The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 2004

Recognition of Homo- and Heterosubtypic Variants of Influenza A Viruses by Human CD8+ T Lymphocytes[1]

Adrianus C. M. Boon; ... et. al

J Immunol (2004) 172 (4): 2453–2460.
https://doi.org/10.4049/jimmunol.172.4.2453

Recognition of Homo- and Heterosubtypic Variants of Influenza A Viruses by Human CD8$^+$ T Lymphocytes[1]

Adrianus C. M. Boon,* Gerrie de Mutsert,* Debbie van Baarle,† Derek J. Smith,‡¶ Alan S. Lapedes,§¶ Ron A. M. Fouchier,* Kees Sintnicolaas,‖ Albert D. M. E. Osterhaus,* and Guus F. Rimmelzwaan[2]*

In the present study, the recognition of epitope variants of influenza A viruses by human CTL was investigated. To this end, human CD8$^+$ CTL clones, specific for natural variants of the HLA-B*3501-restricted epitope in the nucleoprotein ($NP_{418-426}$), were generated. As determined in ^{51}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$^+$ 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)[3] of influenza A virus ($NP_{418-426}$) (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 $NP_{418-426}$ 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 ^{51}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 $NP_{418-426}$. 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$^+$ 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).

*Department of Virology and WHO National Influenza Center, Erasmus MC, Rotterdam, The Netherlands; †Department of Clinical Viro-Immunology, Sanquin Research at Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands; ‡Department of Zoology, University of Cambridge, Cambridge, United Kingdom; §Santa Fe Institute, Santa Fe, NM 87501; ¶Los Alamos National Laboratory, Los Alamos, NM 87545; and ‖Sanquin Bloodbank Rotterdam, Laboratory for Histocompatibility and Immunogenetics, Dordrecht, The Netherlands

Received for publication September 3, 2003. Accepted for publication December 4, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Numico Research BV, European Union Grant QLRT-2001-01034 (Novaflu), and the foundation for Respiratory Virus Infections, notably Influenza.

[2] Address correspondence and reprint requests to Dr. Guus F. Rimmelzwaan, Erasmus MC, Institute of Virology, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail address: g.rimmelzwaan@erasmusmc.nl

[3] Abbreviations used in this paper: NP, nucleoprotein; HAI, hemagglutinin inhibition; EC_{50}, epitope concentration; MDS, multidimensional scaling.

0022-1767/04/$02.00

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°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 $NP_{418-426}$ epitope (designated NP_{ESV}), which was first identified in 1980 (Table II). A second virus, A/Victoria/3/75 (H3N2) containing the LPFDKSTIM variant of the $NP_{418-426}$ epitope (designated NP_{DSI}), 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 LPFDKT TIM variant of the $NP_{418-426}$ epitope (designated NP_{DTI}, 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 $NP_{418-426}$ 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 μM in RPMI 1640 (Invitrogen, Breda, The Netherlands), and stored at −20°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 μg/ml streptomycin, and 100 IU/ml penicillin (R10F). Following a 1-h infection at 37°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 μg/ml streptomycin, 100 IU/ml penicillin, and 20 μM 2-ME (R10H) and added to uninfected PBMC at a ratio of 1:1 in a 25-cm^2 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°C and used as effector cells in a ^{51}Cr release assay or stained with fluorochrome-labeled HLA-B*3501-peptide tetrameric complexes (see below).

Generation of $NP_{418-426}$-specific $CD8^+$ 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 μM of the variant $NP_{418-426}$ epitopes. After two subsequent rounds of nonspecific stimulation with 1.0 μg/ml PHA (Sigma-Aldrich, St. Louis, MO), the ability of the cells to recognize the $NP_{418-426}$ epitope variant used during the initial stimulation was tested in an ELISPOT assay (see below). The CTL clones with specificity for the $NP_{418-426}$ epitope were PHA stimulated in 75-cm^2 culture flasks before cryopreservation at −135°C. The $CD8^+CD3^+$ cell phenotype of the $NP_{418-426}$-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 $NP_{418-426}$ peptide variants in 150 μl of R10F in a 96-well V-bottom plate. Following a 1-min centrifugation at 140 × *g*, the plate was incubated for 2 h at 37°C before the cells were transferred to a 96-well Silent Screen Plate (Nalge Nunc, Rochester, NY) coated with anti-human IFN-γ mAb (Mabtech, Nacka, Sweden). After 4 h at 37°C, the plates were washed and developed according to the manufacturer's recommendations. T cell clones that produced IFN-γ in the presence of peptide-loaded BLCL or virus-infected BLCL cells and failed to produce IFN-γ after stimulation with negative control BLCL cells were selected for further investigation.

