells were incubated for 6 days at  $37^{\circ}\text{C}$  and used as effector cells in a <sup>51</sup>Cr release assay or stained with fluorochrome-labeled HLA-B\*3501-peptide tetrameric complexes (see below).

### Generation of NP<sub>418-426</sub>-specific CD8<sup>+</sup> T cell clones

CTL clones were generated by limiting dilution assay (8) after in vitro stimulation of PBMC infected with influenza A virus (Resvir-9) or pulsed with 10  $\mu\text{M}$  of the variant NP<sub>418-426</sub> epitopes. After two subsequent rounds of nonspecific stimulation with 1.0  $\mu\text{g}/\text{ml}$  PHA (Sigma-Aldrich, St. Louis, MO), the ability of the cells to recognize the NP<sub>418-426</sub> epitope variant used during the initial stimulation was tested in an ELISPOT assay (see below). The CTL clones with specificity for the NP<sub>418-426</sub> epitope were PHA stimulated in 75-cm<sup>2</sup> culture flasks before cryopreservation at  $-135^{\circ}\text{C}$ . The CD8<sup>+</sup>CD3<sup>+</sup> cell phenotype of the NP<sub>418-426</sub>-specific clones was confirmed by flow cytometry, using mAb specific for CD3, CD4, and CD8 (DAKO, Glostrup, Denmark).

### Screening of virus or epitope-specific T cell clones by ELISPOT assay

Approximately  $10^4$  cells of a T cell clone were added to an equal number of HLA-B\*3501-positive BLCL cells that were infected with influenza A virus, uninfected, or loaded with one of the NP<sub>418-426</sub> peptide variants in 150  $\mu\text{l}$  of R10F in a 96-well V-bottom plate. Following a 1-min centrifugation at  $140 \times g$ , the plate was incubated for 2 h at  $37^{\circ}\text{C}$  before the cells were transferred to a 96-well Silent Screen Plate (Nalge Nunc, Rochester, NY) coated with anti-human IFN- $\gamma$  mAb (Mabtech, Nacka, Sweden). After 4 h at  $37^{\circ}\text{C}$ , the plates were washed and developed according to the manufacturer's recommendations. T cell clones that produced IFN- $\gamma$  in the presence of peptide-loaded BLCL or virus-infected BLCL cells and failed to produce IFN- $\gamma$  after stimulation with negative control BLCL cells were selected for further investigation.

### Preparation of target cells for <sup>51</sup>Cr release assay

BLCL were established as previously described (26) and used as target cells in <sup>51</sup>Cr release assays. Cells of an HLA-B\*3501-positive BLCL were infected with Resvir-9, A/Victoria/3/75, or A/Netherlands/306/00 at a multiplicity of infection of three in RPMI 1640 medium containing 0.1% BSA, 2 mM glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 100 IU/ml penicillin (R0.1B). After 1 h at  $37^{\circ}\text{C}$ , the cells were washed once in R10F and cultured in R10F for 16 h at  $37^{\circ}\text{C}$ . To test the ability of CTL clones to recognize synthetic peptides,  $10^6$  cells of a BLCL were loaded with 5  $\mu\text{M}$  of different NP<sub>418-426</sub> epitope variants for 16 h at  $37^{\circ}\text{C}$  in R10F. Radioactively labeling of peptide-loaded and virus-infected BLCL cells was done with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 1 h at  $37^{\circ}\text{C}$ , after which the cells were washed three times with R10F and used as target cells in a <sup>51</sup>Cr release assay. As a measure of functional avidity of CTL clones (27), the minimal peptide concentration was determined for which 50% of the target cells were killed (epitope concentration (EC<sub>50</sub>)). <sup>51</sup>Cr-labeled HLA-B\*3501-positive BLCL cells were distributed in 96-well V-bottom plates. Next, the cells were subsequently incubated with 10-fold serial dilutions of peptide analogues of different NP<sub>418-426</sub> epitope variants in R10F for 1 h at  $37^{\circ}\text{C}$ , washed once in R10F, and used as target cells.

### <sup>51</sup>Cr release assay

<sup>51</sup>Cr release assays were performed as described previously (25). T cell clones were added to  $5 \times 10^3$  <sup>51</sup>Cr-labeled target cells at different E:T cell ratios. Following a 4-h incubation at  $37^{\circ}\text{C}$ , the culture supernatants were harvested (Skatron Instruments, Sterling, VA) and radioactivity was measured by gamma counting. The percentage of specific lysis was calculated with the following formula: ((experimental release - spontaneous release)/(maximum release - spontaneous release))  $\times 100$ . The data are presented as the average percentage of specific lysis of at least three wells.

