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

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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) α 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 $V\beta17^+$ $CD8^+$ 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 $V\beta17^+$ 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

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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 TCRVα, 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)[1] 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 HLA-typed 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 (Rhydlafar, 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 (1×10^4/well), fresh allogeneic PBMC from at least three donors (1×10^5/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 CTL Precursors. 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 χ^2 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-

[1] *Abbreviations used in this paper:* CTLp, CTL precursor; LDA, limiting dilution analysis; MAM, *Mycoplasma arthritidis* derived superantigen.

cial Science/Personal Computer (SPSS/PC) (26), and GLIM 3.77 (Royal Statistical Society, London, UK).

The remaining 50% of cells were continued in culture for a further 7–10 d. Those wells which by LDA originated from a single HLA-A0201/M58-66–specific CTL precursor, and control wells, were subsequently split three ways: (*a*) to confirm M58-66–specific cytotoxicity; (*b*) for $CD8^+$ TCR $V\beta17^+$ usage; and (*c*) total RNA was extracted from HLA-A0201.1/M58-66–specific wells which were shown by dual color immunofluorescence to contain >9.2% $CD8^+$, TCR $V\beta17^+$ cells (mean + 3 SD of $CD8^+$, TCR $V\beta17^+$ PBMC). 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 μCi of a 10 mCi/ml stock) for 1 h. Virus-infected target cells were infected overnight with virus, before ^{51}Cr labeling; while peptide-treated targets were pulsed with peptide (final concentration 250 μg/ml) for 45 min after ^{51}Cr labeling. The targets were then washed and resuspended at 4×10^4/ml in RPMI 1640/FCS-10, and 4×10^3 target cells added to 96-well plates containing 50 μl effector cells in RPMI 1640/FCS-10. After a 3.5-h incubation, the cells were centrifuged, 15 μl supernatant spotted onto glass-fiber mats (Wallac, Milton-Keynes, UK), and the radioactivity released measured on a β-plate counter (Wallac). All assays were performed in triplicate. Target cells incubated with 5% Triton X (Merck, Poole, UK) or medium alone were used to determine maximal and spontaneous release. Percent specific lysis was calculated from the formula: $100 \times (E - M/D - M)$, where E = experimental release; M = release in the absence of CTL; D = maximal release following incubation in 5% Triton X. Spontaneous release averaged 9% in all assays and never exceeded 18%. Percent net ^{51}Cr release was calculated as the difference between percent specific ^{51}Cr release from peptide pulsed and unpulsed targets.

Flow Cytometric Analysis and Antibodies. Flow cytometric analysis of cell surface molecules was performed on a FACScan® 440 flow cytometer (Becton Dickinson & Co., Mountain View, CA). Single-color immunofluorescence was performed as described (27). For two-color analysis, cells were incubated with the first mAb at 4°C for 25 min, washed three times in PBS-1% FCS followed by goat anti–mouse Ig-FITC for 25 min at 4°C. The cells were washed three times and incubated with a murine IgG antibody, to block available goat anti–mouse Ig-FITC binding sites. The PE-labeled second antibody was added and after a 25-min incubation the cells were washed a final three times before fixing in a 2% paraformaldehyde solution.

The following mAb were used: CD8PE (Becton Dickinson & Co.), BMA031 (anti-TCR-α/β) (T Cell Sciences, Lab Impex, Middlesex, United Kingdom), C1 (anti-Vβ17) (28). Vβ17 (anti-Vβ19) (Immunotech International, The Binding Site, Birmingham, UK). The TCR V gene nomenclature used in this paper is based on Choi et al. (29). The commercial Immunotech anti-Vβ17 antibody uses a different nomenclature (30), and by the Choi nomenclature has specificity for Vβ19.

Anchored PCR. Anchored PCR was performed as previously described (12). 1–5 μg of total RNA was used from each sample and cDNA synthesized using a 5′ anchor primer. The PCR products were size selected on an acrylamide gel and cloned into a modified M13mp18 vector using NotI and SalI restriction sites. After transformation of *Escherichia coli*, plaques containing TCR inserts were sequenced using DNA T7 polymerase.