Preparation of target cells for ^{51}Cr release assay

BLCL were established as previously described (26) and used as target cells in ^{51}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 μg/ml streptomycin, and 100 IU/ml penicillin (R0.1B). After 1 h at 37°C, the cells were washed once in R10F and cultured in R10F for 16 h at 37°C. To test the ability of CTL clones to recognize synthetic peptides, 10^6 cells of a BLCL were loaded with 5 μM of different $NP_{418-426}$ epitope variants for 16 h at 37°C in R10F. Radioactively labeling of peptide-loaded and virus-infected BLCL cells was done with $Na_2{}^{51}CrO_4$ for 1 h at 37°C, after which the cells were washed three times with R10F and used as target cells in a ^{51}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_{50})). ^{51}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 $NP_{418-426}$ epitope variants in R10F for 1 h at 37°C, washed once in R10F, and used as target cells.

^{51}Cr release assay

^{51}Cr release assays were performed as described previously (25). T cell clones were added to 5×10^3 ^{51}Cr-labeled target cells at different E:T cell ratios. Following a 4-h incubation at 37°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)] × 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 $NP_{418-426}$ epitope variants within the NP of influenza A

Table II. *Variants and virus origin of $NP_{418-426}$ epitopes*

Epitope Name	Epitope Sequence	Virus Strain	First Year of Introduction	Virus Subtype
NP_{DTI}	LPFDKTTIM	A/Netherlands/306/00	1977	H1N1
NP_{DPI}	LPFDKPTIM	—	1957	H2N2/H3N2
NP_{DSI}	LPFDKSTIM	A/Victoria/3/75	1972	H3N2
NP_{ESV}	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_{ESV}	5
C10	LPFEKSTVM	H3N2/1980	NP_{ESV}	5
15.9	Infection with Resvir-9	H3N2/1980	NP_{ESV}	6
F10	LPFEKSTVM	H3N2/1980	NP_{ESV}	7
G2	LPFEKSTVM	H3N2/1980	NP_{ESV}	7
2384	LPFDKTTIM	H1N1	NP_{DTI}	1
5017	LPFDKTTIM	H1N1	NP_{DTI}	4
3180	LPFDKTTIM	H1N1	NP_{DTI}	5
C4	LPFDKSTIM	H3N2/1972	NP_{DSI}	5

virus (NP_{ESV}, NP_{DSI}, NP_{DTI}, NP_{DPI}) as previously described (20). These natural variants of the $NP_{418-426}$ epitope were selected based on the predominance of these variants since 1957 in H2N2 (NP_{DPI}), since 1968 in H3N2 viruses (NP_{ESV}, NP_{DSI}, NP_{DPI}) and since 1977 in H1N1 viruses (NP_{DTI}). 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 (10^5) were stained in PBS supplemented with 2.0% FCS (P2F) and combinations of two HLA-B*3501-peptide tetrameric complexes with different $NP_{418-426}$ epitope variants at nonsaturating concentrations. After staining for 20 min at 20°C, cells were washed with P2F and at least 10^4 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 10^4 $CD8^+$ cells were acquired. Data were analyzed using the software program CellQuest Pro (BD Biosciences). A proportion tetramer-positive cells in the $CD8^+$ cell fraction of $\geq$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_{50} values of the variant $NP_{418-426}$ 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_{50} 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 $NP_{418-426}$-specific CTL with differentially labeled tetramers

In addition to six previously described CTL clones (Ref. 9 and Table III), obtained after stimulation with two $NP_{418-426}$ epitope variants associated with the H3N2 subtype of influenza A viruses (NP_{ESV} and NP_{DSI}), three additional CTL clones were generated against the currently circulating $NP_{418-426}$ epitope variant of H1N1 viruses (NP_{DTI}). 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 $NP_{418-426}$ epitope present in influenza A viruses of the H3N2 and H1N1 subtype. The CTL activity of the $NP_{418-426}$-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_{ESV} 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 $NP_{418-426}$ epitope. Clone 2384 (Fig. 1) and 5017 (data not shown) reacted with the tetramers containing the NP_{DTI} and NP_{DSI} peptide, but not with those containing the NP_{DPI} or the NP_{ESV} variant of the epitope. CTL clone C4 reacted similarly to clone 2384 and 5017, but also bound the NP_{DPI} 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 $NP_{418-426}$ epitope variants

