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Cross-Recognition of Two Middle T Protein Epitopes by Immunodominant Polyoma Virus-Specific CTL

Christopher S. Wilson,* Janice M. Moser,* John D. Altman,† Peter E. Jensen,* and Aron E. Lukacher2*

We recently identified the immunodominant epitope for polyoma virus-specific CTL as the Dk-associated peptide MT389–397 derived from the middle T (MT) viral oncoprotein. Another Dk-restricted peptide corresponding to residues 236–244 of MT was recognized by nearly all MT389–397-reactive CTL clones, but required concentrations at least 2 logs higher to sensitize syngeneic target cells for lysis. Except for identity at the three putative Dk-peptide anchor residues, MT236–244 shares no homology with MT389–397. Using a novel europium-based class I MHC-peptide binding immunoassay, we determined that MT236–244 bound Dk 2–3 logs less well than MT389–397. Infected with a mutant polyoma virus whose MT is truncated just before the MT389–397 epitope or immunization with MT389–397 or MT236–244 peptides elicited CTL that recognized both MT389–397 and MT236–244. Importantly, infection with a polyoma virus lacking MT389–397 and mutated at an MT236–244 Dk anchor position induced polyoma virus-specific CTL recognizing neither MT389–397 nor MT236–244 epitopes. Despite predominant usage of the Vβ6 gene segment, MT389–397/MT236–244 cross-reactive CTL clones possess diverse complementarity-determining region 3β domains; this is functionally reflected in their heterogeneous recognition patterns of alanine-monosubstituted MT389–397 peptides. Using Dk/MT389–397 tetramers, we directly visualized MT236–244 peptide-induced TCR down-modulation of virtually all MT389–397-specific CD8+ T cells freshly explanted from polyoma-infected mice, suggesting that a single TCR recognizes both Dk-restricted epitopes. The availability of immunodominant epitope-specific CTL capable of recognizing a second epitope in MT, a viral protein essential for tumorigenesis, may serve to amplify the CTL response to the immunodominant epitope and prevent the emergence of immunodominant epitope-loss viruses and virus-induced tumors. The Journal of Immunology, 1999, 162: 3933–3941.

CD8+ CTL are critical components of the adaptive immune response to virus infections. The ligands for Ag receptors on antiviral CD8+ T cells (TCRs) are complexes of class I MHC molecules and 8- to 10-amino acid peptides that are usually derived from endogenously synthesized viral proteins (1, 2). The ligand specificity of the TCR is determined by the composition of its heterodimeric αβ glycoprotein chains. Each of these chains is created by recombining gene segments encoding variable (V), diversity (D; for β-chains), joining (J), and constant (C) elements. The highest level of sequence diversity in the TCR resides in its complementarity-determining region 3 (CDR3), the junctional domain created by rearrangement of individual V, D, and J elements as well as by the introduction of random, template-independent, N-nucleotides (3, 4). The recently solved crystal structures of TCR bound to class I MHC/peptide ligands confirm predictions that CDR3 amino acids primarily make contact with the antigenic peptide, but also indicate interactions between the peptide and CDR1 and CDR2 regions of the Vα and Vβ genes (5, 6).

CD8+ CTL typically express clonally distributed TCRs that possess exquisite specificity for MHC/peptide ligands. A number of recent studies, however, have documented degenerate recognition of MHC/peptide complexes by individual TCRs expressed by class I MHC- and class II MHC-restricted T cells; examples range from T cell recognition of dissimilar peptides by the same MHC molecules to recognition of identical peptides bound to different MHC molecules (7–12). Perhaps the best illustration of degenerate peptide/MHC recognition by single TCRs takes place during positive selection in the thymus, where the interaction of individual TCRs with broad arrays of MHC-bound self-peptides is required for shaping a diverse T cell repertoire (13–18). Homology in sequence or structure between self-T cell epitopes and those of infectious organisms, referred to as molecular mimicry, has been proposed as a mechanism for triggering autoimmunity (19–22). Allen and co-workers recently demonstrated that two structurally dissimilar class II MHC-restricted epitopes within a single protein were cross-reactive at the single T cell level, a phenomenon they termed intramolecular mimicry (23). Whether intramolecular epitope mimicry also occurs among class I MHC-restricted T cells has not been determined.

T cells are essential for conferring protection against tumors induced by the murine Papovavirus, polyoma virus. We recently reported that deletion of polyoma virus-specific CD8+ CTL bearing Vβ6 TCRs by the endogenous Mtv-7-encoded superantigen renders H-2b mice highly susceptible to polyoma virus-induced tumors (24). In resistant H-2k mice, this CTL response is heavily dominated by CTL directed to a Dk-restricted nine amino acid epitope, MT389–397, derived from the viral MT protein (25). Tumor-susceptible Mtv-7+ , H-2k mice possess an approximately 20-fold lower frequency of CTL precursors specific for the MT389–397 epitope compared with syngeneic resistant mice. MT is a 421-amino acid type
II integral membrane protein that associates with and activates an array of host cell enzymes, including Src family tyrosine kinases, phosphatidylinositol 3-kinase, protein phosphatase 2A, and phospholipase C-γ, and interacts with the Hsc adaptors proteins and proteins of the 14-3-3 family (reviewed in Ref. 26). MT is necessary and sufficient for cellular transformation, and wild-type MT is essential for tumor induction and efficient virion assembly (27–29). Given its constitutive expression by cells infected and transformed by polyoma virus, MT is an optimal target protein for CTL-mediated protection against polyoma oncogenesis.