### Flow cytometry and tetramer staining

HLA-B\*3501 molecules were complexed with four different HLA-B\*3501-restricted NP<sub>418-426</sub> epitope variants within the NP of influenza A

Table II. Variants and virus origin of NP<sub>418-426</sub> epitopes

| Epitope Name      | Epitope Sequence | Virus Strain         | First Year of Introduction | Virus Subtype |
|-------------------|------------------|----------------------|----------------------------|---------------|
| NP <sub>DTI</sub> | LPFDKTTIM        | A/Netherlands/306/00 | 1977                       | H1N1          |
| NP <sub>DPI</sub> | LPFDKPTIM        | —                    | 1957                       | H2N2/H3N2     |
| NP <sub>DSI</sub> | LPFDKSTIM        | A/Victoria/3/75      | 1972                       | H3N2          |
| NP <sub>ESV</sub> | LPFEKSTVM        | Resvir-9             | 1980                       | H3N2          |

Table III. CTL clone definition

| CTL Clone | Stimulation             | Influenza A Virus Subtype | Epitope Name      | Donor |
|-----------|-------------------------|---------------------------|-------------------|-------|
| D1        | LPFEKSTVM               | H3N2/1980                 | NP <sub>ESV</sub> | 5     |
| C10       | LPFEKSTVM               | H3N2/1980                 | NP <sub>ESV</sub> | 5     |
| 15.9      | Infection with Resvir-9 | H3N2/1980                 | NP <sub>ESV</sub> | 6     |
| F10       | LPFEKSTVM               | H3N2/1980                 | NP <sub>ESV</sub> | 7     |
| G2        | LPFEKSTVM               | H3N2/1980                 | NP <sub>ESV</sub> | 7     |
| 2384      | LPFDKTTIM               | H1N1                      | NP <sub>DTI</sub> | 1     |
| 5017      | LPFDKTTIM               | H1N1                      | NP <sub>DTI</sub> | 4     |
| 3180      | LPFDKTTIM               | H1N1                      | NP <sub>DTI</sub> | 5     |
| C4        | LPFDKSTIM               | H3N2/1972                 | NP <sub>DSI</sub> | 5     |

virus (NP<sub>ESV</sub>, NP<sub>DSI</sub>, NP<sub>DTI</sub>, NP<sub>DPI</sub>) as previously described (20). These natural variants of the NP<sub>418-426</sub> epitope were selected based on the predominance of these variants since 1957 in H2N2 (NP<sub>DPI</sub>), since 1968 in H3N2 viruses (NP<sub>ESV</sub>, NP<sub>DSI</sub>, NP<sub>DPI</sub>) and since 1977 in H1N1 viruses (NP<sub>DTI</sub>). HLA-B\*3501-peptide complexes were enzymatically biotinylated, fast protein liquid chromatography purified, and tetramerized by addition of allophycocyanin- or PE-conjugated streptavidin (Sanquin Research at CLB, Amsterdam, The Netherlands). Two- or three-color fluorescence analysis was performed as described previously (28). Briefly, CTL clones (10<sup>5</sup>) were stained in PBS supplemented with 2.0% FCS (P2F) and combinations of two HLA-B\*3501-peptide tetrameric complexes with different NP<sub>418-426</sub> epitope variants at nonsaturating concentrations. After staining for 20 min at 20°C, cells were washed with P2F and at least 10<sup>4</sup> events were acquired using a FACSCalibur flow cytometer (BD Biosciences, Alphen a/d Rijn, The Netherlands). Tetramer staining of influenza A virus-stimulated PBMC was performed after 8–10 days of culture. Two hundred thousand cells were stained in P2F with combinations of two different HLA class I tetramers for 20 min at 20°C, followed by a 10-min incubation with anti-CD8 mAb at 20°C. Cells were washed with P2F and on average 10<sup>4</sup> CD8<sup>+</sup> cells were acquired. Data were analyzed using the software program CellQuest Pro (BD Biosciences). A proportion tetramer-positive cells in the CD8<sup>+</sup> cell fraction of ≥0.4% was considered positive.

#### Multidimensional scaling (MDS)

An “antigenic map” based on the reactivity of the CTL clones against the variant peptides was constructed from the EC<sub>50</sub> values of the variant NP<sub>418-426</sub> peptides for each CTL clone (29, 30). The map-making technique is a combination of metric (31) and nonmetric (32) MDS. The distance between two grids in the map corresponded to a 10-fold dilution of the peptide, starting at an EC<sub>50</sub> value of 0.001 nM (the most parsimonious choice for these data). The distance between a peptide and a CTL clone or two peptides is a measure for respectively the avidity between peptides and a CTL clone or antigenic dissimilarity between variant peptides. The clones and the peptides are positioned to minimize the sum of the squared errors between the target distances and the achieved map distances. The average error between target and achieved distances is 0.37 10-fold dilutions (SD = 0.45), indicating a good fit in two dimensions.