The antigenic properties of the epitope variants were studied by measuring the functional avidity of the respective T cell clones as EC_{50} 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_{50} values, an antigenic map was constructed by MDS. Using this mathematical procedure, the antigenic dissimilarity of the peptides and the avidity of $NP_{418-426}$-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 $NP_{418-426}$-specific CTL clones*

CTL Clone	% Specific Lysis[a]		
	Resvir-9 (NP_{ESV})	A/Victoria/3/75 (NP_{DSI})	A/Netherlands/306/00 (NP_{DTI})
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

[a] 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.

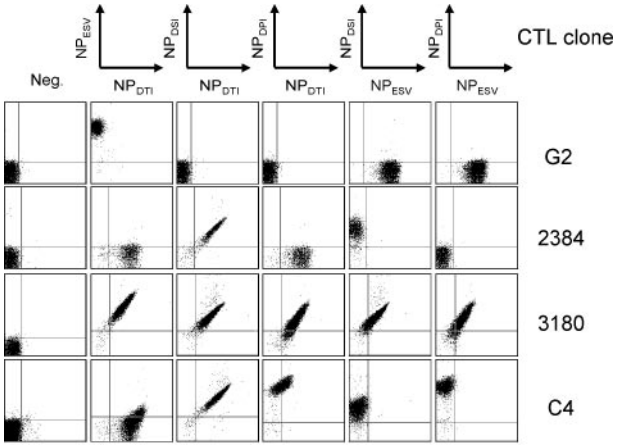

FIGURE 1. Binding of HLA-B*3501-peptide tetrameric complexes containing different $NP_{418-426}$ epitope variants to $NP_{418-426}$-specific CTL clones. The $NP_{418-426}$-specific CTL clones were incubated with five different combinations of tetramers containing four different variants of the $NP_{418-426}$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

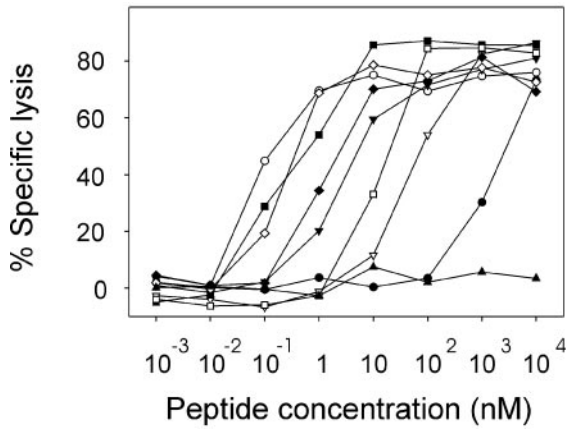

FIGURE 2. Functional avidity of $NP_{418-426}$-specific CTL clone 3180. The ability of CTL clone 3180 to lyse target cells pulsed with 10-fold serial dilutions of eight different variants of the $NP_{418-426}$ epitope was determined. The concentration (nanomolar) at which 50% of the target cells (EC_{50}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

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_{DTI}-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_{ESV}-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_{50} value for this variant epitope increased by a factor of 260 in CTL clone F10. In the antigenic map (Fig. 3), the NP_{ESV}-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 $NP_{418-426}$ epitope.

*Staining of influenza A virus-stimulated PBMC with HLA-B*3501-peptide tetramers that contain different $NP_{418-426}$ variants*

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

Table V. *Functional avidity (EC_{50} value) of the CTL clones for the $NP_{418-426}$ epitope variants*[a]

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

[a] Absence of lytic activity at a concentration of 5 μM peptide is depicted as $>5 \times 10^3$ nM, while a value of $>10^4$ means that the EC_{50} value could not be determined; however, target cells were lysed at a concentration of 5 μM peptide.
[b] Consensus sequence.
[c] EC_{50} value in nanomolar.