Here, we describe intramolecular mimicry between MT₃₈₉–₃₉₇ and another D<sup>+</sup>-restricted MT epitope, MT₂₃₆–₂₄₄, by polyoma virus-specific CD₈<sup>+</sup> CTL. Despite the lack of sequence homology, other than the three predicted MHC anchor positions, the MT₂₃₆–₂₄₄ epitope is recognized by virtually all MT₃₈₉–₃₉₇-specific CTL clones and MT₃₈₉–₃₉₇-specific splenic CD₈<sup>+</sup> T cells from acutely infected mice. We show at the level of CTL clones and primary effector CD₈<sup>+</sup> T cells that a single TCR recognizes both epitopes, and furthermore, that this cross-reactivity is mediated by polyoma virus-specific CTL bearing diverse TCRs. The potential contributions of such intramolecular mimicry to antiviral and antitumor immunity by CD₈<sup>+</sup> CTL are discussed.

**Materials and Methods**

**Animals**

C57Bl/6Jcd and C3H/HeSn were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeNcr and C3H/BiDcR mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD).

**Viruses and virus inoculation**

The wild-type polyoma virus strain A2 and the mutant polyoma virus strain PTA1387T were molecularly cloned and plaque purified, and virus stocks were prepared using primary baby mouse kidney cells as previously described (25). The polyoma virus strain PTA1387T encodes an MT protein lacking the carboxyterminal 37 amino acids (30). The PTA1387T/MT237RH mutation was introduced using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions, with the following primers: CAGTCACCGCCTAAGACTG (forward) and CAGTCTTAGGCGGTTC (reverse) and another D<sup>+</sup>-restricted MT epitope, MT₂₃₆–₂₄₄, by polyoma virus-infected, irradiated, syngeneic splenocytes. T cells were cloned by limiting dilution from day 7 in vitro secondary or tertiary cultures. T cell lymph node cells at approximately 2 wk postinfection were cocultured with infected L929 cells to a ratio of 1:1, and supernatants were removed and counted in a 1470 Wallac gamma counter (Turku, Finland).

For cold target inhibition assays, polyoma-labeled L929 cells were pulsed with MT₃₈₉–₃₉₇ peptide at a concentration of 0.1 μM for 1.5 h at 37°C, washed extensively to remove unbound peptide, and aliquoted at 5000 cells/well into flat-bottom 96-well microtiter plates (Costar). Unlabeled competitor L929 cells pulsed with MT₃₈₉–₃₉₇, MT₂₃₆–₂₄₄ or Flu NP₅₀–₅₇ peptides at 100 μM each were added at ratios of 1:1, 3:1, or 5:1 with respect to the labeled targets. CTL clones were then added at an E:T cell ratio of 5:1, plates were incubated for 4 h at 37°C, and supernatants were counted.

**Spontaneous ⁵¹Cr release from target cells in all assays was 10–20% of the total detergent lysis. The percent specific lysis was calculated as follows: [(% ⁵¹Cr release with effector cells − spontaneous ⁵¹Cr release)/total ⁵¹Cr release with 1% Triton X-100 − spontaneous ⁵¹Cr release)] × 100.** The percent specific lysis values represent the mean values of quadruplicate wells. SEMs were always <5% of the mean values and are omitted.

**Europium fluoroimmunoassay for peptide binding to purified D<sup>k</sup> molecules**

As described in detail previously (31), purified D<sup>k</sup> was incubated with a biotinylated MT₃₈₉–₃₉₇ peptide at a concentration of 0.1 μM and various concentrations of unlabeled competitor peptides. D<sup>k</sup>-biotin-MT₃₈₉–₃₉₇ complexes were captured in wells coated with 16-1-2N mAb, incubated with europium-labeled streptavidin, and quantified by time-resolved fluorescence. The data points represent the mean fluorescent counts per second/1000 (counts per second × 10<sup>−3</sup>) of duplicate or triplicate samples.

**RNA extraction, cDNA synthesis, PCR, and direct sequencing of PCR products**

Viable CTL clones at 6–8 days after Ag restimulation were isolated on LSM (Organon Teknika, Durham, NC) step gradients. Cytoplasmic RNA was extracted from 1–3 × 10<sup>⁶</sup> T cells with TRIzol reagent (Life Technologies, Gaithersburg, MD), and cDNA was synthesized from approximately 1 μg of total RNA with the Superscript Preamplification System (Life Technologies). PCR amplification was conducted on 1 μl of cDNA with a V<sub>b</sub>-specific sense primer and consensus C<sub>b</sub> antisense primer or a V<sub>a</sub>-specific sense primer and consensus C<sub>a</sub> antisense primer, using conditions described previously (32). The following primers (listed 5′ to 3′) were used for PCR amplification and sequence determination: V<sub>b</sub>5, CTCTCAGTCTGACATCTGCC; C<sub>b</sub>-external, CCAGAAGGTAGCAGAGACC; C<sub>a</sub>-external, CTTGGGTTGAGATCGACATTTCTC CATTTCTC. PCR products were gel purified and directly sequenced with the C<sub>b</sub> antisense primer and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) according to the manufacturer’s instructions. Extension products were passed through Micro Bio-Spin 30 chromatography columns (Bio-Rad, Hercules, CA) to remove unincorporated dideoxy terminators, and the samples were spun to dryness in a vacuum centrifuge. Samples were subsequently sequenced on the ABI Prism 377 DNA Sequencer (Perkin Elmer). TCR sequencing for each CTL clone was performed on the products of three independent PCR reactions.