## Results

### Validation of staining of NP<sub>418-426</sub>-specific CTL with differentially labeled tetramers

In addition to six previously described CTL clones (Ref. 9 and Table III), obtained after stimulation with two NP<sub>418-426</sub> epitope variants associated with the H3N2 subtype of influenza A viruses (NP<sub>ESV</sub> and NP<sub>DSI</sub>), three additional CTL clones were generated against the currently circulating NP<sub>418-426</sub> epitope variant of H1N1 viruses (NP<sub>DTI</sub>). The original stimulation protocol and origin of PBMC is given for each CTL clone (Table III). All CTL clones exhibited lytic activity against at least one of the viruses (Table IV). CTL clones D1, C10, 15.9, F10, and G2 lysed target cells infected with Resvir-9, but not cells infected with A/Victoria/3/75 or A/Netherlands/306/00. In contrast, CTL clones 2384, 5017, and C4 recognized both the A/Netherlands/306/00- and A/Victoria/3/75-infected target cells but not the Resvir-9-infected cells. Finally, clone 3180 recognized all three virus-infected target cells to a similar extent, demonstrating the ability of this CTL

clone to recognize homo- and heterosubtypic variants of the NP<sub>418-426</sub> epitope present in influenza A viruses of the H3N2 and H1N1 subtype. The CTL activity of the NP<sub>418-426</sub>-specific CTL clones was used for comparison with staining using HLA-B\*3501-peptide tetrameric complexes (tetramers). The dot plots generated after flow cytometry following incubation with differentially labeled tetramers are shown in Fig. 1. CTL clone G2 (Fig. 1) as well as CTL clones D1, C10, 15.9, and F10 (data not shown) bound to the NP<sub>ESV</sub> tetramer, but did not react with any of the other tetramers, confirming the failure of these T cell clones to recognize homo- and heterosubtypic variants of the NP<sub>418-426</sub> epitope. Clone 2384 (Fig. 1) and 5017 (data not shown) reacted with the tetramers containing the NP<sub>DTI</sub> and NP<sub>DSI</sub> peptide, but not with those containing the NP<sub>DPI</sub> or the NP<sub>ESV</sub> variant of the epitope. CTL clone C4 reacted similarly to clone 2384 and 5017, but also bound the NP<sub>DPI</sub> tetramer (Fig. 1). Finally, clone 3180 bound all four tetramers containing different homo- and heterosubtypic variants of the epitope confirming the cross-reactive nature of this T cell clone. Overall, the ability of the CTL clones to bind tetramers with different peptides correlated well with their ability to kill virus-infected target cells.

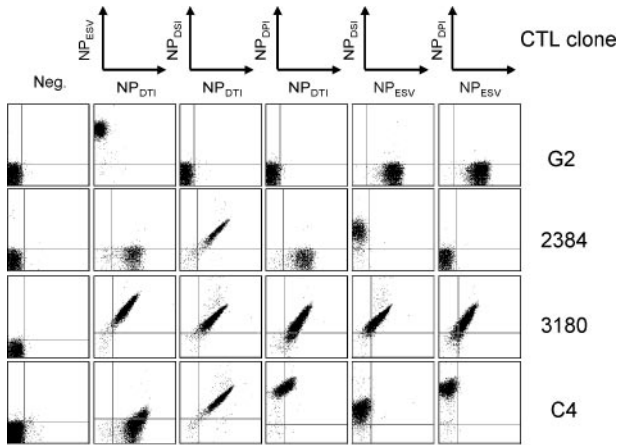
### Antigenic properties of NP<sub>418-426</sub> epitope variants

The antigenic properties of the epitope variants were studied by measuring the functional avidity of the respective T cell clones as EC<sub>50</sub> value for each of the peptides (Table V). An example of such an analysis is shown for CTL clone 3180 (Fig. 2). Using the EC<sub>50</sub> values, an antigenic map was constructed by MDS. Using this mathematical procedure, the antigenic dissimilarity of the peptides and the avidity of NP<sub>418-426</sub>-specific CTL clones for these peptides is visualized by the relative positioning of the peptides and T cell clones in a plot (Fig. 3). The glutamic acid (E) to an aspartic acid (D) substitution at P4 or vice versa reduced functional avidity