Sequencing of $V\beta17^+$ Clones. Total RNA was isolated from clonally derived cultures ($\sim 8 \times 10^5$ cells) using a standard RNA extraction kit (RNAzol B; Biogenesis, Bournemouth, United Kingdom). cDNA synthesis from mRNA was achieved using a poly-T oligonucleotide primer (oligo dT). Oligo-dT_{12-18} (Pharmacia, St. Albans, Hertfordshire, United Kingdom) was annealed to 1–5 μg 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 μl/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 Vβ17 and Cβ 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, Hybaid, Middlesex, UK). Purified products were dried and resuspended in 20–40 μl 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, Inc., 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/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 Vα (Vα10) and 3/4 clones used the same Vβ (Vβ17). Despite having been generated from unrelated individuals in different laboratories, CTL clones were identified which used identical α and β 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 Vβ17 was a frequently used M58-66 specific Vβ chain, we used the TCR Vβ17-specific monoclonal antibody C1 to determine the expansion of $V\beta17^+$ $CD8^+$ 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 $V\beta17^+$ cells in peripheral blood was observed. The mean percent ± SD Vβ17 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 restimulations with the peptide. The development of an

Donor JW			
ZE11	Vβ17	C A S S T R G A Y E Q Y F tgtgccagtagtacccgggggcctacgagcagtacttc	Jβ2.7
ZE11	Vα10.2	Y L C A G A T G N T G K L I F tacctctgtgcaggagctaccggcaacacaggcaaactaatcttt	Jα14.1
JWUB8	Vβ17	C A S S I **R S** S Y E Q Y F tgtgccagtagtatt**aggagc**agctacgagcagtacttc	Jβ2.7
	Vα10.2	Y L C A G G G S Q G N L I F tacctctgtgcaggaggaggaagccaaggaaatctcatcttt	Jα9.11
Donor JM			
JM22	Vβ17	C A S S S **R S** S Y E Q Y F tgtgccagtagttcg**aggagc**tcctacgagcagtacttc	Jβ2.7
	Vα10	C A G A G S Q G N L I F gtgcaggagcgggaagccaaggaaatctcatctttg	Jα9.11
JMc	Vβ4	C S V L Q G S P Y E Q Y F tgcagcgttttacagggaagcccctacgagcagtacttc	Jβ2.7
	Vα2.2	C A V N S Y Y N Q G G K L I F tgtgccgtgaacagctattataaccagggaggaaagcttatcttc	Jα14.2

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 "F-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.

M58-66–specific CTL response was invariably associated with an expansion of the $CD8^+$, TCR $V\beta17^+$ population in CTL lines (15–98%) as compared with freshly isolated PBMC (<7.5%) (Table 1 and Fig. 2), an effect not observed with antigen specific CTL lines of different peptide specificities derived from the same subjects (Table 1). The $CD8^+$, TCR $V\beta17^+$ expansion was particularly apparent in the "blast" population of responding PBMC, (as determined by forward angle and side scatter) which contained a higher proportion (between 17 and 92%) of $V\beta17^+$ $CD8^+$ cells (Table 1).

To determine whether the Vβ17 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 $V\beta17^+CD8^+/CD8^+$ population in excess of 5%. (*b*) CTL lines stimulated with endogenously expressing (M58-66 transfected) CIR.A2 cells also showed expansion of the TCR $V\beta17^+$ population, with a $V\beta17^+CD8^+/CD8^+$ 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 $V\beta17^+CD8^+/CD8^+$ 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 $V\beta17^+CD8^+/CD8^+$ 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%) $V\beta17^+$ $CD8^+$/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-A0201.1/M58-66–specific CTL Response after TCR Vβ17 PBMC 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 $V\beta17^+$ clone (data not shown). An alternative approach was to deplete this subset of cells before in vitro stimulation. We were unable to achieve TCR $V\beta17^-$ populations of sufficient purity with complement or magnetic bead depletion. Therefore TCR $V\beta17^+CD8^+$ 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 $V\beta17^+CD8^+$ 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

Table 1. *Expression of TCR Vβ17⁺ CD8⁺ Cells in PBMC and Peptide-specific Bulk Culture CTL Lines of Varying Duration*

Donors	Culture Duration	Percent net CTL lysis‡ (E/T 5:1)	Percent CD8⁺ TCR Vβ17⁺/total CD8*				
			PBMC	HLA-A2.1/M58-66–specific CTL line		Non-HLA-A2 flu§ peptide–specific CTL line	
				Total population	Blast population	Total population	Blast population
	d						
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.