**Preparation of H-2<sup>D</sup><sup>+</sup> tetramers**

Full-length D<sup>+</sup> cDNA was PCR amplified from cDNA prepared from L929 cells using the 5′ primer CGGCTCGGAGATGGGGGCGATGGTACCA and the 3′ primer GCTCTAGACATCTGACCCGTGACACCATATT and digested with XhoI and XhoI (sites underlined, respectively). DNA encoding the soluble domain of H-2<sup>D</sup>, residues 1–274, was then PCR amplified using the 5′ primer GGAATTCCATATGGGACCACATTCACTAAG and the 3′ primer ATATTTTCGAGACCGTCGTG and various concentrations of unlabeled competitor peptides. D<sup>k</sup>-biotin-MT₃₈₉–₃₉₇ complexes were captured in wells coated with 16-1-2N mAb, incubated with europium-labeled streptavidin, and quantified by time-resolved fluorescence. The data points represent the mean fluorescent counts per second/1000 (counts per second × 10<sup>−3</sup>) of duplicate or triplicate samples.

**Isolation of polyoma virus-specific CTL**

Protocols for establishing polyoma-specific T cell clones and lines were described in detail previously (24). Briefly, draining popliteal and inguinal lymph node cells at approximately 2 wk postinfection were cocultured with virus-infected, irradiated, syngeneic splenocytes. T cells were clones by limiting dilution from day 7 in vitro secondary or tertiary cultures. T cell lines were maintained by weekly restimulation with virus-infected, irradiated, syngeneic splenocytes.

**³¹Cr release assay**

Polyoma virus-infected AG104A and peptide-pulsed AG104A and L929 ⁵¹Cr-labeled target cells were prepared as previously described (25). ⁵¹Cr-labeled target cells were aliquoted at 5000 cells/well into either V- or Untransfected 96-well microtiter plates (Costar, Cambridge, MA). For experiments using virus-infected or peptide-pulsed targets, 100 μl of target cells and 100 μl of T cells were cocultured in each well. In certain experiments, 50 μl of peptides were added at 3 times their final concentration to wells containing 50 μl of ⁵¹Cr-labeled cells, then 1-h incubation at 37°C, 50 μl of T cells were added. The assay medium was Iscove’s modified Dulbecco’s medium and 10% FBS. After a 4-h incubation at 37°C, half the volume of each well was removed and counted in a 1470 Wallac gamma counter (Turku, Finland).

For cold target inhibition assays, polyoma-labeled L929 cells were pulsed with MT₃₈₉–₃₉₇ peptide at a concentration of 0.1 μM for 1.5 h at 37°C, washed extensively to remove unbound peptide, and aliquoted at 5000 cells/well into flat-bottom 96-well microtiter plates (Costar). Unlabeled competitor L929 cells pulsed with MT₃₈₉–₃₉₇, MT₂₃₆–₂₄₄ or Flu NP₅₀–₅₇ peptides at 100 μM each were added at ratios of 1:1, 3:1, or 5:1 with respect to the labeled targets. CTL clones were then added at an E:T cell ratio of 5:1, plates were incubated for 4 h at 37°C, and supernatants were counted.

**Spontaneous ⁵¹Cr release from target cells in all assays was 10–20% of the total detergent lysis. The percent specific lysis was calculated as follows: [(% ⁵¹Cr release with effector cells − spontaneous ⁵¹Cr release)/total ⁵¹Cr release with 1% Triton X-100 − spontaneous ⁵¹Cr release)] × 100.** The percent specific lysis values represent the mean values of quadruplicate wells. SEMs were always <5% of the mean values and are omitted.
IgG1 isotype control Ab (PharMingen; data not shown). No intracellular staining was seen with FITC-conjugated rat anti-mouse CD8 (Caltag, South San Francisco, CA) and allophycocyanin-conjugated rat anti-polyoma virus CTL clones 8-1 and 24-5 were assayed for cytolytic activity against 51Cr-labeled AG104A (H-2k) cells for 4 h at an E:T cell ratio of 5:1 in the presence of the indicated concentration of peptide. The gag88–96 is a Dk-restricted epitope for CTL recognizing an endogenous retrovirus (35), and the other four peptides correspond to MT sequences satisfying a predicted Dk peptide binding motif (25). B, 51Cr-labeled AG104A cells pulsed with MT389–397 or MT236–244 at the indicated peptide concentrations were assayed for lysis by CTL clones 8-1 and 24-5 at an E:T cell ratio of 3:1 in a 4-h 51Cr release assay.

BL21 (DE3) was transformed with the pET23-Dk-BSP plasmid, and expression of Dk was induced with isopropyl β-D-thiogalactoside (IPTG). Human β2m was expressed in the same cell line using the pHN1-β2m plasmid (33). The folding reaction was performed with the MT389–397 peptide. Folding, purification, and biotinylation were performed as previously described (34). Tetramers were made by mixing biotinylated Dk/MT389–397 monomers with allophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR) in a 4:1 molar ratio.