Table IV. Virus specificity of NP<sub>418-426</sub>-specific CTL clones

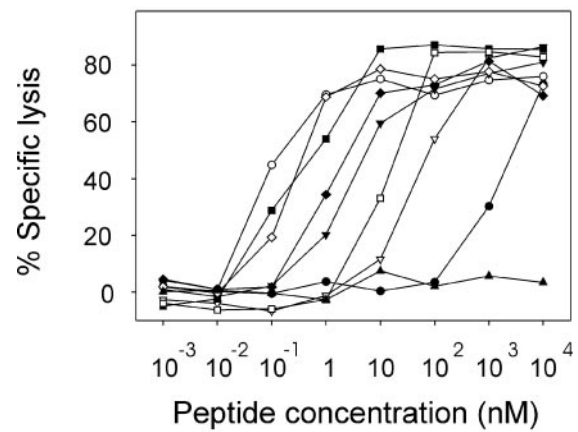
| CTL Clone | % Specific Lysis <sup>a</sup> |                                      |   |
|-----------|-------------------------------|--------------------------------------|---|
|           | Resvir-9 (NP <sub>ESV</sub> ) | A/Victoria/3/75 (NP <sub>DSI</sub> ) | A/Netherlands/306/00 (NP <sub>DTI</sub> ) |
| D1        | 72                            | 3                                    | 14  |
| C10       | 73                            | 0                                    | 6   |
| 15.9      | 63                            | 12                                   | 3   |
| F10       | 61                            | 1                                    | 0   |
| G2        | 91                            | 14                                   | 26  |
| 2384      | 0                             | 58                                   | 63  |
| 5017      | 2                             | 60                                   | 74  |
| 3180      | 62                            | 42                                   | 63  |
| C4        | 0                             | 53                                   | 44  |

<sup>a</sup> Average was calculated from two assays. The values represent the specific lysis minus the background lysis of uninfected target cells. The E:T ratio was 10:1.



**FIGURE 1.** Binding of HLA-B\*3501-peptide tetrameric complexes containing different NP<sub>418-426</sub> epitope variants to NP<sub>418-426</sub>-specific CTL clones. The NP<sub>418-426</sub>-specific CTL clones were incubated with five different combinations of tetramers containing four different variants of the NP<sub>418-426</sub> epitope (NP<sub>ESV</sub>, NP<sub>DSI</sub>, NP<sub>DTI</sub>, NP<sub>DPI</sub>). NP<sub>DTI</sub> tetramer is PE labeled, NP<sub>DSI</sub> and NP<sub>DPI</sub> tetramers are allophycocyanin labeled, and NP<sub>ESV</sub> tetramers are available with PE and allophycocyanin-fluorochrome label. Presented are four CTL clones with different reactivity patterns representative of the remaining five clones.

of six CTL clones (15.9, F10, G2, 2384, 5017, and C4), but not of clone 3180, D1, and C10 (Table V). The large distance in the MDS plot between peptides containing an E and those containing a D at P4 exemplified their antigenic dissimilarity. The lysine (K) to arginine (R) substitution at P5 reduced the functional avidity of CTL clones 2384, 5017, and 3180. As a result, an R at P5 outplaced peptide LPFDRTTIM, confirming the importance of a K at this position for recognition by these NP<sub>DTI</sub>-specific CTL clones (Fig. 3). CTL clone C4 tolerated both the S→T and the S→P substitutions at P6. CTL clones 2384, 5017, and 3180 all tolerated the T→S substitution, but the T→P substitution reduced the avidity of clones 2384 and 5017 more than 10,000-fold and that of clone 3180 300-fold. The S→T substitution at P6 significantly reduced the functional avidity of four of the NP<sub>ESV</sub>-specific CTL clones (D1, C10, 15.9, and F10) when the reactivity to the LPFEKSTIM was compared with that to LPFEKTTIM. CTL clones D1, C10, 15.9, G2, and C4 tolerated a valine (V) to isoleucine (I) substitution at P8, while the EC<sub>50</sub> value for this variant epitope increased by a factor of 260 in CTL clone F10. In the antigenic map (Fig. 3),



**FIGURE 2.** Functional avidity of NP<sub>418-426</sub>-specific CTL clone 3180. The ability of CTL clone 3180 to lyse target cells pulsed with 10-fold serial dilutions of eight different variants of the NP<sub>418-426</sub> epitope was determined. The concentration (nanomolar) at which 50% of the target cells (EC<sub>50</sub> value) at an E:T ratio of 10:1 were killed was determined. Each symbol represents a different NP<sub>418-426</sub> epitope variant, including nonpeptide pulsed target cells as a negative control (▲).

the NP<sub>ESV</sub>-specific T cell clones (D1, C10, 15.9, F10, and G2) were positioned in close proximity to the LPFEKSTVM peptide against which they were raised in vitro. Likewise, clones 2384, 5017, and C4 were positioned in proximity of their homologous peptides, although C4 was situated closer to the heterologous peptide LPFDKTTIM and clones 2384 and 5017 to the heterologous peptide LPFDKSTIM. Finally, clone 3180 was positioned in the middle of the plot, close to the H1N1 peptide and the H3N2 peptides from 1972 and 1980, confirming its ability to recognize hetero- and homosubtypic variants of the NP<sub>418-426</sub> epitope.