10:1) with a 72% CD8⁺Vβ17⁺ population. After the Vβ17 depletion, two lines showed no specific lysis and no cell surface staining for TCR Vβ17. One line showed 20% lysis (E/T 10:1) and was found to have an 18.3% CD8⁺TCR Vβ17⁺ population, which must have been expanded from the residual population of such cells despite initial 96% depletion. The fourth line showed 35% lysis with no detectable CD8⁺ TCR Vβ17⁺ lymphocytes, implying use of a non-Vβ17TCR. Further studies on two different subjects found no non-Vβ17 HLA-A0201/M58-66–specific CTL response in one, and 1/4 non-Vβ17 CTL lines had specific killing in the third subject. To more accurately quantitate the effect of Vβ17 CD8 depletion, this study was repeated under limiting dilution conditions. Control depletion showed an HLA-A0201/M58-66 CTLp of 1/280,000 which became undetectable after Vβ17 depletion (data not shown).

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 CD8/total CD8	Percent net CTL lysis*	Percent Vβ17 CD8/total CD8	Percent net CTL lysis	Percent Vβ17 CD8/total CD8	Percent net CTL lysis
Cord						
Day 1	3.9		4		3.2	
Day 18	7	0	7	0	8	0
Mother						
Day 1			4.5		1.9	
	non-HLA-A2					
Day 18			18	37	27	34

* Percent net CTL lysis was calculated as the difference between percent specific ^{51}Cr 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 $CD8^+$ $V\beta17^+$ 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 $V\beta17^+$ Proportion of the HLA-A0201/M58-66-specific CTL Response. To quantitate the proportion of $CD8^+V\beta17^+$ 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.

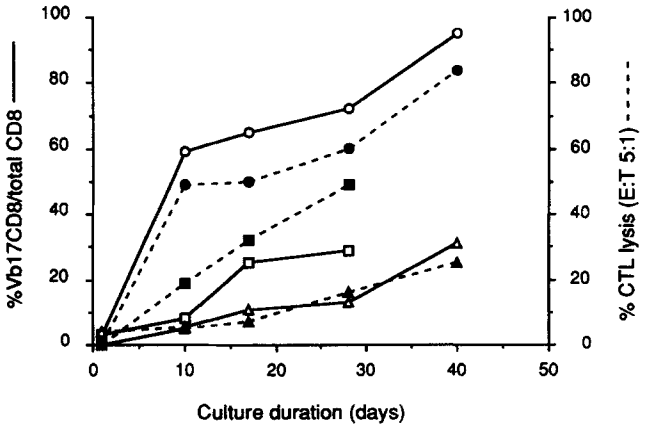

Figure 2. $V\beta17^+$ $CD8^+$ CTL expand after secondary in vitro stimulation and continued culture. The proportion of $V\beta17^+$ $CD8^+$ T cells (——), and peptide-specific cytotoxicity (- - - -) of high (—○—, - -●- -), medium (—□—, - -■- -) and poor (—△—, - -▲- -) responder bulk culture lines was measured after fresh PBMC were cultured in vitro with influenza A–infected cells and pulsed at weekly intervals with M58-66 peptide and feeder cells. The effector/target ratio was fixed at 5:1.

