Human HLA-A0201-restricted Cytotoxic T Lymphocyte Recognition of Influenza A Is Dominated by T Cells Bearing the Vβ17 Gene Segment

By Paul J. Lehner,* Eddie C. Y. Wang,* Paul A. H. Moss,† Sheila Williams,* Kaye Platt,* Steven M. Friedman,§ John I. Bell,‖ and Leszek K. Borysiewicz∗

From the *Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff, CF4 4XN; †Molecular Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; and the §Hospital for Special Surgery, Cornell University, New York 10021

Summary

The major histocompatibility complex class I-restricted cytotoxic T lymphocyte (CTL) response is important in the clearance of viral infections in humans. After influenza A infection, a peptide from the matrix protein, M58-66, is presented in the context of the MHC allele HLA-A0201 and the resulting CTL response is detectable in most HLA-A0201 subjects. An initial study suggested that M58-66-specific CTL clones show conserved T cell receptor (TCR) cα and β gene segments. We have addressed the significance of this observation by determining the expression of Vβ17 during the development of M58-66-specific CTL lines in 21 unrelated HLA-A0201 subjects, and analyzing TCR usage by M58-66-specific CTL clones. TCR Vβ17 was the dominant Vβ segment used and CD8 Vβ17 expansion correlated with M58-66-specific lysis. Limiting dilution analysis from five subjects showed the M58-66 CTL precursor frequency to vary between 1/54,000 and less than 1/250,000, and that up to 85% of the matrix peptide (M58-66)-specific CTL used the Vβ17 gene segment. The M58-66 specific CTL response was dependent on previous viral exposure and specific Vβ17 expansion, as it was not found in cord blood, despite a readily expandable Vβ17+ CD8+ T cell subpopulation. Sequence analysis of 38 M58-66-specific Vβ17 transcripts from 13 subjects revealed extensive conservation in the CDR3 region including conservation of an arginine-serine motif. To test the dependence of this CTL response on the Vβ17 gene segment, peripheral blood lymphocytes were depleted of CD8+ TCR Vβ17+ cells, before the generation of M58-66-specific CTL. In most cases such depletion blocked or severely reduced the generation of the M58-66-specific response, and under limiting dilution conditions could abolish M58-66-specific CTL precursors. These studies reveal the dependence of this natural human immune response on a particular TCR gene segment.

Virus-specific CTL play a role in viral clearance after both acute and persistent virus infections in humans (1, 2) and experimental infections (3, 4). Viral proteins are processed to peptides and translocated via peptide transporters to the endoplasmic reticulum where they access class I molecules (5–7). The diverse array of peptides, presented by the six potential class I molecules, is balanced by the specificity of the T cell response encoded within the TCR-α/β. While the host may benefit from a broad antigen-selected T cell repertoire, decreasing the chance that a rapidly mutating organism will evade immune detection, TCR diversity will be limited by a number of factors, including positive and negative selection and interaction with the peptide/MHC complex.

Both TCR heterogeneity (8) and conservation (9–13) to a specific MHC class I peptide complex has been described. Whether immunity after natural infection leads to restrictions on TCR usage remains unclear. Furthermore recurrent exposure of an outbred population to a pathogen may elicit a different response to that seen in experimental systems.

We have studied the TCR usage of CTL specific for the influenza A matrix protein-derived peptide (M58-66), presented by the HLA-A0201 molecule. This immune response is of considerable interest. First, influenza A is a common viral infection of humans and the majority of adults have serological evidence of previous influenza A exposure. Second, HLA-A0201 is one of the most frequent class I alleles being
present in 40% of the Caucasoid population. The influenza A virus–specific CTL response is of special significance as, unlike other virus-derived peptide epitopes (14), no other HLA-A0201-restricted influenza A-specific epitopes have been isolated. This CTL response was first described using vaccinia virus recombinants (15) and the optimal matrix peptide 9 mer (M58-66) subsequently defined (16, 17). The HLA-A0201 peptide complex has since become one of the best studied in humans such that recent X-ray crystallographic studies have defined the orientation of each amino acid in the MHC class I binding cleft (18).

The conservation of TCR usage in a large human population after natural infection has not been determined. In an initial study we described conserved TCR α, Vβ, and Jα usage in seven clonally derived lines from two unrelated individuals of similar haplotype (12). These preliminary investigations raised several questions: how far is the restricted usage of Vβ17 observed in a wider HLA-A0201 population, and how is Vβ17 usage influenced by in vitro culture? To what extent is TCR conservation also retained across the proposed peptide binding CDR3 region (19), and can non-Vβ17 CTL substitute for Vβ17 in the HLA-A0201/M58-66 CTL response?

We found that (a) long-term CTL clones show conservation of both α and β chain usage. (b) Using a clonotypic Vβ17-specific mAb there is dominant use of the TCR Vβ17 segment in both bulk culture lines and CTL clones; limiting dilution culture suggests that between 55 and 85% of all M58-66-specific CTL clones use this Vβ chain. (c) Direct TCR sequencing of limiting dilution analysis (LDA) derived clones shows a conserved arginine-serine motif in the predicted CDR3 region, and conserved length of the CDR3 loop for each Jβ chain identified. (d) Finally, Vβ17 depletion of PBMC in some subjects can abrogate the CTL response to the M58-66 peptide, suggesting that manipulation of human class I restricted CTL responses by Vβ-specific mAbs may be a possible approach to immunotherapy.

Materials and Methods

Donors. Donors were healthy human volunteers (n = 23) or subjects with juvenile rheumatoid arthritis (n = 6). No significant differences with respect to the HLA-A0201–restricted CTL response were observed between the groups.

Cell Lines. EBV-transformed lymphoblastoid B cell lines were established by conventional methods using supernatant from the EBV-producing marmoset cell line B95.8 (type 1 isolate). CIR.A2 was a kind gift from Professor A. McMichael (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK). CIR.A2 expresses a transfected genomic clone of HLA-A2.1 (20). It was grown in RPMI 1640 with 10% FCS and 400 µg/ml G418 (GIBCO BRL, Gaithersburg, MD). CIR.A2 M57-68 and CIR.A2 M58-66 are CIR.A2 cells transfected with minigene DNA encoding influenza virus matrix peptides B57-68 and M58-66, respectively (17, 21).

They were a kind gift from Dr. H. Zweerink (Merck, Sharpe and Dohme, Rahway, NJ).

Virus. Influenza A (AX/31) virus was a kind gift from Dr. D. B. Thomas and Dr. J. Skehel (National Institute for Medical Research, London, UK). The virus had been grown in the allantoic cavity of embryonated chicken eggs. The viral hemagglutination titre, determined by fowl red cell agglutination, was 1,000 HA U/50 µl.

Synthetic Peptide. Peptide M58-66 was synthesized commercially (Neosystem Laboratoire, Strasbourg, France). Purity was 92% by HPLC analysis.

Mycoplasma arthritidis–derived superantigen (MAM) was a kind gift from Dr. B. C. Cole (University of Utah College of Medicine, Salt Lake City, UT). MAM was isolated from M. arthritidis culture supernatants, as previously described (22).

HLA Typing. HLA typing from all donors was performed by the Welsh National Blood Transfusion Service (Rhydalafr, Cardiff, UK) using a standard microlymphocyte toxicity test.