*Staining of influenza A virus-stimulated PBMC with HLA-B\*3501-peptide tetramers that contain different NP<sub>418-426</sub> variants*

The specificity of NP<sub>418-426</sub> tetramer staining was confirmed with well-defined CTL clones and this technology was then used to evaluate the specificity of NP<sub>418-426</sub>-specific human polyclonal CTL responses using PBMC stimulated with influenza A virus. Following stimulation of PBMC of five HLA-B35<sup>+</sup> donors with Resvir-9, a H3N2 virus containing the NP<sub>ESV</sub> epitope, the percentage of NP<sub>ESV</sub>-specific cells ranged from 1.1 to 18.5% of the CD8<sup>+</sup>

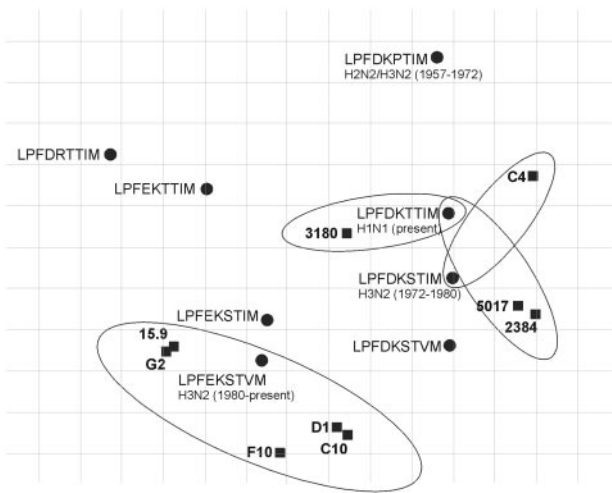
Table V. Functional avidity (EC<sub>50</sub> value) of the CTL clones for the NP<sub>418-426</sub> epitope variants<sup>a</sup>

| Peptide Variants             | CTL Clones                     |                      |                      |                      |                      |                           |                      |      |                                |  |
|------------------------------|--------------------------------|----------------------|----------------------|----------------------|----------------------|---------------------------|----------------------|------|--------------------------------|--|
|                              | H3N2/1980 (NP <sub>ESV</sub> ) |                      |                      |                      |                      | H1N1 (NP <sub>DTI</sub> ) |                      |      | H3N2/1972 (NP <sub>DSI</sub> ) |  |
| LPF - - - T - M <sup>b</sup> | D1                             | C10                  | 15.9                 | F10                  | G2                   | 2384                      | 5017                 | 3180 | C4                             |  |
| —DKT-I-                      | >5 × 10 <sup>3</sup>           | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | 0.7                       | 0.1                  | 0.08 | 2 <sup>c</sup>                 |  |
| —DKP-I-                      | >5 × 10 <sup>3</sup>           | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | >10 <sup>4</sup>          | >10 <sup>4</sup>     | 26   | 0.8                            |  |
| —DKS-I-                      | 18                             | 17                   | >10 <sup>4</sup>     | >5 × 10 <sup>3</sup> | >5 × 10 <sup>3</sup> | 0.1                       | 0.1                  | 0.5  | 2                              |  |
| —DKS-V-                      | 0.8                            | 0.8                  | >10 <sup>4</sup>     | 12                   | >5 × 10 <sup>3</sup> | 0.1                       | 0.1                  | 11   | 2                              |  |
| —DRT-I-                      | >10 <sup>4</sup>               | >10 <sup>4</sup>     | 80                   | >5 × 10 <sup>3</sup> | 100                  | >5 × 10 <sup>3</sup>      | >10 <sup>4</sup>     | 1100 | >5 × 10 <sup>3</sup>           |  |
| —EKS-I-                      | 0.8                            | 1.0                  | 0.1                  | 8                    | 0.8                  | >10 <sup>4</sup>          | 500                  | 1.1  | >5 × 10 <sup>3</sup>           |  |
| —EKS-V-                      | 0.2                            | 0.8                  | 0.1                  | 0.03                 | 0.1                  | >5 × 10 <sup>3</sup>      | >5 × 10 <sup>3</sup> | 0.5  | >5 × 10 <sup>3</sup>           |  |
| —EKT-I-                      | >5 × 10 <sup>3</sup>           | >5 × 10 <sup>3</sup> | 10                   | >5 × 10 <sup>3</sup> | 8                    | >5 × 10 <sup>3</sup>      | >5 × 10 <sup>3</sup> | 3    | >5 × 10 <sup>3</sup>           |  |

<sup>a</sup> Absence of lytic activity at a concentration of 5 μM peptide is depicted as >5 × 10<sup>3</sup> nM, while a value of >10<sup>4</sup> means that the EC<sub>50</sub> value could not be determined; however, target cells were lysed at a concentration of 5 μM peptide.