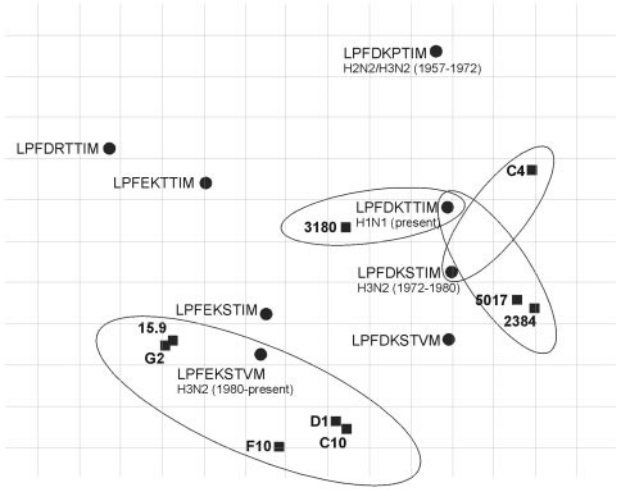

FIGURE 3. An antigenic map visualizing functional avidity between the variant $NP_{418-426}$ peptides and the CTL clones. The functional avidity between the peptides and CTL clones was calculated as EC_{50} values (Table V). The CTL clones are depicted by squares while circles represent the variant peptides. The large circles contain the CTL clones and the $NP_{418-426}$ epitope variant used for in vitro stimulation of these clones. The distance between two grids corresponds to a 10-fold dilution in EC_{50} value. For example, the EC_{50} 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 LPFEK STIM when constructing the map was 2 U.

cells (Fig. 4*a* and data not shown). In the same PBMC cultures, the percentage of NP_{DTI}-, NP_{DSI}-, and NP_{DPI}-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_{DTI} and NP_{ESV} double-reactive $CD8^+$ cells were observed (0.4%), demonstrating the presence of heterosubtypic reactive $CD8^+$ cells (Fig. 4*a*). In the PBMC of the remaining four donors, no NP_{DTI}-specific cells were detected after stimulation with Resvir-9. The ability to recognize homosubtypic variants of the $NP_{418-426}$ epitope following stimulation with a virus containing the NP_{ESV} variant peptide (Resvir-9) was variable between donors. In donors 1 and 2 a fraction of the NP_{ESV}-specific cells also bound to NP_{DSI} tetramers (Fig. 4*a*), while in donors 3–5 the NP_{ESV}-specific cells did not bind to NP_{DSI} tetramers (Fig. 4*a* and data not shown). Finally, in vitro expanded NP_{ESV}-specific $CD8^+$ cells did not bind to the NP_{DPI} tetramer (Fig. 4*a*). Similar experiments were performed with PBMC following stimulation with influenza viruses A/Victoria/3/75 (H3N2, NP_{DSI}) or A/Netherlands/306/00 (H1N1, NP_{DTI}). Of each of the five donors tested, 0.4–4.5% of the $CD8^+$ cells that were expanded after stimulation with influenza virus A/Victoria/3/75 bound to the homologous NP_{DSI} tetramers. In some donors, a large fraction of the NP_{DSI}-specific cells also bound to the NP_{DTI} 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_{DSI}-specific cells from donor 2 however, cross-reacted with the NP_{ESV} tetramer and not with the NP_{DTI} tetramer, confirming the existence of homosubtypic cross-reactive CTL in this donor. Upon stimulation with influenza virus A/Netherlands/306/00, NP_{DTI}-specific $CD8^+$ cells were detected in some PBMC cultures (range, 0.0–6.2%, Fig. 4*c*). Most of the NP_{DTI}-specific $CD8^+$ cells also bound the NP_{DSI} tetramers (donors 1, 3, and 4, Fig. 4*c* and data not shown). In addition, a portion of the NP_{DTI}-specific cells from donor 1 cross-reacted with NP_{ESV} tetramers (Fig. 4*c*), confirming the existence of these cells following stimulation with Resvir-9 virus. Finally, only in donor 3 some NP_{DTI}-specific cells in the PBMC cultures stimulated with A/Netherlands/306/00 also bound to the NP_{DPI} tetramers (Fig. 4*c*). Thus, tetramer staining provided evidence for the existence of CTL cross-reactive with current H3N2 (NP_{ESV}) and H1N1 (NP_{DTI}) 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_{DSI}) and current H1N1 (NP_{DTI}) viruses.