Flow cytometry
Single cell suspensions of spleen were prepared, erythrocytes were lysed (RBC lysing buffer, Sigma, St. Louis, MO), and 1 × 10^6 cells were stained in phenol red-free RPMI 1640 (Life Technologies) containing 2% FBS and 0.01% sodium azide (FACS buffer) for 1 h at 4°C, followed by three washes in FACS buffer and fixation in PBS containing 1% paraformaldehyde. Samples were acquired on a FACSCalibur (Becton Dickinson, San Jose, CA), and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Intracellular IFN-γ staining
RBC-lysed spleen cells were cultured for 7 h in 96-well flat-bottom microtiter plates (Costar) at 1 × 10^6 cells/well in 0.2 ml/well Iscove’s modified Dulbecco’s medium (Life Technologies) containing 10% FBS, 50 μM 2-ME, and penicillin/streptomycin and supplemented with 1 μl/ml brefeldin A (Golgiplug, PharMingen, San Diego, CA), 50 μ/ml human rIL-2 (PharMingen), and synthetic peptides at the indicated concentrations. Cells were then surface-stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8α Ab (Caltag, South San Francisco, CA) and allophycocyanin-conjugated Dk/MT389 tetramers. After washing, cells were permeabilized and stained for intracellular IFN-γ with FITC-conjugated monoclonal rat anti-mouse IFN-γ antibody (clone XMG1.2; PharMingen) using the Cytofix/Cytoperm kit according to the manufacturer’s instructions (PharMingen). No intracellular staining was seen with FITC-conjugated rat IgG1 isotype control Ab (PharMingen; data not shown).

Results
Cross-recognition of two nonoverlapping MT epitopes by anti-polyoma CTL
We previously reported that H-2k mice resistant to tumors induced by polyoma virus mount a strong immunodominant CTL response to the Dk-restricted MT389–397 epitope derived from the MT protein of the virus (25). In the course of screening four potential Dk-binding peptides derived from polyoma early region proteins as candidate epitopes for anti-polyoma CTL cloned lines, another MT-derived peptide, MT236–244, was found to sensitize syngeneic target cells for lysis by nearly all the MT389–397-reactive CTL clones. This cross-reactivity, however, was revealed only at peptide concentrations approximately 2 logs higher than that of the immunodominant MT389–397 peptide. Fig. 1 confirms and extends these observations using two representative polyoma-virus specific CTL clones, 8-1 and 24-5. Each of the four MT peptides, selected on the basis of a predicted consensus motif for Dk-binding peptides (8–9 mer peptides with P2 basic, P5 basic, carboxyl-terminal leucine) (25), bound Dk (Fig. 2). Except for identity at each of the three putative Dk anchor residues, MT389–397 and MT236–244 share no conserved amino acids.

Because MT389–397 is able to sensitize target cells for anti-polyoma CTL recognition at significantly lower concentrations than MT236–244, we asked whether these peptides bind the Dk molecule with different affinities. A fluorimmunoassay using europium-streptavidin and time-resolved fluorometry was developed to measure binding of biotinylated peptides to purified Dk molecules (31). We determined that the synthetic peptide MT102–109, biotinylated at its natural cysteine at P4, specifically bound to Dk. Purified Dk molecules were incubated with biotinylated MT102–109 and the concentration-dependent competition of unlabeled peptides with MT102–109 for binding to Dk was determined. As shown in Fig. 2, the immunodominant anti-polyoma CTL peptide, MT389–397, had an approximately 10-fold higher affinity for Dk than gag88–96, a Dk-restricted epitope for CTL recognizing an endogenous retrovirus (35). Flu NP50–57, a Kk-restricted epitope (36), did not compete with MT102–109 for binding to Dk. T6–14 also bound Dk, but at roughly 1 log lower affinity than gag88–96. Notably, the cross-reactive MT236–244 peptide bound to Dk with an affinity 2–3 logs lower than that of MT389–397. This affinity difference parallels the difference in minimum concentrations of MT389–397 and MT236–244 needed to sensitize target cells for lysis by MT389–397-specific CTL clones (Fig. 1B) (25). Because MT389–397 and MT236–244 peptides are of equal size and have identical residues at the three putative primary anchors, secondary
MT_{236-244} primes MT_{389-397}-reactive CTL

Since MT_{236-244} is recognized in vitro by anti-polyoma CTL clones specific for the immunodominant MT_{389-397} epitope, we wanted to determine whether MT_{236-244} plays a functional role in shaping the anti-polyoma immune response in vivo. As a first step to determine whether the MT_{236-244} epitope was immunogenic for MT_{389-397}-specific CTL, we attempted to prime for CTL reactive to MT_{389-397} by immunization with the MT_{236-244} peptide. Because peptide immunogenicity for class I MHC-restricted T cell responses can be augmented by coinjection with a class II MHC-restricted T helper epitope (38–40), the MT_{236-244} and MT_{389-397} synthetic peptides were each emulsified in IFA with the IA^{b}-restricted T helper epitope HEL_{48-62} and injected s.c. into C3H/HeN mice. T cell lines were independently established from the draining lymph nodes of six mice by in vitro restimulation with wild-type virus-infected syngeneic stimulators. As shown in Fig. 3, priming with either MT_{236-244} or MT_{389-397} peptide elicited polyoma virus-specific CTL that recognized target cells pulsed with either peptide, but not the gag_{88-96} peptide. No lysis of infected or peptide-pulsed targets was seen by draining lymph node cells from mice injected s.c. with IFA and restimulated in vitro with virus-infected stimulators (data not shown).