<sup>b</sup> Consensus sequence.

<sup>c</sup> EC<sub>50</sub> value in nanomolar.



**FIGURE 3.** An antigenic map visualizing functional avidity between the variant NP<sub>418–426</sub> peptides and the CTL clones. The functional avidity between the peptides and CTL clones was calculated as EC<sub>50</sub> values (Table V). The CTL clones are depicted by squares while circles represent the variant peptides. The large circles contain the CTL clones and the NP<sub>418–426</sub> epitope variant used for *in vitro* stimulation of these clones. The distance between two grids corresponds to a 10-fold dilution in EC<sub>50</sub> value. For example, the EC<sub>50</sub> value for clone 15.9 with peptide LPFEKSTIM is 0.1 nM; this is two 10-fold dilutions from the assumed baseline titer of 0.001 nM, thus the “target” distance from clone 15.9 to peptide LPFEKSTIM when constructing the map was 2 U.

cells (Fig. 4*a* and data not shown). In the same PBMC cultures, the percentage of NP<sub>D<sub>TI</sub></sub>, NP<sub>D<sub>SI</sub></sub>, and NP<sub>D<sub>PI</sub></sub>-specific cells was also determined using HLA-B\*3501-peptide tetrameric complexes containing the respective peptides. In the *in vitro* expanded PBMC of donor 1 small numbers of NP<sub>D<sub>TI</sub></sub> and NP<sub>ESV</sub> double-reactive CD8<sup>+</sup> cells were observed (0.4%), demonstrating the presence of heterosubtypic reactive CD8<sup>+</sup> cells (Fig. 4*a*). In the PBMC of the remaining four donors, no NP<sub>D<sub>TI</sub></sub>-specific cells were detected after stimulation with Resvir-9. The ability to recognize homosubtypic variants of the NP<sub>418–426</sub> epitope following stimulation with a virus containing the NP<sub>ESV</sub> variant peptide (Resvir-9) was variable between donors. In donors 1 and 2 a fraction of the NP<sub>ESV</sub>-specific cells also bound to NP<sub>D<sub>SI</sub></sub> tetramers (Fig. 4*a*), while in donors 3–5 the NP<sub>ESV</sub>-specific cells did not bind to NP<sub>D<sub>SI</sub></sub> tetramers (Fig. 4*a* and data not shown). Finally, *in vitro* expanded NP<sub>ESV</sub>-specific CD8<sup>+</sup> cells did not bind to the NP<sub>D<sub>PI</sub></sub> tetramer (Fig. 4*a*). Similar experiments were performed with PBMC following stimulation with influenza viruses A/Victoria/3/75 (H3N2, NP<sub>D<sub>SI</sub></sub>) or A/Netherlands/306/00 (H1N1, NP<sub>D<sub>TI</sub></sub>). Of each of the five donors tested, 0.4–4.5% of the CD8<sup>+</sup> cells that were expanded after stimulation with influenza virus A/Victoria/3/75 bound to the homologous NP<sub>D<sub>SI</sub></sub> tetramers. In some donors, a large fraction of the NP<sub>D<sub>SI</sub></sub>-specific cells also bound to the NP<sub>D<sub>TI</sub></sub> tetramers (donors 1, 3, and 4, Fig. 4*b* and data not shown), indicating a high degree of cross-reactivity between these epitopes. The NP<sub>D<sub>SI</sub></sub>-specific cells from donor 2 however, cross-reacted with the NP<sub>ESV</sub> tetramer and not with the NP<sub>D<sub>TI</sub></sub> tetramer, confirming the existence of homosubtypic cross-reactive CTL in this donor. Upon stimulation with influenza virus A/Netherlands/306/00, NP<sub>D<sub>TI</sub></sub>-specific CD8<sup>+</sup> cells were detected in some PBMC cultures (range, 0.0–6.2%, Fig. 4*c*). Most of the NP<sub>D<sub>TI</sub></sub>-specific CD8<sup>+</sup> cells also bound the NP<sub>D<sub>SI</sub></sub> tetramers (donors 1, 3, and 4, Fig. 4*c* and data not shown). In addition, a portion of the NP<sub>D<sub>TI</sub></sub>-specific cells from donor 1 cross-reacted with NP<sub>ESV</sub> tetramers (Fig. 4*c*), confirming the existence

of these cells following stimulation with Resvir-9 virus. Finally, only in donor 3 some NP<sub>D<sub>TI</sub></sub>-specific cells in the PBMC cultures stimulated with A/Netherlands/306/00 also bound to the NP<sub>D<sub>PI</sub></sub> tetramers (Fig. 4*c*). Thus, tetramer staining provided evidence for the existence of CTL cross-reactive with current H3N2 (NP<sub>ESV</sub>) and H1N1 (NP<sub>D<sub>TI</sub></sub>) viruses in one donor, whereas in the other four donors only influenza A virus subtype-specific cells were demonstrated or cells cross-reactive with previous H3N2 (NP<sub>D<sub>SI</sub></sub>) and current H1N1 (NP<sub>D<sub>TI</sub></sub>) viruses.