Virus- and Peptide-specific CTL Lines and Clones. Virus- and peptide-specific CTL lines were generated as previously described (23). To establish peptide-specific clones, PBMC were infected with virus and on day 7 after culture the cells were centrifuged on a Ficoll-Hypaque density gradient and clones set up in limiting dilution culture with replicate input cell numbers at 1, 2, 5, 10, 15, 20, and 50 cells/well. Replicates for each input cell number ranged from 400 (1 cell/well) to 25 (15 cells/well). The cultures were stimulated with peptide-pulsed irradiated autologous B cells (1 x 10⁶/well), fresh allogeneic PBMC from at least three donors (1 x 10⁶/well), PHA (2 µg/ml) (Sigma Chemical Co., St. Louis, MO) and lymphocult-T (Biotest, Dreieich, Germany); this protocol was repeated every 7 d. Fresh medium (RPMI 1640/10% human serum) and lymphocult-T were added every 3–4 d. Positive wells, as determined by phase contrast microscopy, were screened from day 14. Only wells that came from plates within the conventional clonal distribution of outgrowth, which by LDA were found to be wells with an initial input number of <5 cells/well were expanded. Positive wells which fell below this input cell number were screened for peptide-specific cytotoxic activity and positive cultures transferred into 24-well plates.

Limiting Dilution Cultures for Peptide-specific CTLPrecursors. Fresh PBMC infected with influenza A virus were inactivated with human serum, and added to freshly isolated PBMC to give a final concentration of 10% infected cells. Replicate microcultures (25–50 replicates/input cell number) of PBMC and 10% virus infected PBMC were set up in 96-well round bottomed plates, with PBMC dilutions varying from 5,000–150,000 cells/well. After 7 d each well was fed with fresh medium, recombinant human IL-2 (10 IU/ml) (Boehringer Mannheim Corp., Indianapolis, IN) and M58-66 peptide-pulsed B cells at a feeder to responder ratio of 1:3. Each well was refed on day 14 and given fresh medium and IL-2 every 3–4 d. The LDA was assayed on day 18. Split-well analysis was performed on two sets of targets (untreated or M58-66 peptide–treated CIR.A2 cells). To perform the assay half the resuspended well volume was divided into two aliquots and each aliquot was assayed for cytotoxicity on the respective targets. Maximal and spontaneous release was measured for each plate. Analysis against untreated and peptide-pulsed CIR.A2 cells was performed as described (24), using a threshold of 10% specific lysis. Where the single-hit Poisson model was fulfilled (a straight line relationship demonstrated by the χ² value for goodness of fit) the regression line was calculated by the maximum likelihood method (25). The peptide-specific CTLp frequency was estimated from the initial responder cell number at which 37% of the wells were negative for cytotoxicity. Statistical analysis was performed using Statistical Package for So-

Abbreviations used in this paper: CTLp, CTL precursor; LDA, limiting dilution analysis; MAM, Mycoplasma arthritidis derived superantigen.
CD8+ TCR V\(\beta\)317+ cells (mean + 3 SD of CD8+ TCR V\(\beta\)317+ cytotoxicity; (b) for CD8+ TCR V\(\beta\) usage; and (c) total RNA cDNA synthesis and PCR was subsequently performed on these samples for sequencing studies (see below).

**Cytotoxicity Assays.** Target cells (EBV-transformed B cells or CIR.A2 cells) were pelleted, resuspended in minimal volume, and loaded with \(^{51}\)Cr (Amersham International, Little Chalfont, UK) (100 \(\mu\)Ci of a 10 mCi/ml stock) for 1 h. Virus-infected target cells were infected overnight with virus, before \(^{51}\)Cr labeling; while PBMC). cDNA synthesis and PCR was subsequently performed in triplicate. Target cells incubated with 5% Triton X-100, and 4 \(\times\) 10\(^3\) target cells added to 96-well plates containing 0.8–1.5 \(\mu\)g total RNA at 70°C for 5 min and snap chilled on ice for 3 min before addition of reaction buffer. Reactions were performed using 2 mM dNTP (Promega, Southampton, UK), 20 mM dithiothreitol (DTT), 10 mM Tris-HCl, 50 mM KCl, 10 mM MgCl\(_2\), 5 U human placental RNAase inhibitor (Gibco, Paisley, UK) and 100 U murine Moloney leukemia virus reverse transcriptase (Gibco) at 42°C for 1 h, before inactivation of murine Moloney leukemia virus reverse transcriptase for 5 min at 95°C. cDNA (2 \(\mu\)l/reaction) was then subjected to PCR using 2.5 U Taq polymerase (Promega) on a Techne Thermal Cycler in a final concentration of 2.3 mM MgCl\(_2\). PCR mix was placed on ice immediately after adding template, then transferred directly to a hot (95°C) PCR block to begin denaturation for 3 min at 95°C. Cycle parameters were 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles. The VB17 and \(\beta\)5 PCR primers used were as described (28).

The amplification products from four PCR reactions (for each clone) were then pooled and purified using a standard PCR purification kit (QiAquick-spin; QIAGEN, Hilden, Germany). Purified products were dried and resuspended in 20–40 \(\mu\)l distilled water for sequencing. Direct cycle sequencing was performed on a Techne Thermal Cycler, using the PRISM™ Ready Reaction DyeDeoxy™ Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA), and sequences read on an automated sequencer (Applied Biosystems, Inc.). Primers used for sequencing were the same as used during amplification.

**Results**

Conservation of the HLA-A0201/1/M58-66–specific CTL Response. Using anchored PCR, we studied the TCR usage of HLA-A0201/1/M58-66–specific clones and clonally derived lines from a further two unrelated individuals (Fig. 1). In 3/4 cases these clones used the same V\(\alpha\) (V\(\alpha\)10) and 3/4 clones used the same V\(\beta\) (V\(\beta\)17). Despite having been generated from unrelated individuals in different laboratories, CTL clones were identified which used identical \(\alpha\) and \(\beta\) gene joining segments to those previously reported (12). In the previous and present study, clones from all four individuals had been maintained in vitro for more than 2 mo, requiring repeated stimulation with virus-infected or peptide-pulsed stimulator cells.

Thus as TCR V\(\beta\)17 was a frequently used M58-66 specific V\(\beta\) chain, we used the TCR V\(\beta\)17-specific monoclonal antibody C1 to determine the expansion of V\(\beta\)17+ CD8+ cells in CTL bulk culture cell lines from 10 to >100 d (Tables 1 and 2). In 11 of the 21 individuals where PBMC were examined before secondary in vitro stimulation, no significantly expanded population of TCR V\(\beta\)17+ cells in peripheral blood was observed. The mean percent \pm SD V\(\beta\)17 CD8+ total CD8 from freshly isolated PBMC in HLA-A0201 individuals was 4.23 ± 1.54 (\(n = 11\)), and in age-matched non-HLA-A0201 individuals was 4.17 ± 1.18 (\(n = 10\); not shown). After in vitro culture, all 21 HLA-A0201–positive individuals were eventually able to generate an M58-66–specific response, although poor responders required up to two re-stimulations with the peptide. The development of an
prolonged in vitro culture with peptide restimulation, we determined by forward angle and side scatter) which contained a higher proportion (between 17 and 92%) of VB17+ CD8+ cells (Table 1).