To determine whether the MT_{236-244} epitope presented during polyoma virus infection induced MT_{389-397}-reactive CTL, we immunized mice with the mutant polyoma virus PTA1387T, which encodes a stop codon at residue 384 of the MT protein (30) and, consequently, lacks the MT_{389-397} epitope. As shown in Fig. 4A, a T cell line established from PTA1387T virus-immune mice by in vitro selection with wild-type virus-infected syngeneic stimulators efficiently lysed not only wild-type virus-infected targets, but, notably, recognized target cells coated with either MT_{389-397} or MT_{236-244}. In vitro selection of the same PTA1387T-immune T cells with PTA1387T virus-infected stimulators generated a T cell line that recognized wild-type virus-infected targets, but not target cells coated by MT_{389-397} or MT_{236-244} peptides. To directly demonstrate that the MT_{236-244} epitope is presented in polyoma-infected mice and is responsible for priming MT_{389-397}-reactive CTL, we replaced histidine for arginine at residue 237 of the MT protein of PTA1387T virus, with the intention of disrupting a primary D^k anchor in MT_{236-244}. A T cell line established from a mouse immunized with this mutant virus, designated PTA1387T.237RH, by in vitro restimulation with wild-type virus-infected syngeneic spleen cells specifically lysed wild-type virus-infected target cells, but did not recognize target cells coated with either MT_{389-397} or MT_{236-244} peptide; a T cell line generated under the same in vitro selection conditions, but from a mouse inoculated with the parental PTA1387T virus, recognized both peptides (Fig. 4B). Taken together, these findings indicate that the MT_{236-244} epitope is presented in vivo during the course of polyoma virus infection, and that it primes but is unable to drive expansion of MT_{389-397} CTL.

MT_{389-397}-specific CTL possess heterogeneous CDR3\(\beta\) regions and fine specificity

Because of the strong preferential expression of the V\(\beta\)6 gene segment by polyoma-specific CTL that cross-react with MT_{389-397} and MT_{236-244} (24, 25), we asked whether their CDR3\(\beta\) domains possessed shared structural features. cDNA was prepared from 11 V\(\beta\)6\(^+\) MT_{389-397}-specific CTL clones isolated from 10 different H-2^d, Mtv7-negative mice infected by wild-type polyoma virus. Clones 6-6, 8-1, 9-12, 10-11, and 11-1 were derived by independent limiting dilution analyses from five C57BR/cdJ mice, and the other clones were isolated by limiting dilution analyses from five individual C3H/HeSnJ mice (25) (data not shown). Of these MT_{389-397}-specific CTL clones, only 14-1 lacks cross-reactivity for MT_{236-244} (25). As shown in Table I, the CDR3\(\beta\) regions of

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Peptide binding to H-2^D. Purified D^k was incubated with 1 \(\mu\)M biotinylated MT_{102-109} peptide in the presence or the absence of unlabeled competitor peptides over the indicated concentration ranges. D^k-biotin-peptide complexes were captured on assay plates coated with an anti-H-2^D mAb and incubated with europium-labeled streptavidin, and % specific Iodine by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/}

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Immunization with either MT_{389-397} or MT_{236-244} peptides elicits polyoma virus-specific CTL that recognize both peptides. T cell lines established from mice coimmunized with IFA-emulsified MT_{389-397} and the IA^b T helper epitope HEL_{48-62} or IFA-emulsified MT_{236-244} and HEL_{48-62} were tested for cytolytic activity against uninfected, virally infected, or peptide-pulsed AG104A target cells at an E:T cell ratio of 10:1 in a 4-h \(^{51}\)Cr release assay.
the MT 389 –397 -specific CTL clones are heterogeneous in both length and sequence. CDR3β lengths range from six to nine amino acids and eight of 12 possible Jβ segments are used. Within this diversity, four clones (6-6, 9-12, 10-11, and 16-3) share the CDR3β sequence glutamine-glycine-alanine, situated at position 96 for CDR3β regions seven amino acids in length (clones 6-6 and 16-3) and at position 98 for nine-amino acid-long CDR3βs (clones 9-12 and 10-11). The nongermline-encoded glycine at position 97 is frequently found among class I MHC-restricted TCRs of different specificities and presumably confers flexibility to the CDR3β loop (41, 42). Of these 11 Vβ61 CTL clones, 9-12 and 10-11 exhibit CDR3β regions with the highest sequence similarity, differing in only two of nine amino acids. It is interesting to note that 14-1, the only MT389–397-specific CTL clone isolated to date that fails to recognize MT 236 –244, has the shortest CDR3β length of the 11 Vβ6+ clones sequenced.

In view of their diverse CDR3βs, we investigated the fine specificities of each of the MT 389 –397 -specific CTL clones listed in Table I. Clonotypic differences in TCR interactions with Dk/MT 389 –397 complexes were suggested by our previous finding that each CTL clone exhibited distinct patterns of reactivity to truncated versions of the MT389–397 epitope (25). A TCR functional fingerprint for each clone was revealed by its capacity to differentially recognize target cells presenting each of a panel of MT389–397 peptides bearing consecutive single alanine substitutions. For any given alanine-substituted MT 389 –397 peptide analogue, at least one of the CTL clones was capable of recognizing it (Fig. 5 and data not shown), indicating that each substituted peptide bound Dk. As determined by the Dk europium fluorimunoassay, each of the nine alanine-substituted peptides bound Dk with an affinity similar to that of MT 389–397 (data not shown). With three primary anchor residues in the predicted motif for peptides binding to Dk, alanine substitution of a single primary anchor residue is apparently insufficient to affect peptide binding.