#### *Influenza A virus-specific Ab titers*

To correlate the NP<sub>418–426</sub>-specific CTL response in the PBMC of donors to the history of influenza A virus infections, serum HAI titers specific for A/H1N1 and A/H3N2 viruses were determined.

Serum Abs were detected in donor 1 against the 1978 vaccine strain (H1N1), which cross-reacted with later vaccine strains, and the 1991 vaccine strain. Abs specific for H3N2 viruses were observed against A/Hong Kong/1/68 (NP<sub>D<sub>PI</sub></sub>) and the 1995 vaccine strain (NP<sub>ESV</sub>). These findings indicate that donor 1 was exposed to influenza A viruses of the H1N1 subtype around 1978 and 1991 and to the H3N2 viruses around 1968 and 1995 (Fig. 4*d*). In donor 2, Abs specific for 1968, 1977, and 1995 vaccine strains of the H3N2 subtype were readily detected (Fig. 4*d*). These findings suggest that this donor has experienced multiple exposures to influenza A viruses of the H3N2 subtype but not to those of the H1N1 subtype. Donor 3 had serum Abs against the 1968 and 1995 strain of the H3N2 subtype (Fig. 4*d*) and low levels of Abs against the 1978 and 1983 strains of the H1N1 subtype. Donor 4 was found to have serum Abs to viruses of the A/H3N2 (1977, 1979, 1982, 1987, and 1995) and the A/H1N1 subtype (1978, 1983, and 1996), whereas in donor 5 solely Abs against viruses of the H3N2 subtype from 1977, 1979, 1995, and 1998 were detected (data not shown).

## Discussion

In the present study, the recognition of natural variants of an HLA-B\*3501-restricted CTL epitope within the NP of influenza A viruses by human clonal and polyclonal CTL populations was investigated. Using classical <sup>51</sup>Cr release assays and direct visualization of specific T cells with tetramers, CTL were identified that were cross-reactive with homo- and heterosubtypic variants of the epitope. The use of CTL clones specific for the respective epitope variants allowed the identification of residues involved in TCR recognition. Based on the finding that cross-reactive CTL were found at low frequencies in PBMC stimulated with influenza virus, we hypothesize that in response to influenza virus infections, HLA-B\*3501-positive individuals develop a CTL response against the homologous epitope variant of which a small proportion may be cross-reactive with other variants. These cross-reactive cells may then be further expanded upon a subsequent infection with viruses carrying a heterologous variant epitope. It was assumed that the study subjects were infected in the past with a homogeneous virus population with regard to the epitope NP<sub>418–426</sub>, based on epidemiological data on the circulation of these variants (24).

The results obtained in <sup>51</sup>Cr release assays with virus-infected target cells and direct visualization with labeled tetramers correlated well, confirming the specificity of these procedures and the antigenic dissimilarity of the epitopes. Using serial dilutions of the peptides, the avidity of the CTL clones for the peptides was determined in <sup>51</sup>Cr release assays and the effect of the amino acid substitutions was assessed. *In vitro* three T cell clones were established after stimulation with peptide NP<sub>D<sub>TI</sub></sub> (5017, 2384, and 3180) and one after stimulation with NP<sub>D<sub>SI</sub></sub> (C4). Although these clones had high avidity for these peptides, it is not clear which variant of the epitope had primed these CTL *in vivo*. As can be judged from



epitope. In contrast to NP<sub>418-426</sub>-specific CTL, CTL specific for an HLA-B\*3501-restricted epitope of dengue virus (NS<sub>500-508</sub>) tolerated an amino acid substitution at position 8 poorly (33).