To determine whether the VB17 expansion is specific for the HLA-A0201/M58-66 complex and is not a reflection of prolonged in vitro culture with peptide restimulation, we have performed the following investigations: (a) After flow cytometric analysis of 12 CD8+ influenza A virus-specific (non-peptide defined), 7 CD8+, influenza A-specific, class I restricted lines (HLA-B27/N383-91, HLA-B8/N380-89; or HLA-A3/NP265-273–specific) and 6 CD8+ nonspecific lines, only one line showed a VB17+CD8+/CD8+ population in excess of 5%. (b) CTL lines stimulated with endogenously expressing (M58-66 transfected) CIR.A2 cells also showed expansion of the TCR Vβ17+ population, with a Vβ17+CD8+/CD8+ population of 28% and specific CTL lysis of 39% (E/T 3:1, day 18) indicating that expansion is not conditional on the presence of synthetic peptide. (c) Comparison of identical CTL lines expanded with either virus-infected, or peptide-pulsed, autologous B cells showed the virus-stimulated cells to have a TCR Vβ17+CD8+/CD8+ population of 13.5% and specific CTL lysis of 25% (E/T ratio 1:1) (day 18), as opposed to the CTL expanded with peptide-pulsed B cells which showed a Vβ17+CD8+/CD8+ population of 25% and specific CTL lysis of 44%. The Vβ17 population is therefore expanded after restimulation with both virus- and HLA-A0201–specific viral peptide, though the expansion was consistently greater with M58-66 peptide alone. (d) An HLA-A0201/M58-66–specific CTL line (GT, Table 1) generated in a different laboratory (kindly provided by Dr. H. Zweerink) (31) also showed a greatly expanded (83%) Vβ17+ CD8+/total CD8+ population (Table 1). (e) Using PBMC from subjects who made a good HLA-A0201/M58-66–specific CTL response, occasional restimulation would fail to generate CTL. When this occurred no Vβ17 expansion was observed.

**Generation of HLA-A0201/M58-66–specific CTL Response after TCR Vβ17 PMBC Depletion.** Since Vβ17 is commonly used by HLA-A0201/M58-66–specific CTL it was important to determine whether other TCR V gene segments were able to either contribute, or substitute for Vβ17 in the HLA-A0201/M58-66–specific response. The Vβ17–specific mAb C1 only partially inhibited cytolytic activity of a TCR Vβ17+ clone (data not shown). An alternative approach was to deplete this subset of cells before in vitro stimulation. We were unable to achieve TCR Vβ17+ populations of sufficient purity with complement or magnetic bead depletion. Therefore TCR Vβ17+CD8+ T cells were depleted from freshly prepared PBMC by flow cytometry (FACS® 440, Becton Dickinson & Co.), and then restimulated in vitro with influenza AX/31 virus–infected cells. This was performed on three individuals with a representative experiment shown in Fig. 3. Depletion of a Vβ17+CD8+ population from 4.8% to 0.18% (96.4%) was achieved. Parallel control depletions were performed with a TCR Vβ19 specific antibody. CTL lysis and flow cytometric analysis were examined after 15-d culture (Fig. 3). Four CD8 TCR Vβ17–depleted lines from the same individual were examined. The control CD8 TCR Vβ19–depleted line showed 61% CTL lysis (E/T ratio

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**Figure 1.** Nucleotide and predicted amino acid sequences of the TCR α and β chains from anchored PCR-amplified HLA-A0201/M58-66–specific independent clones from two unrelated donors, JW and JM. The Jα nomenclature follows that of Moss et al. (52). Each β chain sequence runs from the conserved cysteine to the first phenylalanine in the "P-G-X-T" motif found in the J region (where X is any amino acid), and are aligned to that conserved region. The conserved CDR3 arginine-serine motif is highlighted. These sequence data are available from EMBL/Genbank/DDBJ under accession numbers Z35683-6 and Z35690-3.

**Table 1.** CD8+ TCR VB17§ population of responding PBMC, (as determined by forward angle and side scatter) which contained a higher proportion (between 17 and 92%) of VB17+ CD8+ cells. The control CD8+ TCR VB17§ population is therefore expanded after restimulation with both virus- and HLA-A0201–specific viral peptide, though the expansion was consistently greater with M58-66 peptide alone. Parallel control depletions were performed with a TCR Vβ19 specific antibody. CTL lysis and flow cytometric analysis were examined after 15-d culture (Fig. 3). Four CD8 TCR Vβ17–depleted lines from the same individual were examined. The control CD8 TCR Vβ19–depleted line showed 61% CTL lysis (E/T ratio

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**Table 2.** CD8+ TCR VB17§ population of responding PBMC, (as determined by forward angle and side scatter) which contained a higher proportion (between 17 and 92%) of VB17+ CD8+ cells. The control CD8+ TCR VB17§ population is therefore expanded after restimulation with both virus- and HLA-A0201–specific viral peptide, though the expansion was consistently greater with M58-66 peptide alone.
Table 1. Expression of TCR Vβ17+ CD8+ Cells in PBMC and Peptide-specific Bulk Culture CTL Lines of Varying Duration

| Donors | Culture | Percent net CTL lysis* (E/T 5:1) | HLA-A2.1/M58-66-specific CTL line | Non-HLA-A2 flu5 peptide-specific CTL line |
|--------|---------|---------------------------------|-----------------------------------|----------------------------------------|
|        | PBMC    | Total population                | Blast population                  | Total population                      | Blast population |
| SP     | 10      | 41                              | –                                 | 30                                     | 52               | –               | –               |
| EC     | 10      | 40                              | –                                 | 30                                     | 40               | 3.1             | 2.2             |
| KD     | 10      | 20                              | –                                 | 15                                     | 17               | –               | –               |
| JM     | 12      | 56                              | 2.6                               | 65                                     | 75               | –               | –               |
| DDC    | 21      | 60                              | 3.6                               | 76                                     | –               | –               | –               |
| ND     | 21      | 53                              | –                                 | 61                                     | 57               | –               | –               |
| MD     | 21      | 30                              | –                                 | 61                                     | 82               | –               | –               |
| KP     | 21      | 24                              | 3.6                               | 45                                     | 69               | –               | –               |
| LB     | 21      | 28                              | –                                 | 38                                     | 49               | –               | –               |
| ID     | 21      | 49                              | 7.35                              | 47                                     | 69               | –               | –               |
| JD     | 21      | 41                              | –                                 | 32                                     | 51               | –               | –               |
| MJ     | 28      | 23                              | 4.1                               | 13                                     | 29               | –               | –               |
| PL     | 28      | 37                              | 5.3                               | 43                                     | 61               | 5.7             | 8.26            |
| GW     | 36      | 60                              | –                                 | 65                                     | 92               | –               | –               |
| AF     | 36      | 50                              | –                                 | 25                                     | 57               | –               | –               |
| MP     | 42      | >80                             | 6.03                              | 98                                     | –               | 2.6             | 3.5             |
| SW     | 42      | >80                             | 3.5                               | 85                                     | –               | –               | –               |
| GT†    | >28     | 75                              | –                                 | 83                                     | –               | –               | –               |
| JW     | >100    | >80                             | 4.1                               | 87                                     | –               | 0.75            | –               |
| LM‡    | 10      | –                               | 3.5                               | –                                      | –               | 3.5             | 2.8             |

* PBMC and peptide-specific CTL lines were analyzed by two-color immunofluorescence staining for distribution of TCR Vβ17+ staining within the CD8+ population. Data are expressed as percent TCR Vβ17+ CD8+ cells within the total CD8+ subset.