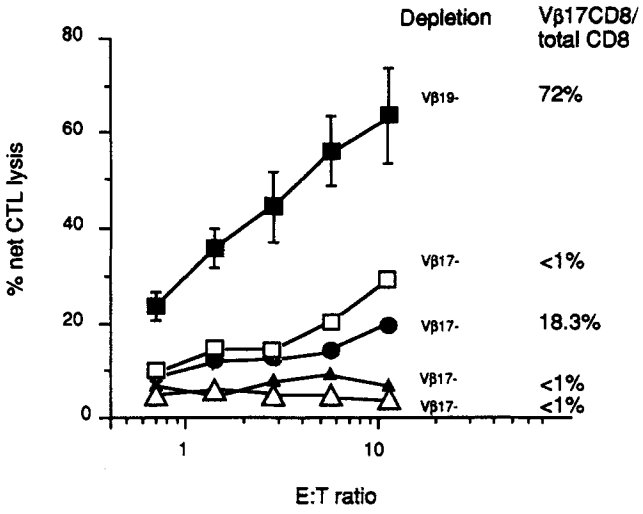

Figure 3. TCR $V\beta17^+$ $CD8^+$ depletion reduces M58-66 peptide-specific CTL generation but allows detection of $V\beta17^-$ CTL. TCR $V\beta17^+CD8^+$ and control (TCR $V\beta19^+CD8^+$) T cells were depleted from PBMC population by cell sorting. Remaining cells were stimulated with influenza A virus and plated in 96-well flat-bottomed plates at 1 × 10^6/well, to ensure HLA-A0201/M58-66 CTLp were present in the microculture (Table 3). A 96% $V\beta17^+CD8^+$ depletion of a-4.8% $V\beta17^+$ $CD8^+$ staining population was achieved. Net CTL lysis and the proportion of $V\beta17^+$ $CD8^+$ 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-ve. One line (—●—) showed 10% lysis but was found to have an 18.3% $V\beta17^+$ $CD8^+$ population, despite the initial 96% depletion. The remaining two Vβ17-depleted lines (—▲— —△—) showed <10% lysis, and contained <1% $V\beta17^+$ $CD8^+$ 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‡	Non-Vβ17 wells	CD8⁺ Vβ17⁺ wells	Non-Vβ17 wells
		%	%	%	%
DDC	1/54,000	5/9 (55.5)	4/9 (44.5)	0/4 (0)	4/4 (100)
SW	1/105,000	24/35 (68.5)	11/35 (31.4)	0/3 (0)	4/4 (100)
AF	1/170,000	14/20 (70)	7/20 (30)	2/38 (5.3)	36/38 (94.7)
PL	<1/250,000	9/12 (75)	3/12 (25)	0/2 (0)	2/2 (100)
JW§	1/215,000	23/32 (71.8)	9/32 (28.2)	1/22 (4.5)	21/22 (95.5)
JW (1)	1/275,000 (approximately)	7/9 (77.7)	2/9 (22.2)	0/4 (0)	4/4 (100)
JW (2)	–	8/9 (88.8)	1/9 (11.2)	3/34 (8.8)	31/34 (91.2)

* 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.
‡ 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.

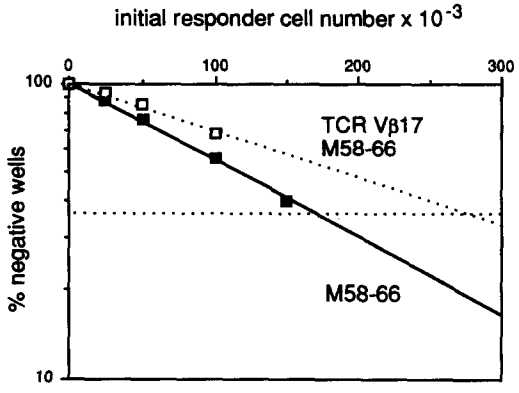

Figure 4. Estimation of frequency of total, and TCR Vβ17⁺ CTL precursors against the HLA-A0201/M58-66 complex. Fresh PBMC (from AF) were cocultured with 10% influenza-infected autologous PBMC and restimulated on day 7 with hIL-2 and peptide-pulsed B cells. On day 18 the cultures were assayed for cytotoxicity against uninfected and peptide-pulsed CIR.A2 cells as described. M58-66-specific wells indicative of single precursor cell origin were allowed to expand in culture for a further 5 d, and reexamined for cytotoxicity and CD8, Vβ17 dual staining. The CTL precursor frequency is estimated from the intercept of the regression line at 37% negative wells. M58-66-specific CTLp (1/170,000 PBMC, upper and lower 95% confidence intervals, calculated by the method of maximum likelihood: 1/236,673 and 1/130,197, χ^2 = 0.156), TCR Vβ17⁺ M58-66-specific CTLp (1/280,000 PBMC, upper and lower 95% confidence intervals: 1/194,000 and 1/410,000, χ^2 = 0.5).