Influenza A virus-specific Ab titers

To correlate the $NP_{418-426}$-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_{DPI}) and the 1995 vaccine strain (NP_{ESV}). 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 ^{51}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 $NP_{418-426}$, based on epidemiological data on the circulation of these variants (24).

The results obtained in ^{51}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 ^{51}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_{DTI} (5017, 2384, and 3180) and one after stimulation with NP_{DSI} (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

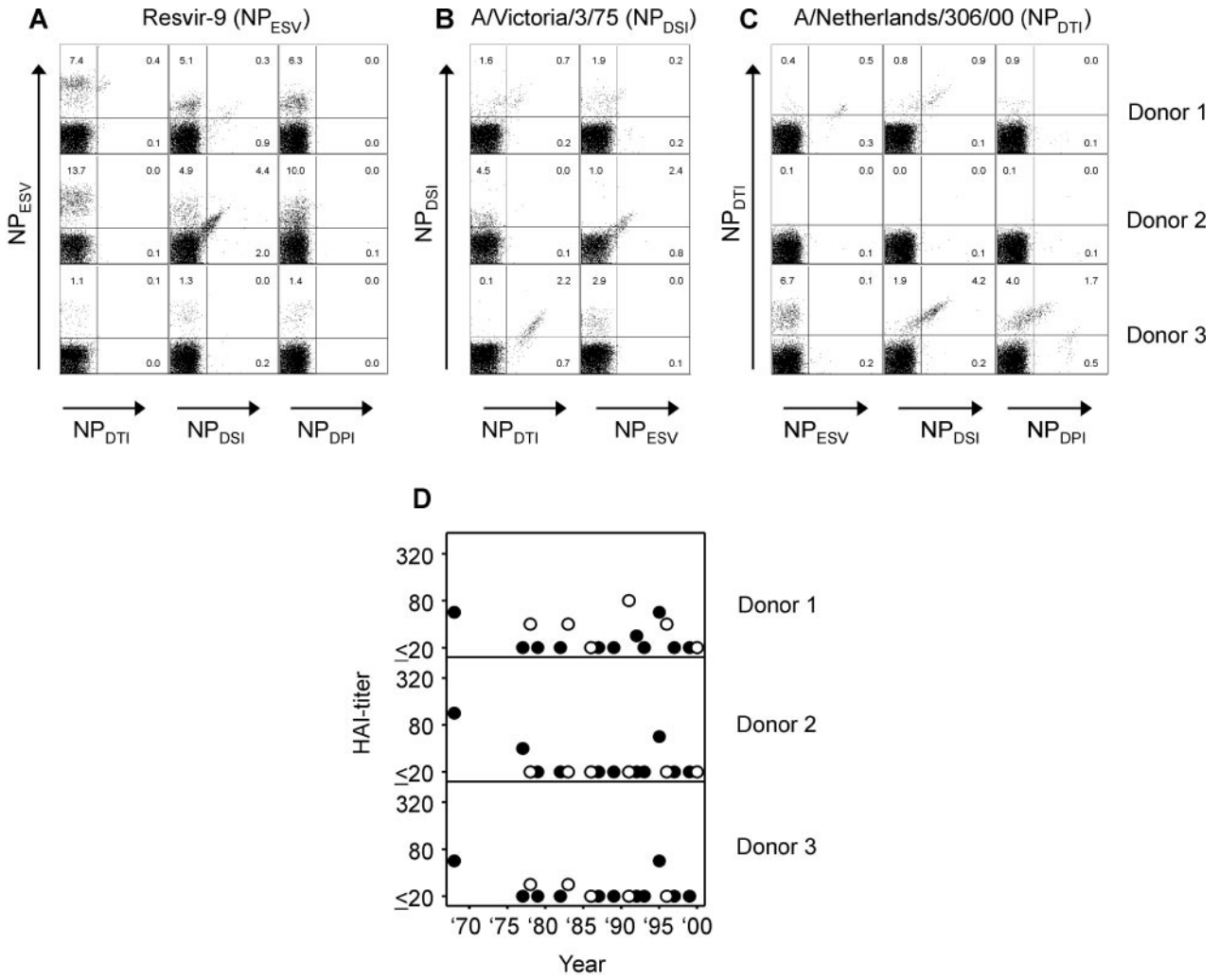

FIGURE 4. Tetramer-staining of polyclonal $NP_{418-426}$-specific CTL response in PBMC stimulated with influenza A virus. PBMC of donors 1–5 were stimulated in vitro with Resvir-9 (NP_{ESV}), A/Victoria/3/75 (NP_{DSI}), or A/Netherlands/306/00 (NP_{DTI}) for 8–10 days before tetramer staining with combinations of two $NP_{418-426}$ epitope variant-containing tetramers conjugated to two different fluorochromes. Depicted are donors 1, 2, and 3, respectively, stained with NP_{ESV} tetramers (vertical) and NP_{DTI}, NP_{DSI}, or NP_{DPI} tetramers after stimulation with Resvir-9 (*A*) or NP_{DSI} tetramers (vertical) and NP_{DTI} or NP_{ESV} tetramers following stimulation with A/Victoria/3/75 (*B*) or NP_{DTI} tetramers (vertical) and NP_{ESV}, NP_{DSI} or NP_{DPI} tetramers following stimulation with A/Netherlands/306/00 (*C*). Staining profiles of donors 4 and 5 were not shown, since they resembled those of donor 3 (donor 4) or had low to undetectable levels of tetramer-positive cells. The percentages indicate the percentage of tetramer-positive cells in 10^4 $CD8^+$ cells. *D*, Influenza A virus-specific HAI titers in plasma of HLA-$B35^+$ blood donor HAI assays were performed on plasma obtained from donors 1–5 using 6 different H1N1 vaccine strains (○) and 11 different H3N2 vaccine strains (●) used since 1968. The HAI titers specific for each vaccine strain are depicted for donors 1–3 against the years in which the vaccine strains were isolated.