† CTL lysis is net lysis measured at an E/T ratio of 5:1.

§ Non-HLA-A0201/M58-66-specific CTL lines were influenza peptide-specific CTL lines, from the same subject recognizing influenza A-derived peptides restricted through either HLA-A3, B8, or B27.

¶ CTL line provided by Dr. H. Zweerink.

£ CTL line from non-HLA-A0201 individual.

While the Vβ17 response appears to be dominant, some subjects are able to generate a non-Vβ17, HLA-A0201/M58-66-specific CTL response. The low precursor frequency of these non-Vβ17, M58-66-specific CTL (see below) has made it difficult to clone out this population and only one such clone has been established to date (Fig. 1). This clone used Vβ4 and Vα2.2 variable region genes, with no recognizable junctional sequence similarity to the Vβ17, Vα10 TCR clones.

Primary or Secondary Immune Response. The ubiquitous nature of the M58-66-specific CTL response in 21 HLA-A0201 individuals studied, raised the question whether this was a true memory response to influenza A, or if the naturally processed 9-mer peptide could induce a primary CTL response in vitro. Cord blood from HLA-A2 phenotyped neonates (as detected by mAb MA2.1) and a corresponding maternal sample were stimulated for more than 3 wk in vitro with virus- or
Table 2. Comparison of M58-66-specific Lysis and CD8 TCR Vβ17 Expansion in HLA-A2 Maternal and Paired Cord Blood Samples

|       | Pair 1 |       | Pair 2 |       | Pair 3 |
|------|--------|------|--------|------|--------|
|       | Percent Vβ17 | Percent net CTL lysis* | Percent Vβ17 | Percent net CTL lysis | Percent Vβ17 | Percent net CTL lysis |
|       | CD8/total CD8 | | CD8/total CD8 | | CD8/total CD8 | |
| Cord | Day 1 | 3.9 | 4 | 3.2 | |
|      | Day 18 | 7 | 0 | 7 | 0 |
|      | Mother | Day 1 | 4.5 | 4.5 | 1.9 | |
|      | Day 18 | 18 | 37 | 27 | 34 | |
|      | non-HLA-A2 | |

* Percent net CTL lysis was calculated as the difference between percent specific 51Cr release from peptide-pulsed and unpulsed targets.

peptide-infected cells. No HLA-A0201 M58-66 peptide-specific killing or TCR Vβ17-expanded cells in 3/3 cord blood samples was found, whilst 2/2 matching (HLA-A2) maternal samples showed peptide-specific lysis and expansion of the CD8+Vβ17+ cells (Table 2). The lack of cord blood response was not a result of an inability to expand the Vβ17 repertoire as MAM-treated cord blood samples rapidly expanded TCR Vβ17 expressing cells from 4-26% of the total population after 7 d in culture. Despite MAM-induced CD8 and CD4 TCR Vβ17 expansion in cord blood there was no peptide specific killing. However, it should be noted that MAM-induced Vβ17 expansion from adult donors also does not result in specific expansion of an M58-66-specific CTL population (P. J. Lehner, unpublished observations).

Quantitation of the CD8+ TCR Vβ17+ Proportion of the HLA-A0201/M58-66-specific CTL Response. To quantitate the proportion of CD8+Vβ17+ lymphocytes in CTL in the HLA-A0201/M58-66 specific CTL response, we used LDA to determine the M58-66-specific CTLp frequency and then analyzed the Vβ17 CD8 cells of all microcultures from plates with input cell numbers indicative of single precursor cell origin. Only HLA-A0201/M58-66-specific wells with input cell number <150,000 were analyzed.

![Figure 3](https://via.placeholder.com/150)

Figure 3. TCR Vβ17+ CD8+ depletion reduces M58-66 peptide-specific CTL generation but allows detection of Vβ17- CTL. TCR Vβ17+CD8+ and control (TCR Vβ19+CD8+) T cells were depleted from PBMC population by cell sorting. Remaining cells were stimulated with influenza A virus and plated in 96-well flat-bottomed plates at 1 x 10^5/well, to ensue HLA-A0201/M58-66 CTLp were present in the microculture (Table 3). A 96% Vβ17+CD8+ depletion of a 4.8% Vβ17+CD8+ staining population was achieved. Net CTL lysis and the proportion of Vβ17+CD8+ cells were estimated after 15-d culture. Eight lines were examined. Four TCR Vβ19-depleted CTL lines (■) all recognized M58-66. One of four TCR Vβ17-depleted lines (□) recognized M58-66 and was TCR Vβ17-. One line (△) showed 10% lysis but was found to have an 18.3% Vβ17+CD8+ population, despite the initial 96% depletion. The remaining two Vβ17-depleted lines (▲) showed <10% lysis, and contained <1% Vβ17+CD8+ cells.
Table 3. Quantitative Analysis of the CD8⁺ TCR Vβ17⁺ Contribution to the HLA-A2.1/M58-66-specific CTL Response

|                      | HLA-A0201/M58-66-specific CTL clonal cultures | Non-HLA-A0201/M58-66-specific CTL clonal cultures |
|----------------------|-----------------------------------------------|---------------------------------------------------|
| Frequency of HLA-A0201/M58-66-specific CTLp in PBMC* |                                              |
|                      | CD8⁺ Vβ17⁺ wells                           | CD8⁺ Non-Vβ17⁺ wells                                |
|                      | %                                             | %                                                  |
|                      | %                                             | %                                                  |
| DDC                  | 1/54,000                                     | 5/9 (55.5)                                        |
|                      | 0/4 (0)                                      | 4/4 (100)                                         |
| SW                   | 1/105,000                                    | 24/35 (68.5)                                     |
|                      | 11/35 (31.4)                                 | 0/3 (0)                                           |
|                      | 4/4 (100)                                    | 36/38 (94.7)                                     |
| AF                   | 1/170,000                                    | 14/20 (70)                                        |
|                      | 7/20 (30)                                    | 2/38 (5.3)                                        |
|                      | 3/12 (25)                                    | 2/2 (100)                                         |
| PL                   | <1/250,000                                   | 9/12 (75)                                         |
|                      | 3/12 (25)                                    | 0/2 (0)                                           |
|                      | 2/2 (100)                                    | 21/22 (95.5)                                     |
| JW (1)               | 1/275,000 (approximately)                    | 23/32 (71.8)                                      |
|                      | 9/32 (28.2)                                  | 1/22 (4.5)                                        |
|                      | 36/38 (94.7)                                 | 21/22 (95.5)                                     |
| JW (2)               | -                                            | 7/9 (77.7)                                        |
|                      | 2/9 (22.2)                                   | 0/4 (0)                                           |
|                      | 4/4 (100)                                    | 3/4 (8.8)                                         |

* LDA were established and assayed for M58-66-specific CTL activity as described in Materials and Methods. Clonally derived HLA-A0201/M58-66-specific CTL and M58-66-negative controls were expanded in culture and the proportion of CD8⁺ TCR Vβ17⁺ cells in the microcultures determined.