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

Jβ2.7

JWZE11*	C A S S	T R G A Y E Q Y F
	tgtgccagtagt	acccgggggcctacgagcagtacttc
MPB6*	C A S S	S R S A Y E Q Y F
	tgtgccagtagt	agcaggtccgcctacgagcagtacttc
JM22*	C A S S	S R S S Y E Q Y F
	tgtgccagtagt	tcgaggagctcctacgagcagtacttc
JWUB8*	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	attaggagcagctacgagcagtacttc
MODG5	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	atccgcagctcctacgaacagtacttc
HLE18	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	atcaggagctcgtacgagcagtacttc
DDD4	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	atccggagctcctacgagcagtacttc
DDD8	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	attcggagctcctacgagcagtacttc
DDE1	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	atccggagctcgtacgagcagtacttc
NMH8	C A S S	I R S S Y E Q Y F
	tgtgccagtagt	ataaggtccagctacgagcagtacttc
MODG4	C A S S	M R S S Y E Q Y F
	tgtgccagtagt	atgaggtcatcctacgagcagtacttc
KEF9	C A S S	I R S A Y E Q Y F
	tgtgccagtagt	atcaggagcgcctacgagcagtacttc
DDE5	C A S S	M R S S Y E Q Y F
	tgtgccagtagt	atgcgatcgagctacgagcagtacttc
BDE8	C A S S	I R S A D E Q Y F
	tgtgccagtagt	atacggtccgccgacgagcagtacttt
JNJ1	C A S S	M R S S Y E Q Y F
	tgtgccagtagt	atgaggagctcctacgagcagtacttc
SWA13	C A S S	M R S G P E Q Y F
	tgtgccagtagt	atgaggtccgggcccgaacagtacttc
SWA10	C A S S	S R A A V E Q Y F
	tctgccagttcg	tctcgggcagcggtcgaqcaqtacttc

Jβ2.3

MPB3*	C A S S	P R S T D T Q Y F
	tgtgccagtagt	cctaggagcacagatacgcagtatttt
MPB7*	C A S S	T R S T D T Q Y F
	tgtgccagtagt	acaaggagcacagatacgcagtacttc
MPB8*	C A S S	S R S T D T Q Y F
	tgtgccagtagt	agtcggagcacagatacgcagtatttt
SWA20	C A S S	I R S S D T Q Y F
	tgtgccagtagt	atccggagttcagatacgcagtatttt
KDA24	C A S S	M R S T D T Q Y F
	tgtgccagtagt	atgaggagcacagatacacagtatttt
HLE19	C A S S	T R S T D T Q Y F
	tgtgccagtagt	acccgaagcacagatacgcagtatttt
HLE3	C A S S	G R S T D T Q Y F
	tgtgccagtagt	ggccggagtacagatacgcagtatttt
JN5K2	C A S S	M R S T D T Q Y F
	tgtgccagtagt	atgaggagcacagatacgcagtacttt
NMH12	C A S S	I H G A D T Q Y F
	tgtgccagtagt	attcatggtgcagatacgcagtatttt

Jβ2.2

MPB5*	C A S S	I R S S G E L F F
	tgtgccagtagt	ataagaagcagcggggagctgttttt
KEF11	C A S S	I R S D G E L F F
	tgtgccagtagt	atacggagcgacggggagctgttttt

Jβ2.1

CLH3	C A S S	I R S N G E Q F F
	tgtgccagtagt	atacgtagtaatggtgagcagtttttc
JNBJ12	C A S S	V S S Y N E Q F F
	tgtgccagtagt	gtcagctcctacaatgagcagttcttc

Jβ1.5

BDE20	C A S S	M R S S E Q H F
	tgtgccagtagt	atgcggagctccgagcagcacttt
JEC17	C A S S	Y Y R N Q P Q H F
	tgtgccagtagt	tattatagaaatcagccccagcatttt