the EC_{50} values (Table V) and the MDS plot (Fig. 3), some clones exhibited the highest avidity for a heterologous peptide. For example, clone C4 may have been directed to NP_{DPI} and clone 2384 to NP_{DSI}. In contrast, T cell clones raised in vitro to peptide NP_{ESV} all showed the highest avidity to this peptide, suggesting that indeed it was the homologous peptide responsible for the activation of these cells in vivo. MDS proved to be a useful aide in the interpretation of EC_{50} values and allowed the direct visualization of recognition patterns of the respective CTL clones and the antigenic differences between epitopes.

The $NP_{418-426}$ epitope was found to vary in influenza viruses isolated during the past 70 years at positions 4, 5, 6, and 8 of the 9-mer epitope. All peptides retained the anchor residues at positions 2 and 9, indicating that they bind to HLA-B*3501 with equal affinity, as has been demonstrated previously in an HLA peptide-binding assay (9). Substitutions at P4 and P5 were found to have the largest effect on recognition by specific T cells as can be judged from the EC_{50} values and therefore the antigenic distance in the MDS plot. The $D_{421}E$ substitution in 1980 allowed the virus to escape from NP_{DSI}-specific CTL (9). These findings coincide with data obtained with dengue virus-specific CTL, confirming the importance of position 4 for the recognition of an HLA-B*3501-restricted epitope (33). Although a mutation at P4 had a major impact on TCR recognition, CTL do exist (3180) that recognize peptides containing either an E or a D at position 4. These CTL may have been selected for by repeated infections with different influenza A viruses containing different variants of the epitope. The $K_{422}R$ substitution also had a dramatic effect on the recognition of the epitope. However the R at amino acid position 422 on the NP is rare and has only been found in influenza A viruses isolated in 1933 and 1934 and in several human isolates of avian origin. Substitutions at P6 and P8 had relatively little impact on TCR recognition, although for most NP_{ESV}-specific T cell clones the T→S substitution at P6 reduced the TCR avidity for the