1 A positive CD8⁺ TCR Vβ17⁺ well was defined as a well with a CD8⁺ TCR Vβ17⁺ population >9.2% (mean ± 3 SD) of the total CD8⁺ population.

Individual JW was tested for the CD8⁺ TCR Vβ17⁺ contribution on three separate occasions over a 6-mo period.

It is difficult to ascertain a cut-off for Vβ17 positivity. The mean percent + 3 SD Vβ17 CD8/total CD8 from freshly isolated PBMC in 25 subjects was 9.2%. While the percent Vβ17 CD8/total CD8 in LDA cultures ranged from <1 to 98%, 102/108 (94.4%) M58-66-negative cultures showed a Vβ17 CD8/total CD8 <10%. Therefore in the LDA analysis a well was considered to be Vβ17 positive if >10% Vβ17 CD8 cells were present.

Five individuals were studied (Table 3, Fig. 4). Microcultures originating from a single CTLp, in which the CD8⁺ Vβ17⁺ population exceeded 10% of the total CD8 population were observed in 55.5–89% of microcultures with M58-66-specific cytotoxicity. In contrast, only 6/108 (5.6%) M58-66 CTL negative wells had >10% CD8⁺ Vβ17⁺ usage. Repeat analyses were performed on three separate occasions in the same subject (JW) over a 6-mo period. The CD8 Vβ17 CTL varied between 72 and 89% of the total HLA-A0201/M58-66-specific CTL response.

**Direct Sequencing of LDA-derived Vβ17⁺ Clones.** LDA studies confirmed the dominance of Vβ17⁺ CD8⁺ CTL in the HLA-A0201-restricted M58-66 peptide-specific response. However a question remained as to whether the TCR Vβ17⁺ chains showed restriction in their CDR3 region, encoded within the junctional (V-D-J) region of the β chain. We used the mAb C1 to identify which wells among all CTL recognizing this peptide were Vβ17⁺ and these wells were allowed to expand by further in vitro culture. Using only those cultures originating from input cell numbers which indicated that the culture was clonal, Vβ17-specific primers were used to amplify, clone, and sequence the dominant Vβ17 sequence in these wells. Although “non-M58-66-specific” polyclonal Vβ17 TCR-β chains will also be present in these wells, any dominant clonal population is preferentially expanded by primer selection for direct sequencing. Reverse transcriptase-PCR was performed on 43 M58-66-specific, Vβ17-positive microcultures from 11 individuals. Sequences were obtained from 30 of these LDA-derived clones and shown, together with the anchored PCR-derived Vβ17-positive transcripts, in Fig. 5. The results show that 26/38
Direct sequences obtained from non-matrix peptide-specific, VB17-expanded wells are shown for comparison. These sequence data are available from J/~ usage, each sequence runs from the conserved cysteine in the VB17 region to the first phenylalanine in the "F-G-X-T" motif found and V/~17-amplified LDA-derived HLA-A0201/M58-66-specific CTL clones from 13 donors (see Materials and Methods for further details). Clones in the J region (where X is any amino acid), and are aligned to that conserved region. The conserved CDR3 arginine-serine motif is highlighted.

Figure 5. Nucleotide and predicted amino acid sequences of the VB17~ chains from anchored PCR-amplified independent (marked by asterisk), EMBL/GenBank/DDBJ under accession numbers Z35670-Z35715.

(68%) of the TCR VB17 clones use either the Jb2.7 or Jb2.3 gene. Five different J~ genes were less commonly used. Of particular interest was the conserved "arginine-serine motif" found in the CDR3 region in clones from all 13 individuals, and this motif was found in 28/38 (74%) of all the sequenced clones. Seven clones from five subjects used identical junctional amino acid sequences, all of which differed at the nucleotide level, providing evidence for selection of this rearrangement. The junctional region was of constant length in all clones using the J~81.2 gene segment. None of these clones showed the arginine-serine motif.


Discussion

The major aims of this study were: (a) to determine the degree of TCR \( \alpha \) and \( \beta \) chain conservation in the human CTL response to the influenza A-specific HLA-A0201/M58-66 complex, in a wide range of HLA-A0201 individuals; (b) to quantitate and sequence the dominant \( \beta \) chain used in the CTL response to this naturally occurring human virus; and (c) to determine the extent to which the HLA-A0201/M58-66 CTL response is dependent on V\( \beta \)17 and adjacent D-\( \gamma \) segments.

V\( \beta \)17 is the dominant V\( \beta \) segment in the CTL response to the HLA-A0201/M58-66 complex, as demonstrated by anchored PCR on isolated CTL clones, and the V\( \beta \)17 expansion of M58-66-specific bulk culture CTL lines. M58-66-specific TCR sequences from two unrelated individuals, of similar haplotype, have previously shown conservation of V\( \beta \) (V\( \beta \)17 8/9 sequences), V\( \alpha \) (V\( \alpha \)10 8/12), and J\( \alpha \) gene usage. In the present study, sequences of established long-term clones from two further individuals had direct chain pairing of V\( \beta \)17 and V\( \alpha \)10, J\( \alpha \) conservation and independent use of V\( \beta \)17 and V\( \alpha \)10. The lack of available V\( \alpha \)10 TCR mAb has precluded further studies of V\( \alpha \) usage in bulk culture CTL lines. However, V\( \beta \)17 gene usage was not identified in any of five HLA-A2 restricted, influenza A-specific CTL clones from an individual studied by de Waal Malefyt et al. (32). V\( \alpha \)10.2 was used by two of these individuals, for which no corresponding \( \beta \) chain was identified. In view of the predominant use of V\( \beta \)17 in the present study, this discrepancy may reflect the different matrix peptides used (M59-68 vs. M58-66), or the unusual phenotype (HLA-2.1, HLA-2.2F) of their CTL donor (Q66) (33, 34).

TCR gene usage in human class I-restricted CTL (12, 13) has been examined in only a few studies, with small numbers of subjects. Conclusions based on a limited number of long-term clones from a few individuals may be neither representative of short-term bulk culture CTL lines, nor of the in vivo situation. We have therefore examined the kinetics of in vitro expansion of the M58-66-specific CTL response from initiation to 3 mo. All individuals generating an M58-66-specific response expanded the V\( \beta \)17+ T cells, especially in the CD8+ blast population. These cells were detectable early and in higher numbers in those individuals with a strong M58-66 CTL response. This was not dependent on the presence of synthetic peptide as it was also observed after stimulation with virus-infected cells or CIR-A2 cells expressing the minigenome encoding M58-66. The V\( \beta \)17 expansion was consistently less with virus-infected cells, as the HLA-A0201/ M58-66-specific CTL response may be competing with other class I restricted viral peptides presented at the cell surface. CTL lines restricted through HLA-A0201 but with different peptide specificities have not shown a V\( \beta \)17 preference, making it unlikely that the class I-restricting element alone is responsible for the V\( \beta \)17 preponderance (12).