Jβ1.1

MODG2	C A S S	T R S N T E A F F
	tgtgccagtagt	acccgctcgaacactgaagctttcttt

Jβ1.2

SWA3	C A S S	I G N Y G Y T F
	tgtgccagtagt	atcgggaactatggctacaccttc
SWA9	C A S S	Q G S Y G Y T F
	tgtgccagtagt	caggggagctatggctacaccttc
SWA18	C A S S	T G S Y G Y T F
	tgtgccagtagt	acaggatcctatggctacaccttc
MODG9	C A S S	T G S Y G Y T F
	tgtgccagtagt	acgggcagctatggctacaccttc
KDA1	C A S S	I G N Y G Y T F
	tgtgccagtagt	atcgggaactatggctacaccttc

Vβ17 non-killers

BDE11	C A S S	I V G T E A F F
	tgtgccagtagt	atagtcgggacagaagctttcttt
DPBK9	C A S S	V A L A T E A F F
	tgtgccagtagt	gtggccttggccactgaagctttcttt
CLH7	C A S S	S G S E T Q Y F
	tgtgccagtagt	agcgggagtgagacccagtacttc
NMH9	C A S S	M G G S Y E Q Y F
	tgtgccagtagt	atgggaggctcgtacgagcagtacttt

Figure 5. Nucleotide and predicted amino acid sequences of the Vβ17 β chains from anchored PCR-amplified independent (marked by asterisk), and Vβ17-amplified LDA-derived HLA-A0201/M58-66-specific CTL clones from 13 donors (see Materials and Methods for further details). Clones are listed by Jβ usage, each sequence runs from the conserved cysteine in the Vβ17 region to the first phenylalanine in the "F-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. Direct sequences obtained from non-matrix peptide-specific, Vβ17-expanded wells are shown for comparison. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers Z35670–Z35715.

(68%) of the TCR Vβ17 clones use either the Jβ2.7 or Jβ2.3 gene. Five different Jβ 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 Jβ2.3 and 2.7 clones, but was a single amino acid shorter in clones using the Jβ1.2 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 α and β 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 β 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 Vβ17 and adjacent D-J segments.

Vβ17 is the dominant Vβ segment in the CTL response to the HLA-A0201/M58-66 complex, as demonstrated by anchored PCR on isolated CTL clones, and the Vβ17 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 Vβ (Vβ17 8/9 sequences), Vα (Vα10 8/12), and Jα gene usage. In the present study, sequences of established long-term clones from two further individuals had direct chain pairing of Vβ17 and Vα10, Jα conservation and independent use of Vβ17 and Vα10. The lack of available Vα10 TCR mAb has precluded further studies of Vα usage in bulk culture CTL lines. However, Vβ17 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). Vα10.2 was used by two of these clones, for which no corresponding β chain was identified. In view of the predominant use of Vβ17 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 Vβ17$^+$ 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 minigene encoding M58-66. The Vβ17 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 Vβ17 preference, making it unlikely that the class I–restricting element alone is responsible for the Vβ17 preponderance (12).

We have used LDA to both quantitate the proportion of TCR Vβ17 in the M58-66–specific CTL response and generate M58-66–specific CTL clones for PCR amplification and sequencing. Analysis of five individuals showed Vβ17 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% Vβ17 usage in long-lived bulk culture lines and the 15–45% "non-Vβ17" 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-2K^d–restricted HLA-CW3 170–179 antigen has shown a marked TCR preference for Vβ10 and a unique Jα 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 Vβ10 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 Vβ10$^+$ 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 Vβ17 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 Vβ17 gene segment, several features of the CDR3 region conservation are of note. There appears to be relatively little conservation of the Jβ segment, and although Jβ2.3 and Jβ2.7 were used in 65% of all transcripts, these gene segments are amongst the most commonly used in the TCR-β 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 Jβ1.2 gene segment. There are several examples of conservation of this residue in peptide-specific responses and in the study of TCR usage in H-2K^d–restricted CTL recognizing an HLA-CW3–derived peptide, a glycine was found at this position in all TCR-β 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 GLLGFVGTL. 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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