Upon stimulation of PBMC with different influenza A viruses, various recognition patterns were observed that correlated partially with the history of infections of the respective donors (see below). In three donors (donors 1, 3, and 4), CTL expanded after stimulation with influenza virus A/Netherlands/306/00 (of the H1N1 subtype containing NP<sub>DTI</sub>) were cross-reactive with the NP<sub>DSI</sub> variant present in influenza viruses of the H3N2 subtype, circulating between 1972 and 1980 and vice versa. Thus, also stimulation with A/Victoria/3/75 (H3N2) led to the expansion of NP<sub>DSI</sub>-specific cells cross-reactive with NP<sub>DTI</sub>. This finding resembles the cross-reactivity observed with CTL specific for epitopes in HIV-1, dengue virus, and SIV (33-35). HIV-1-specific CTL could recognize variants of epitopes present in different clades of this virus. An important difference between influenza virus and HIV-1 infections is that individuals usually are not infected with HIV from different clades whereas for influenza, repeated infections with variant viruses of distinct or the same subtype is very common. For that reason, repeated infections with variant viruses may select for cross-reactive CTL from the pool of NP<sub>418-426</sub>-specific CTL induced during the primary infection. To correlate the presence of cross-reactive CTL with the history of influenza A virus infections of the respective donors, serology was performed. Because of the relative insensitivity of the HAI assay and waning Ab titers over time, failure to detect serum Abs against a certain virus may not be conclusive regarding the lack of exposure to this virus in the past. However, a positive result in this assay was considered indicative for a history of infection. Donor 1 was infected with an influenza A virus of the H1N1 subtype and subsequently with an H3N2 virus. This is in agreement with the observation in this donor of NP<sub>DTI</sub>- and NP<sub>ESV</sub>-specific cells. A small proportion of these cells cross-reacted with NP<sub>ESV</sub> and NP<sub>DTI</sub>. The strong staining with the NP<sub>ESV</sub> tetramers (NP<sub>ESV</sub><sup>high</sup>) and the relatively weak staining with the NP<sub>DTI</sub> tetramer (NP<sub>DTI</sub><sup>low</sup>) of these double-reactive CTL detectable after stimulation of PBMC with Resvir-9 and A/Netherlands/306/00 suggests that these NP<sub>ESV</sub><sup>high</sup>NP<sub>DTI</sub><sup>low</sup> CTL have a stronger avidity for the NP<sub>ESV</sub> variant of the epitope. However, since the tetramers were used at nonsaturating concentrations it is impossible to draw firm conclusions regarding the avidity of the CD8<sup>+</sup> cells for the respective tetramers. Donors 2 and 5 were found to be exposed to viruses of the H3N2 subtype circulating in 1977 and 1995 and not to H1N1 viruses. As a result, NP<sub>ESV</sub> monospecific CTL were found in both donors and not T cells reactive with the NP<sub>DTI</sub> (H1N1) variant of the epitope. In addition, homo-subtypic cross-reactive CTL were found in donor 2. In donor 4, the serology correlated with the presence of NP<sub>ESV</sub>- and NP<sub>DTI</sub>-specific cells, although cross-reactive cells were not observed in this donor. A similar reaction pattern was observed in donor 3, although H1N1-specific Ab titers were very low. The absence of heterosubtypic cross-reactive CTL may be explained by the long period between infection with an H1N1 virus and an H3N2 virus. Thus, in two of five donors tested, there was evidence for the existence of T cells cross-reactive with homo- and heterosubtypic variants of the NP<sub>418-426</sub> epitope. The heterogeneity in the NP<sub>418-426</sub>-specific responses in these donors of similar age can be the result of differences in history of infection as well as many other factors influencing the CTL response in these donors.

In mice it has been demonstrated *ex vivo* (17) and *in vitro* (18) that consecutive infections with influenza viruses containing different variants of the same epitope (NP<sub>366-374</sub>) resulted in the expansion of CTL able to recognize both variants of the epitope, whereas infection with the same virus resulted in the induction of

monospecific cells. This expansion of cross-reactive CTL may even take place after consecutive infections with unrelated viruses as has also been observed in mice (36, 37). We hypothesize that also in humans cross-reactive CTL can be selected for against variable epitopes by multiple infections with influenza viruses carrying variant epitopes (apart from true cross-reactive responses against conserved epitopes).

The percentage of heterosubtypic cross-reactive T cells specific for the NP<sub>418-426</sub> epitope is relatively small in the subjects compared with those observed *in vivo* in mice. In some of the donors, there was no evidence for exposure to both subtypes, explaining the absence of cross-reactive CTL. The timing of the infections may be crucial as the frequency of cross-reactive CTL in the pool of memory cells may have declined to undetectable levels, unable to dominate the response to the variant epitope.

We conclude that upon influenza virus infections a small proportion of the NP<sub>418-426</sub>-specific CTL can cross-react with variants of the epitope. Apparently the flexibility of the TCR of this subset of CTL allows the recognition of naturally occurring variants of the epitope that may be escape mutants selected for by CTL-mediated immune pressure. This subset of cross-reactive CTL may increase after repeated infections with heterologous viruses and may contribute to protective immunity against arising mutant viruses.

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