Table I. CDR3β sequences of Vβ6+ MT 389–397-specific CTL clones*

<table>
<thead>
<tr>
<th>CTL Clone</th>
<th>CDR3</th>
<th>Jβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-6</td>
<td>S Q G A T E V</td>
<td>1.1</td>
</tr>
<tr>
<td>8-1</td>
<td>S M G N T G Q L</td>
<td>2.2</td>
</tr>
<tr>
<td>9-12</td>
<td>S M G Q G A E T L</td>
<td>2.3</td>
</tr>
<tr>
<td>10-11</td>
<td>S I G Q G A Q T L</td>
<td>2.3</td>
</tr>
<tr>
<td>11-1</td>
<td>S I G T G R Y E Q</td>
<td>2.6</td>
</tr>
<tr>
<td>12-2</td>
<td>S M G A G N T L</td>
<td>1.3</td>
</tr>
<tr>
<td>14-1</td>
<td>S M G G E Q</td>
<td>2.6</td>
</tr>
<tr>
<td>16-3</td>
<td>S Q G A G Q L</td>
<td>2.2</td>
</tr>
<tr>
<td>24-1</td>
<td>S T G T G A E Q</td>
<td>2.6</td>
</tr>
<tr>
<td>24-5</td>
<td>S M G G L D T Q</td>
<td>2.5</td>
</tr>
<tr>
<td>25-2</td>
<td>S M G Q N T L</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* cDNA prepared from each CTL cloned line was PCR-amplified using a Vβ6 primer and a Cβ antisense primer, and PCR products were directly sequenced using an internal Cβ primer. The amino acid sequences of the CDR3β regions begin at amino acid 95 according to the TCR numbering system proposed by Chothia et al. (61).
but not those pulsed with the K\textsuperscript{k}-binding Flu NP\textsuperscript{50–57} peptide, inhibited MT\textsuperscript{389–397} specificity by 8-1 and 24-5 in the absence of cold targets. This result argues that the MT\textsuperscript{389–397} peptide induces D\textsuperscript{k} tetramer down-modulation of CD8\textsuperscript{+} T cells that stimulated IFN-\gamma production coincides with the MT\textsuperscript{389–397} peptide doses that stimulated IFN-\gamma production by the CD8\textsuperscript{+} T cells. As shown in Fig. 7, the absence of peptide or the presence of gag\textsubscript{88–96} peptide, approximately 17% of the CD8\textsuperscript{+} T cells bound the D\textsuperscript{k}/MT389 tetramer. The MT\textsuperscript{389–397} peptide induced a dose-dependent reduction of tetramer staining, with TCR down-modulation being just detectable at 10\textsuperscript{−8} M and maximal by 10\textsuperscript{−6} M. This titration curve for MT\textsuperscript{389–397} peptide-triggered down-modulation of tetramer staining coincides with the MT\textsuperscript{389–397} peptide doses that stimulated IFN-\gamma production by the CD8\textsuperscript{+} T cells. In a parallel fashion, MT\textsuperscript{236–244} also induced D\textsuperscript{k}/MT389 tetramer down-modulation of CD8\textsuperscript{+} T cells and IFN-\gamma production, but at 2–3 logs higher peptide concentrations than MT\textsuperscript{389–397}. Seven days after s.c. inoculation with wild-type polyoma virus, the peak of the MT\textsuperscript{389–397} and MT\textsuperscript{236–244} peptide responses were cultured for 7 h in the presence of graded concentrations of MT\textsuperscript{389–397} and MT\textsuperscript{236–244} or gag\textsubscript{88–96} Peptides. Cells were then surface stained with the D\textsuperscript{k}/MT389 tetramer and anti-CD8 Ab, and stained for intracellular IFN-\gamma. As shown in Fig. 6, in the absence of peptide or the presence of gag\textsubscript{88–96} peptide, approximately 17% of the CD8\textsuperscript{+} T cells bound the D\textsuperscript{k}/MT389 tetramer. The MT\textsuperscript{389–397} peptide induced a dose-dependent reduction of tetramer staining, with TCR down-modulation being just detectable at 10\textsuperscript{−8} M and maximal by 10\textsuperscript{−6} M. This titration curve for MT\textsuperscript{389–397} peptide-triggered down-modulation of tetramer staining coincides with the MT\textsuperscript{389–397} peptide doses that stimulated IFN-\gamma production by the CD8\textsuperscript{+} T cells. In a parallel fashion, MT\textsuperscript{236–244} also induced D\textsuperscript{k}/MT389 tetramer down-modulation of CD8\textsuperscript{+} T cells and IFN-\gamma production, but at 2–3 logs higher peptide concentrations than MT\textsuperscript{389–397}. The maximum TCR down-modulation induced by MT\textsuperscript{389–397} and MT\textsuperscript{236–244}, the mean fluorescence intensity for D\textsuperscript{k}/MT389 tetramer staining of the CD8\textsuperscript{+} T cells decreased approximately sevenfold from that of CD8\textsuperscript{+} T cells incubated in the absence of peptide. This difference in peptide doses between MT\textsuperscript{389–397} and MT\textsuperscript{236–244} for triggering TCR down-modulation and IFN-\gamma production by CD8\textsuperscript{+} T cells taken directly ex vivo matched that required to sensitize target cells for lysis by MT\textsuperscript{389–397} specific CTL clones (25) (Fig. 1A). The finding that the MT\textsuperscript{236–244} peptide triggered TCR down-regulation by virtually all the MT\textsuperscript{389–397} specific CD8\textsuperscript{+} T cells in the spleens of acutely infected mice strongly argues that a single TCR recognizes both
MT epitopes and accounts for the low frequency of MT\textsubscript{389–397} specific CTL clones that do not cross-react with MT\textsubscript{236–244}.