We have used LDA to both quantitate the proportion of TCR V\( \beta \)17 in the M58-66-specific CTL response and generate M58-66-specific CTL clones for PCR amplification and sequencing. Analysis of five individuals showed V\( \beta \)17 to account for between 55.5 and 89% of the TCR usage in the HLA-A0201/M58-66-specific CTL response. This remained relatively constant in a single individual tested on three separate occasions over a 6-mo period. This adaptation of LDA complements information gained from the generation of long-term CTL clones, by allowing the quantitation of peptide-specific TCR usage under culture conditions which allow clonal expansion of M58-66-specific CTL precursors. This technique allows the generation of a large number of CTL clones for sequence analysis, and comparison of sequences obtained from established long-term and LDA-derived clones shows the data to be consistent.

The discrepancy between the 90% V\( \beta \)17 usage in long-lived bulk culture lines and the 15–45% “non-V\( \beta \)17” M58-66-specific clones suggests a selective maturation of high affinity TCR clones after bulk culture stimulation, which will be precluded under limiting dilution conditions. Whether CTL TCR selection and maturation occurs in vivo is important to determine. Murine studies on the CTL response to the H-2Kd-restricted HLA-CW3 170–179 antigen has shown a marked TCR preference for V\( \beta \)10 and a unique J\( \alpha \) segment in 23 independent in vitro derived CTL clones (35). This in vitro TCR restriction was paralleled by an in vivo study which showed marked expansion of V\( \beta \)10 in PBMC, spleen, and draining lymph nodes of mice immunized with this antigen, and direct sequencing confirmed similar J and junctional chain usage in these conserved V\( \beta \)10+ clones (36). It is not known whether the TCR expansion after immunization with an immunodominant synthetic peptide reflects TCR expansion after natural infection. Future studies in humans will need to examine both peripheral blood and respiratory tract lymphocytes for TCR V\( \beta \)17 expansion after acute influenza infection.

The TCR recognition of influenza matrix peptide exhibits the most conserved V region segment usage of any studied class I-restricted human immune response, and given the requirement for the V\( \beta \)17 gene segment, several features of the CDR3 region conservation are of note. There appears to be relatively little conservation of the J\( \beta \) segment, and although J\( \beta \)2.3 and J\( \beta \)2.7 were used in 65% of all transcripts, these gene segments are amongst the most commonly used in the TCR-\( \beta \) repertoire (37).

More dramatic is the conservation of amino acids in the CDR3 region, with the most conserved residue (arginine at position 97) used in 30 of the 40 transcripts (for residue nomenclature see Chothia et al. [38]). 5 of the 10 transcripts without an arginine at this position use a J\( \beta \)1.2 gene segment. There are several examples of conservation of this residue in peptide-specific responses and in the study of TCR usage in H-2Kd-restricted CTL recognizing an HLA-CW3-derived peptide, a glycine was found at this position in all TCR-\( \beta \) sequences (35). In the absence of a three-dimensional structure of the ternary complex it is impossible to determine where the conserved arginine may contact the MHC/peptide complex. Around this arginine other amino acids show strong conservation. A serine is found at position 98 and to a lesser
extent at position 99 in the majority of transcripts. Isoleucine or methionine is usually located at position 96. In contrast to the TCR-α sequences used in this immune response the length of the β chain CDR3 region is virtually always conserved at 11 amino acids between the conserved cysteine and phenylalanine residues of V and J segments, respectively. The only exceptions are the five transcripts using the Jβ1.2 segment and the sequence BDE20. While several features of the pattern of amino acid conservation have been reported in other murine and human T cell responses there is no similar example of such striking TCR conservation in a natural human immune response to a common pathogen.

The analysis of many different TCR sequences gives insight into the degree of amino acid diversity that is acceptable at each position in the CDR3 loop and acts as a physiological correlate of murine transgenic analysis (39). As the structure of the HLA-A0201/M58-66 peptide complex has been solved at the three-dimensional levels these TCR sequences should eventually assist in the interpretation of the TCR contacts with the MHC:peptide once the crystal structure of the TCR is solved.

Examples of restricted Vβ gene usage with more heterogeneous Vα usage have also been found with human class II restricted tetanus toxoid-specific T cell clones (40). These clones were unusual in that the tetanus toxoid–derived peptide was promiscuous, being recognized in association with several HLA-DR molecules, as has also been reported for other class II restricted clones (41, 42). In the report by Boitel et al. a particular Vβ gene product was selected irrespective of whether the peptide was presented by four different HLA-DR alleles, implying shared MHC molecules were not required for shared Vβ gene usage by TCRs specific for the peptide. The M58-66 HLA-A0201 restricted peptide is not known to bind any other human MHC molecule and although class I peptide promiscuity has also been found with both human and murine HIV–specific CTL clones (43, 44), in neither study was the TCR usage reported.

The presence of an M58-66–specific CTL response in all HLA-A0201–positive adults raised the question as to whether the in vitro response was a memory response, or whether stimulation of CD8 cells with the optimal 9-mer peptide is capable of activating a primary CTL response in vitro. The absence of an M58-66–specific CTL response in cord blood, despite a readily expandable Vβ17 population of lymphocytes, and the inability of either MAM or TCR Vβ17 mAb-activated adult or cord PBMC to generate M58-66–specific killing supports the view that the M58-66 response represents a memory response acquired by primed individuals in an antigen specific manner. The generation of antigen-specific CTL requires both in vivo exposure to virus, and the expansion of specific junctional and Jβ receptors to generate CTLp, a process which cannot be mimicked by polyclonal stimulation of the dominant TCR Vβ population. The absence of influenza viral persistence (45), and the lack of reported clinical influenza cases in our geographical area during this study, suggests that the population is either continually exposed to subclinical infection or that CTL memory is retained for considerable periods in the absence of persistent viral antigen.

To test the requirement of TCR Vβ17 for the M58-66–specific CTL response, TCR Vβ17 was deleted from freshly isolated PBMC before in vitro expansion. In 10/12 depleted CTL lines from three individuals the CTL response was completely abolished, and under limiting dilution conditions a low CTLp frequency became undetectable after Vβ17 depletion. However, analysis of M58-66–positive limiting dilution cultures suggested that between 10 and 40% of the response may be contributed by Vβ17-negative CTL. This discrepancy could result from a lack of sensitivity in the culture assay, difficulties with the depletion assay or differences in donors. While TCR Vβ17 is clearly the predominant Vβ gene segment in the human M58-66–specific CTL response, other non-Vβ17 gene segments can contribute to this response, though only a single Vβ17 negative clone has been established. These results suggest that while TCR-directed gene therapy is a possibility, the role of subdominant TCR requires further investigation, in particular whether these clones assume greater importance after depletion of the dominant CTL.

Influenza A is a natural, repeatedly encountered infection (46). Conserved TCR usage may therefore reflect in vivo maturation and selection for optimal high affinity CTL clones. However such marked restrictions on TCR usage has not been found for other flu CTL epitopes (13), and other factors also have to be considered. Based on an analysis of reported studies of TCR conservation against several class I and class II peptides, Casanova and Maryanski (47) have suggested that the current conflicting results on TCR conservation may reflect restrictions on TCR usage which would occur when strong homology exists between a presented endogenous self and foreign peptide. In these cases tolerance to self-proteins may trim the range of potential TCRs used in the CTL response. However a single amino acid change in a peptide has been shown to convert a homogenous TCR response into a heterogeneous one and the type of TCR response may therefore be a function of the peptide itself (48). These observations are not exclusive; the restricted TCR usage seen in the human M58-66 CTL response is not as evident in the M58-66–specific CTL response in the HLA-A0201 transgenic mouse (49), suggesting that endogenous factors may influence TCR selection.