epitope. In contrast to $NP_{418-426}$-specific CTL, CTL specific for an HLA-B*3501-restricted epitope of dengue virus ($NS_{500-508}$) 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_{DTI}) were cross-reactive with the NP_{DSI} 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_{DSI}-specific cells cross-reactive with NP_{DTI}. 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 $NP_{418-426}$-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_{DTI}- and NP_{ESV}-specific cells. A small proportion of these cells cross-reacted with NP_{ESV} and NP_{DTI}. The strong staining with the NP_{ESV} tetramers (NP_{ESV}^{high}) and the relatively weak staining with the NP_{DTI} tetramer (NP_{DTI}^{low}) of these double-reactive CTL detectable after stimulation of PBMC with Resvir-9 and A/Netherlands/306/00 suggests that these $NP_{ESV}^{high}NP_{DTI}^{low}$ CTL have a stronger avidity for the NP_{ESV} 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 $CD8^+$ 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_{ESV} monospecific CTL were found in both donors and not T cells reactive with the NP_{DTI} (H1N1) variant of the epitope. In addition, homosubtypic cross-reactive CTL were found in donor 2. In donor 4, the serology correlated with the presence of NP_{ESV}- and NP_{DTI}-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 $NP_{418-426}$ epitope. The heterogeneity in the $NP_{418-426}$-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 ($NP_{366-374}$) 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 $NP_{418-426}$ 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 $NP_{418-426}$-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.

Acknowledgments

We acknowledge Liane van de Kemp for growing, purifying, and sequencing the influenza A viruses and Marianne Baars for serology. Furthermore, we thank Wilfried Levering for performing the HLA typing, Saskia Sakko for organizing the blood collection of the blood donors (Sanquin Bloodbank Rotterdam), and Gijs van Schijndel for preparing the HLA class I-peptide tetrameric complexes. Dr. R. A. M. Fouchier is a fellow of the Royal Dutch Academy of Arts and Sciences.

References

1. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science 259:1745.*
2. Kuwano, K., M. Tamura, and F. A. Ennis. 1990. Cross-reactive protection against influenza A virus infections by an NS1-specific CTL clone. *Virology 178:174.*
3. Yap, K. L., G. L. Ada, and I. F. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature 273:238.*
4. Fu, T. M., L. Guan, A. Friedman, T. L. Schofield, J. B. Ulmer, M. A. Liu, and J. J. Donnelly. 1999. Dose dependence of CTL precursor frequency induced by a DNA vaccine and correlation with protective immunity against influenza virus challenge. *J. Immunol. 162:4163.*
5. Bender, B. S., T. Croghan, L. Zhang, and P. A. Small. 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med. 175:1143.*
6. McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare. 1983. Cytotoxic T-cell immunity to influenza. *N. Engl. J. Med. 309:13.*
7. Mackenzie, C. D., P. M. Taylor, and B. A. Askonas. 1989. Rapid recovery of lung histology correlates with clearance of influenza virus by specific $CD8^+$ cytotoxic T cells. *Immunology 67:375.*
8. Voeten, J. T., T. M. Bestebroer, N. J. Nieuwkoop, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan. 2000. Antigenic drift in the influenza A virus (H3N2) nucleoprotein and escape from recognition by cytotoxic T lymphocytes. *J. Virol. 74:6800.*
9. Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan. 2002. Sequence variation in a newly identified HLA-B35-restricted epitope in the influenza A virus nucleoprotein associated with escape from cytotoxic T lymphocytes. *J. Virol. 76:2567.*
10. Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med. 160:814.*
11. Kuwano, K., V. E. Reyes, R. E. Humphreys, and F. A. Ennis. 1991. Recognition of disparate HA and NS1 peptides by an $H\text{-}2K^d$-restricted, influenza specific CTL clone. *Mol. Immunol. 28:1.*
12. Zweerink, H. J., B. A. Askonas, D. Millican, S. A. Courtneidge, and J. J. Skehel. 1977. Cytotoxic T cells to type A influenza virus; viral hemagglutinin induces A-strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol. 7:630.*

13. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature 267:354*.
14. Lamb, J. R., D. D. Eckels, M. Phelan, P. Lake, and J. N. Woody. 1982. Antigen-specific human T lymphocyte clones: viral antigen specificity of influenza virus-immune clones. *J. Immunol. 128:1428*.
15. Braciale, T. J., M. E. Andrew, and V. L. Braciale. 1981. Heterogeneity and specificity of cloned lines of influenza-virus specific cytotoxic T lymphocytes. *J. Exp. Med. 153:910*.
16. Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink. 1977. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med. 145:557*.
17. Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher. 1999. Selective expansion of cross-reactive $CD8^+$ memory T cells by viral variants. *J. Exp. Med. 190:1319*.
18. Townsend, A. R., and J. J. Skehel. 1984. The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells. *J. Exp. Med. 160:552*.
19. Jameson, J., J. Cruz, M. Terajima, and F. A. Ennis. 1999. Human $CD8^+$ and $CD4^+$ T lymphocyte memory to influenza A viruses of swine and avian species. *J. Immunol. 162:7578*.
20. Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:94*.
21. Masurel, N., P. Ophof, and P. de Jong. 1981. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. *J. Hyg. 87:201*.
22. Palmer, D., W. Dowdle, M. Coleman, and G. Schild. 1975. Haemagglutination inhibition test. In *Advanced Laboratory Techniques for Influenza Diagnosis: Procedural Guide.* Anonymous, ed. U.S. Department of Health, Education, and Welfare, Atlanta, p. 25.
23. Rimmelzwaan, G. F., M. Baars, E. C. Claas, and A. D. Osterhaus. 1998. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. *J. Virol. Methods 74:57*.
24. Macken, C., H. Lu, J. Goodman, and L. Boykin. 2001. The value of a database in surveillance and vaccine selection. In *Options for the Control of Influenza IV*. A. D. M. E. Osterhaus, N. Cox, and A. W. Hempson, eds. Elsevier Science, Amsterdam, pp. 103–106.
25. Boon, A. C., G. de Mutsert, Y. M. Graus, R. A. Fouchier, K. Sintnicolaas, A. D. Osterhaus, and G. F. Rimmelzwaan. 2002. The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype. *J. Virol. 76:582*.
26. Rimmelzwaan, G. F., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. E. Beyer, D. Maher, J. Bates, and A. D. Osterhaus. 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines. *Vaccine 19:1180*.
27. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc. Natl. Acad. Sci. USA 93:4102*.
28. van Baarle, D., E. Hovenkamp, M. F. Callan, K. C. Wolthers, S. Kostense, L. C. Tan, H. G. Niesters, A. D. Osterhaus, A. J. McMichael, M. H. van Oers, and F. Miedema. 2001. Dysfunctional Epstein-Barr virus (EBV)-specific $CD8^+$ T lymphocytes and increased EBV load in HIV-1 infected individuals progressing to AIDS-related non-Hodgkin lymphoma. *Blood 98:146*.
29. Lapedes, A., and R. Farber. 2001. The geometry of shape space: application to influenza. *J. Theor. Biol. 212:57*.
30. Perelson, A. S., and G. F. Oster. 1979. Theoretical studies of clonal selection: minimal antibody repertoire size and reliability of self-non-self discrimination. *J. Theor. Biol. 81:645*.
31. Shepherd, R. 1963. Analysis of proximities as a technique for the study of information processing in man. *Hum. Factors 5:33*.
32. Kruskal, J. 1964. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. *Psychometrika 29:1*.
33. Zivny, J., I. Kurane, A. M. Leporati, M. Ibe, M. Takiguchi, L. L. Zeng, M. A. Brinton, and F. A. Ennis. 1995. A single nine-amino acid peptide induces virus-specific, $CD8^+$ human cytotoxic T lymphocyte clones of heterogeneous serotype specificities. *J. Exp. Med. 182:853*.
34. Cao, H., P. Kanki, J. L. Sankale, A. Dieng-Sarr, G. P. Mazzara, S. A. Kalams, B. Korber, S. Mboup, and B. D. Walker. 1997. Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. *J. Virol. 71:8615*.
35. Buseyne, F., M. L. Chaix, C. Rouzioux, S. Blanche, and Y. Riviere. 2001. Patient-specific cytotoxic T-lymphocyte cross-recognition of naturally occurring variants of a human immunodeficiency virus type 1 (HIV-1) p24gag epitope by HIV-1-infected children. *J. Virol. 75:4941*.
36. Chen, H. D., A. E. Fraire, I. Joris, M. A. Brehm, R. M. Welsh, and L. K. Selin. 2001. Memory $CD8^+$ T cells in heterologous antiviral immunity and immunopathology in the lung. *Nat. Immunol. 2:1067*.
37. Brehm, M. A., A. K. Pinto, K. A. Daniels, J. P. Schneck, R. M. Welsh, and L. K. Selin. 2002. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat. Immunol. 3:627*.