**Discussion**

In this report, we show that polyoma virus-specific CD8\textsuperscript{+} CTL directed to the immunodominant epitope in the MT viral oncprotein cross-react with another, nonoverlapping MT-derived epitope presented by the same class I MHC molecule. Immunization with either the dominant MT\textsubscript{389–397} peptide or the cross-reactive MT\textsubscript{236–244} peptide or infection with a mutant polyoma virus lacking the MT\textsubscript{389–397} epitope elicits polyoma virus-specific CTL that recognize both epitopes. Using soluble class I MHC tetramers containing the MT\textsubscript{389–397} peptide, we directly visualized MT\textsubscript{236–244}-peptide-induced TCR down-modulation of virtually all D\textsuperscript{α}/MT389 tetramer\textsuperscript{+} CD8\textsuperscript{+} T cells from spleens of polyoma-infected mice. Moreover, we show that cross-reactivity for these two MT epitopes is mediated by TCRs with highly heterogeneous CDR3\textbeta{} regions. Thus, individual, diverse TCRs are responsible for class I MHC epitope intramolecular mimicry by these immunodominant anti-polyoma CTL.

Degeneracy in epitope recognition by mature class I MHC- and class II MHC-restricted T cells has been described in a variety of systems and points to the flexibility in TCR interaction with its MHC/peptide ligand. While such reduced stringency in TCR recognition is well documented for positive selection of thymocytes by self MHC/peptide complexes and by T cell recognition of peptides bound to allogeneic and xenogeneic MHC molecules, it is also evident in the ability of peptides bearing single or multiple substitutions at TCR contact residues to activate their cognate T cells. Evavold et al. (7) have shown that conservation of as few as a single TCR contact residue in a class II MHC-bound peptide may be sufficient for T cell activation. The MT\textsubscript{389–397} and MT\textsubscript{236–244} epitopes share no sequence homology other than identity at the three putative D\textsuperscript{α} anchor residues at positions 2, 5, and 9. Similar to the results described by Hagerty and Allen (23) using T cells cross-reactive for two human α\textsubscript{1}-antitrypsin peptides complexed to I-A\textsuperscript{A}, increasing sequence identity between MT\textsubscript{389–397} and MT\textsubscript{236–244} by single substitutions of amino acids in MT\textsubscript{236–244} with the corresponding residues in MT\textsubscript{389–397} completely ablated CTL recognition of the MT\textsubscript{236–244} epitope (data not shown). This finding lends further support to the concept that structural mimicry between intramolecular epitopes with minimal sequence homology is responsible for TCR cross-recognition (23).

Several lines of evidence indicate that the MT\textsubscript{236–244} epitope primes but fails to trigger expansion of polyoma virus-specific CTL that cross-react with MT\textsubscript{389–397}. MT\textsubscript{389–397}-specific CTL are recovered from mice infected with the PTA1387T polyoma virus mutant, whose MT terminates at amino acid 384, after restimulation in vitro with polyoma virus-infected spleen cells. In contrast, in vitro restimulation of PTA1387T virus-immune T cells with PTA1387T virus-infected stimulators elicits only non-MT\textsubscript{389–397}/MT\textsubscript{236–244}-reactive, polyoma virus-specific CTL. The inability of PTA1387T virus infection to expand MT\textsubscript{389–397}-reactive CTL in vivo was directly visualized by the absence of D\textsuperscript{α}/MT389 tetramer staining of CD8\textsuperscript{+} T cells in the spleens of mice acutely infected with the PTA1387T virus; this result was recently confirmed using a wild-type A2 strain polyoma virus bearing mutations in the MT\textsubscript{389–397} sequence that abrogated MT\textsubscript{389–397}-specific CTL recognition (C. S. Wilson et al., manuscript in preparation). Recent studies indicate that the density of cell surface MHC/peptide complexes correlates with the magnitude of the CTL response to that epitope (45, 46). Different threshold levels of MHC/peptide complexes, which result in different levels of TCR occupancy, have been shown to elicit different responses from class I MHC-restricted CTL clones and lines, with sensitization for target cell lysis requiring lower peptide concentrations than proliferation (47, 48). Although we have not determined off-rates for the MT\textsubscript{389–397} and MT\textsubscript{236–244} peptides from D\textsuperscript{α} molecules, peptide competition studies indicate roughly 1000-fold higher affinity of D\textsuperscript{α} for MT\textsubscript{389–397} than MT\textsubscript{236–244} (Fig. 2). It is interesting to note that in the only other report of intramolecular mimicry, involving two I-A\textsuperscript{A}-restricted epitopes in the human α\textsubscript{1}-antitrypsin protein, a 3-log higher concentration of the cross-reactive epitope than the dominant epitope was also required to achieve equivalent levels of T cell stimulation (23). While the differences in binding affinity clearly fit the concentration differences between MT\textsubscript{389–397} and
MT\(^{236-244}\) in sensitizing target cells for CTL lysis (Fig. 1) and inducing TCR down-modulation (Fig. 7), the relative efficiencies for intracellular processing of these two epitopes from MT and their respective availability for binding to newly synthesized D\(^{b}\) molecules in the endoplasmic reticulum may also vary, as described for CTL epitopes generated from the same Listeria monocytogenes protein (49). We propose that MHC/peptide levels below the threshold for triggering proliferation in vivo may be sufficient to drive naïve CTL precursors to memory CTL that are capable of expansion when exposed to a strong agonist MHC/peptide ligand. The phenomenon of MT\(^{236-244}\) and MT\(^{389-397}\) intramolecular mimicry presented here extends this concept in that a weak agonist ligand (i.e., D\(^{3}/\)MT\(^{236-244}\)) triggers antiviral CTL precursors to memory CTL whose differentiation to effector CTL depends on engagement of a strong agonist ligand (i.e., D\(^{3}/\)MT\(^{389-397}\)) derived from the same viral protein.