To evaluate whether the TCR restriction found in the M58-66–specific response reflects the requirement to differentiate M58-66 from an endogenous homologue, a Swissprot database search was performed to look for endogenous proteins with sequence homology to the matrix peptide and identified the endogenous sequence GLLGFGVT. This 9 mer resides within the RING 11 encoded human transporter protein (TAP) 2 (50), at position 57–65, and shares 7/9 residues with M58-66, with changes at position 2 and 7 of the peptide. The conservative change from isoleucine to leucine at position 2 favors HLA-A0201 binding and does not affect CTL lysis (51). We have shown that the synthetic transporter peptide binds HLA-A0201, but is not recognized by matrix peptide specific human CTL clones (P. J. Lehner, E. C. Y. Wang, S. Williams, and L. K. Borysiewicz, manuscript in preparation). Whether this transporter-derived peptide is actually presented on HLA-A0201–expressing cells remains to be determined, as processing restrictions may prevent the peptide
accessing HLA-A0201. This peptide is, however, a potential candidate restricting a diverse T cell response to the HLA-A0201/M58-66 complex.

In summary, the CTL response to the naturally occurring HLA-A0201-restricted influenza-derived peptide shows conservation of both TCR-α and β chain usage. TCR Vβ17 accounts for between 55 and 85% of all β chains used in this response, and is associated with a conserved junctional region in the majority of subjects examined. PBMC deletion of the TCR Vβ17 gene segment can abolish the M58-66-specific CTL response. Thus under these conditions there is an overwhelming requirement for TCR Vβ17 to establish HLA-A0201/M58-66-specific CTL.

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Address correspondence to Paul Lehner, Howard Hughes Medical Institute Research Laboratories, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510.

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References
1. McMichael, A., F. Gotch, G.R. Noble, and P.A.S. Beare. 1983. Cytotoxic T-cell immunity to influenza. N. Engl. J. Med. 309:13–17.
2. Reussers, S.R., S.D. Riddell, S.D. Meyers, and P.D. Greenberg. 1991. Cytotoxic T lymphocyte response to cytomegalovirus following human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood. 78:1373–1380.
3. Reddehase, M.J., W. Mutter, K. Munch, H.K. Buring, and U. Kosinowski. 1987. CD8+ lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. J. Virol. 61:3102–3108.
4. Bender, B.S., T. Croghan, L. Zhang, A. Parker, and J. 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. 175:1143–1145.
5. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. Annu. Rev. Immunol. 7:601–624.
6. Monaco, J.J. 1992. A molecular model of MHC class I restricted antigen presentation. Immunol. Today. 13:173–179.
7. Neefjes, J.J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. Science (Wash. DC). 261:769–771.
8. Casanova, J.L., P. Romero, C. Widmann, P. Kourilsky, and J.L. Maryanski. 1991. T cell receptor genes in a series of class I major histocompatibility complex-restricted cytotoxic T lymphocyte clones specific for a Plasmodium berghei nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. J. Exp. Med. 174:1371–1383.
9. Aeberscher, T., S. Oelen, and H. Hengartner. 1990. Preferential usage of Vβ4 and Vβ10 T cell receptor genes by lymphocytic choriomeningitis virus glycoprotein-specific H-2Db-restricted cytotoxic T cells. Eur. J. Immunol. 20:523–531.
10. Chen, Z.W., H. Yamamoto, D.J. Watkins, G. Levinson, and N. Letvin. 1992. Predominant use of a T cell receptor Vβ gene family in simian immunodeficiency virus gag-specific cytotoxic T lymphocytes in a rhesus monkey. J. Virol. 66:3913–3917.
11. Yanagi, Y., R. Maekawa, T. Cook, and M.B.A. Oldstone. 1990. Restricted V-segment usage in T-cell receptors from cytotoxic T lymphocytes specific for a major epitope of lymphoectytic choriomeningitis virus. J. Virol. 64:5919–5926.
12. Moss, P.A., R.J. Moots, W.M. Rosenburg, S.J. Rowland-Jones, H.C. Bodmer, A.J. McMichael, and J.I. Bell. 1991. Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. Proc. Natl. Acad. Sci. USA. 88:8987–8990.
13. Bowness, P., P.A. Moss, S. Rowland-Jones, J.I. Bell, and A.J. McMichael. 1993. Conservation of T cell receptor usage by HLA B27-restricted influenza-specific cytotoxic T lymphocytes suggests a general pattern for antigen-specific major histocompatibility complex class I-restricted responses. Eur. J. Immunol. 23:1417–1421.
14. Nixon, D.F., K. Broliden, G. Ogg, and P.A. Broliden. 1992. Cellular and humoral antigenic epitopes in HIV and SIV. Immunology. 76:515–534.
15. Gotch, F., A. McMichael, G. Smith, and B. Moss. 1987. Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes. J. Exp. Med. 165:408–416.
16. Morrison, J., J. Elvin, F. Latron, F. Gotch, R. Moots, J.L. Strominger, and A. McMichael. 1992. Identification of the nonamer peptide from influenza A matrix protein and the role of pockets of HLA-A2 in its recognition by cytotoxic T lymphocytes. Eur. J. Immunol. 22:903–907.
17. Bednarek, M.A., S.Y. Sauma, M.C. Gammon, J.A. Linquist, G. Porter, S. Tamhankar, A.R. Williams, and H. Zweerink. 1991. The minimum peptide epitope from the influenza virus matrix protein: extra and intracellular loading of HLA-A2. J. Immunol. 147:4047–4053.
18. Madden, D.R., D.N. Garboczi, and D.C. Wiley. 1993. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. Cell. 75:693–708.
19. Davis, M.M., and P.J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. Nature (Lond.). 334:395-402.

20. Storkus, W.J., D.N. Howell, R.D. Salter, J.R. Dawson, and P. Cresswell. 1997. NK susceptibility varies inversely with target cell class I HLA antigen expression. J. Immunol. 138:1657-1659.

21. Anderson, K.P., P. Cresswell, M. Gammon, J. Hermes, A. Williamson, and H. Zweierink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. J. Exp. Med. 174:489-492.

22. Atkin, C.L., B.C. Cole, G.J. Sullivan, L.R. Washburn, and B.B. Wiley. 1986. Stimulation of mouse lymphocytes by a mitogen derived from Mycoplasma arthritidis. V. A small basic protein from culture supernatants is a potent T cell mitogen. J. Immunol. 137:1581-1589.

23. Warren, A.P., D.H. Ducrocq, P.J. Lehner, and L.K. Borsyiewicz. 1994. Human cytomegalovirus infected cells have unstable assembly of MHC class I complexes and are resistant to lysis by cytotoxic T lymphocytes. J. Virol. 68:2822-2829.

24. Carmichael, A., X. Jin, P. Sissons, and L.K. Borysiewicz. 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. J. Exp. Med. 177:249-256.