A number of studies describe shifts in CTL epitope hierarchies following infection with dominant epitope-loss viruses and point toward a general strategy for uncovering subdominant epitopes recognized by antiviral CTL (50, 51). Immunization and in vitro restimulation with the MT\(^{389-397}\) epitope-loss mutant polyoma virus PTA1387T select polyoma virus-specific CTL recognizing a class I MHC-restricted subdominant epitope(s). CTL clones recognizing viral epitopes other than the immunodominant MT\(^{389-397}\) epitope are infrequently isolated from H-2\(^{b}\) mice infected by wild-type polyoma virus (25). CTL directed to subdominant viral epitopes may be primed early in the course of an antiviral CTL response, but fail to expand because the dominant epitope-specific CTL response emerges rapidly and clears the source of Ag, i.e., virus-infected cells (38, 50). Studies are in progress to define these subdominant polyoma epitopes.

The unique fine specificity patterns among the MT\(^{389-397}\)-specific CTL clones reveals that different modifications in this immunodominant epitope are tolerated by TCRs bearing heterogeneous CDR3\(^{b}\) regions. Analogous to the structural and functional TCR diversity among these anti-polyoma CTL, murine CTL responses to dominant class I MHC-restricted vesicular stomatitis virus and Sendai virus epitopes are characterized by diverse TCRs with distinct fine specificities (52, 53). Such diversity in TCR recognition among monospecific antiviral CTL clones within an individual would be advantageous in impeding selection of immunodominant CTL epitope-loss mutant viruses. Although several solvent-exposed side chains in the MHC-bound peptide may form TCR contacts, a single peptide residue often dominates TCR engagement (7). For the MT\(^{389-397}\) epitope, alanine substitution of the threonine at position 6 in MT\(^{389-397}\) is the only substitution that uniformly impairs recognition by all the MT\(^{389-397}\)-specific CTL clones examined (Fig. 5 and data not shown). Because this substitution does not affect peptide binding to D\(^{b}\) (data not shown), threonine 394 is likely to be a major TCR contact residue in the MT\(^{389-397}\) epitope.

TCR structure-function studies confirm the critical contribution of the \(\alpha\) and \(\beta\) CDR3 functional domains to peptide specificity (54, 55). In some instances the CDR3\(^{b}\) has been implicated in playing a primary role in peptide recognition by TCRs (5, 56), whereas in others it has been found to have negligible direct contact with peptides complexed to class I MHC molecules (6). In the latter case, the unusual stretch of four glycines in the 2C \(\beta\)-chain CDR3 region may mitigate its capacity to interact with its antigenic peptide. For the CDR3\(^{b}\) domains of the MT\(^{389-397}\)-specific CTL, except for the single glycine at position 97, a common feature of class I MHC-restricted TCRs (41), there are no glycine runs. In addition, the incorporation of a D gene segment in the \(\beta\)-chain, but not the \(\alpha\)-chain, CDR3 affords greater potential for diversity in the \(\beta\)-chain CDR3 and a larger contribution of the CDR3\(^{b}\) to peptide specificity. The lack of MT\(^{236-244}\) cross-reactivity by MT\(^{389-397}\)-specific CTL clone 14-1 may reflect limitations in plasticity of TCR recognition imposed by its short CDR3\(^{b}\) domain. In addition, the identical recognition profile for alanine-monosubstituted MT\(^{389-397}\) peptides by CTL clones 9-12 and 10-11, whose CDR3\(^{b}\) regions are of identical lengths and differ in only two residues points toward a dominant role for the V\(\beta\)-chain in MT\(^{389-397}\) peptide recognition by anti-polyoma CTL.

The capacity of immunodominant antiviral CTL to cross-react with another epitope from the same viral antigen could conceivably confer strong protection against CTL escape variant viruses. Viruses mutated in immunodominant CTL epitopes have been identified in situations where the anti-viral CTL response is functionally monospecific (57–59). The pronounced immunodominance of polyoma virus-specific CTL directed to the middle T protein epitope MT\(^{389-397}\) would likewise favor selection for viruses mutated in this epitope. Because the MT\(^{389-397}\) sequence is situated at the cytoplasmic-plasma membrane interface, the virus can tolerate certain amino acid substitutions in this region without deficits in transformation-competence or infectivity, but which abrogate recognition by MT\(^{389-397}\)-specific CTL (60) (C. S. Wilson and A. E. Lukacher, unpublished observations). Because constitutive expression of fully functional MT is required to maintain cellular transformation, induce tumors, and permit efficient virion assembly, CTL epitope intramolecular mimicry within MT would be expected to prevent emergence of not only immune-escape viruses but immune-escape tumors as well.

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References