25. Fazekas de St. Groth, S. 1982. The evaluation of limiting dilution analysis. J. Immunol. Methods. 49:R11.

26. Norusis, M.J. 1988. Statistical Package for Social Sciences/Personal Computer (SPSS/PC) + V2.0. SPSS Inc., Chicago, IL. 214 pp.

27. Ormerod, M.G. 1990. An introduction to fluorescence technology. In Flow Cytometry: A Practical Approach. M.G. Ormerod, editor. IRL Press at Oxford University Press, Oxford, United Kingdom. 29-44.

28. Friedman, S.M., M.K. Crow, J.R. Tumang, M. Tumang, Y. Xu, A.S. Hodtsev, B.C. Cole, and D.N. Posnett. 1991. Characterization of human T cells reactive with the Mycoplasma arthritidis-derived superantigen (MAM): generation of a monoclonal antibody against Vβ7, the T cell receptor gene product expressed by a large fraction of MAM-reactive human T cells. J. Exp. Med. 174:891-900.

29. Choi, Y., B.L. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. Proc. Natl. Acad. Sci. USA. 86:8941-8945.

30. Wilson, R.K., E. Lai, P. Concannon, R.K. Barth, and L.E. Hood. 1988. Structure, organization and polymorphism of human and mouse T-cell receptor α and β chain gene families. Immunol. Rev. 101:149-172.

31. Bednarek, M.A., S.A. Engl, M.C. Gammon, J.A. Lingist, G. Porter, A.R. Williamson, and H. Zweierink. 1991. Soluble HLA-A2.1 restricted peptides that are recognised by influenza virus specific cytotoxic T lymphocytes. J. Immunol. Methods. 139:41-47.

32. de-Waal-Malefyt, R., S. Verma, M.-T. Bejarano, M. Ranes-Goldberg, M. Hill, and H. Spits. 1993. CD2/LFA-3 or LFA-1/ICAM-1 but not CD28/B7 interactions can augment cytotoxicity by virus-specific CD8+ cytotoxic T lymphocytes. Eur. J. Immunol. 23:418-424.

33. Hogan, K.T., N. Shimojo, S.F. Walk, V.H. Engelhard, W.L. Maloy, J.E. Coligan, and W.E. Biddison. 1988. Mutations in the alpha 2 helix of HLA-A2 affect presentation but do not inhibit binding of influenza virus matrix peptide. J. Exp. Med. 168:725-736.

34. Shimojo, N., E.P. Cowan, V.H. Engelhard, W.L. Maloy, J.E. Coligan, and W.E. Biddison. 1989. A single amino acid substitution in HLA-A2 can alter the selection of the cytotoxic T lymphocyte repertoire that responds to influenza virus matrix peptide 55-73. J. Immunol. 143:558-564.

35. Casanova, J.-L., J.-C. Cerottini, M. Matthaes, A. Necker, H. Gournier, C. Burra, C. Widmann, H.R. Macdonald, P. Kourilsky, F. Lemonnier, et al. 1992. H-2 restricted cytolytic T lymphocytes specific for HLA display T cell receptors of limited diversity. J. Exp. Med. 176:439-447.

36. Robson MacDonald, H., J.-L. Casanova, J.L. Maryanski, and J.-C. Cerottini. 1993. Oligoclonal expression of major histocompatibility complex class I-restricted cytolytic T lymphocytes during a primary immune response in vivo: direct monitoring by flow cytometry and polymerase chain reaction. J. Exp. Med. 177:1487-1492.

37. Rosenberg, W.M., P.A. Moss, and J.I. Bell. 1992. Variation in human T cell receptor V beta and J beta repertoire: analysis using anchor polymerase chain reaction. Eur. J. Immunol. 22:541-549.

38. Chothia, C., D.R. Boswell, and A.M. Lesk. 1988. The outline structure of the T-cell alpha beta receptor. EMBO (Eur. Mol. Biol. Organ.) J. 7:3745-3755.

39. Jorgensen, J.L., U. Esser, B.F. de-St-Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T-cell receptor-peptide contacts by peptide vaccination using single-chain transgenics. Nature (Lond.). 355:224-230.

40. Boitl, B., M. Ermonoval, P. Panina-Bordignon, R.A. Mariuzza, A. Lanzavecchia, and O. Acuto. 1992. Preferential Vβ gene usage and lack of junctional sequence conservation among human T cell receptors specific for a tetanus toxin-derived peptide: evidence for a dominant role of a germline-encoded V region in antigen/major histocompatibility complex recognition. J. Exp. Med. 175:765-777.

41. Sinigaglia, F., M. Gutttinger, J. Kilgus, D.M. Doran, H. Ma tile, H. Ettinger, D. Trzeciak, D. Gillessen, and J.R.L. Pink. 1988. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. Nature (Lond.). 336:778-780.

42. Panina-Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia. 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. Eur. J. Immunol. 19:2237-2242.

43. Shirai, M., C.D. Pendleton, and J.A. Berzofsky. 1992. Broad recognition of cytotoxic T cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules. J. Immunol. 148:1657-1667.

44. Safrit, J.T., C.A. Andrews, T. Zhu, D.D. Ho, and R.A. Koup. 1994. Characterization of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte clones isolated during acute seroconversion: recognition of autologous virus sequences within a conserved immunodominant epitope. J. Exp. Med. 179:463-472.

45. Eichelberger, M.C., M. Wang, W. Allan, R.G. Webster, and P.C. Doherty. 1991. Influenza virus RNA in the lung and lymphoid tissue or immunologically intact and CD4-depleted mice. J. Gen. Virol. 72:1695-1698.

46. Stuart-Harris, C.H., G.C. Schild, and J.S. Oxford. 1985. Influenza: The Viruses and the Disease. Edward Arnold Ltd., 90 Human CTL Recognition of Influenza A Matrix Protein
47. Casanova, J.-L., and J.L. Maryanski. 1993. Antigen-selected T-cell receptor diversity and self-nonself homology. *Immunol. Today.* 14:391-394.
48. Chien, Y.H., and M.M. Davis. 1993. How αβ T-cell receptors "see" peptide/MHC complexes. *Immunol. Today.* 14:597-602.
49. Man, S., J.P. Ridge, and V. Englehard V. 1994. Diversity and dominance among TCR recognizing HLA-A2.1+ influenza matrix peptide in human MHC class I transgenic mice. *J. Immunol.* In press.
50. Powis, S.H., I. Mockridge, A. Kelly, L.A. Kerr, R. Glynne, U. Gileadi, S. Beck, and J. Trowsdale. 1992. Polymorphism in a second ABC transporter gene located within the class II region of the human major histocompatibility complex. *Proc. Natl. Acad. Sci. USA.* 89:1463-1467.
51. Parker, K.C., M.A. Bednarek, L.K. Hull, B. Cunningham, H.J. Zweerink, W.E. Biddison, and J.E. Coligan. 1992. Sequence motifs important for peptide binding to the human MHC class I molecule, HLA-A2. *J. Immunol.* 149:3580-3587.
52. Moss, P.A.H., W.M.C. Rosenberg, E. Zintzaras, and J.I. Bell. 1993. Characterization of the human T-cell receptor alpha chain repertoire and demonstration of a genetic influence on Vα usage. *Eur. J. Immunol.* 23:1153-1159.