

Nonconventional (TL-encoded) Major Histocompatibility Complex Molecules Present Processed Viral Antigen to Cytotoxic T Lymphocytes

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Summary

A large number of class I-like genes are located distal to the K and D regions of the murine major histocompatibility complex (MHC) within the Q and TL region. The function of the molecules encoded within this region is obscure since unlike conventional MHC gene products, these molecules have not been reported to present processed environmental antigens to T cells. In the present report, we demonstrate that a peptide corresponding to processed influenza virus hemagglutinin can be recognized by CD8⁺ T cell receptor α/β -positive cytotoxic T lymphocytes (CTL) in association with a MHC class I-like product encoded within the TL region. Thus, nonconventional class I MHC molecules can bind and present processed environmental antigens, and TCR- α/β CTL directed to such peptide MHC complexes are represented in the mature T cell pool. Our data imply that Q/TL region products may be charged by peptides generated through an antigen processing and presentation pathway distinct from the pathway used by conventional MHC molecules and not normally available to environmental antigens.

Murine H-2 region-encoded class I MHC molecules are cell surface glycoproteins associated noncovalently with β_2 -microglobulin (β_2m).¹ These highly polymorphic molecules are expressed ubiquitously, and function both as transplantation antigens and as restriction elements for CD8⁺ T lymphocytes (1, 2). Nonconventional class I MHC molecules of the Q and TL regions are structurally similar to H-2 class I MHC molecules, but differ in tissue expression and level of polymorphism (3, 4). With few notable exceptions (5, 6), these molecules have not been reported to serve as restriction elements for foreign antigen-specific T lymphocytes. Recent work has suggested a possible role for these molecules as restriction elements in recognition of phylogenetically conserved self-antigens by TCR- γ/δ ⁺ T lymphocytes (7-9). In this report, we have identified a site on influenza virus hemagglutinin (HA) definable as a short synthetic peptide that is recognized by CD8⁺, TCR- α/β ⁺ T lymphocytes restricted by a TL region-encoded class I MHC molecule. This finding implies that nonconventional class I MHC molecules can bind processed foreign antigen fragments and have the potential to present environmental antigens to conventional TCR- α/β ⁺ T cells.

¹ Abbreviations used in this paper: β_2m , β_2 -microglobulin; HA, hemagglutinin.

Materials and Methods

Mice. BALB/cByJ (H-2^d), CBA/J (H-2^k), and C57BL/6 (H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C3H/HeN, A.SW, B10.A, B10.BR, A.TL, A/J, and B10.M mice were purchased from the Jewish Hospital of St. Louis Animal Facility (St. Louis, MO). B6.AC1, B6.Tla^a, A.CA, and B6.AC3 mice were bred and maintained in the Laboratory of Immunology, Wadsworth Center for Laboratories and Research (Albany, NY).

Cell Lines. The P815 (H-2^d) mastocytoma cell line and the S49.35 (H-2^d) lymphoma were maintained in DME (Gibco Laboratories, Grand Island, NY) and supplemented with 10% (vol/vol) FCS and 1% (wt/vol) glutamine. L929 (H-2^k) fibroblasts were grown in DME and 10% FCS. The EL-4 (H-2^b) thymoma, the β_2m -deficient line R1.E (H-2^k), and its parental line R1.1 were maintained in RPMI (Gibco Laboratories), 10% FCS, and 1% glutamine. The S49.35, R1.E, and R1.1 cell lines were the kind gift of Dr. Ted Hansen (Washington University School of Medicine, St. Louis, MO).

Antibodies. Affinity-purified antibody H57-579 (10) (pan-reactive anti TCR- α/β antibody) was obtained from Dr. Jeffrey Lake (Jewish Hospital, St. Louis, MO). The 3A10 anti-TCR- γ/δ antibody (11) was obtained as a hybridoma supernatant from Dr. Osami Kanagawa (Washington University School of Medicine). Anti-CD8 (53.6.2) and anti-CD4 (GK1.5) antibodies were used as hybridoma supernatants.

Peptides. Nested peptides corresponding to residues 170-199 of

the A/JAP/305/57 HA glycoprotein were produced manually on a rapid amino acid multiple peptide synthesizer system (DuPont Co., Wilmington, DE).

Assays for Cell-mediated Cytotoxicity. Standard ^{51}Cr release assays and the resultant data were analyzed as previously described (12, 13). Cytolysis of lymphoblast targets by HA 173-190-reactive cytolytic lymphocytes was measured by release of radiolabeled (^{125}I -IUdR) DNA fragments from detergent permeabilized nuclei as described (14). Briefly, splenocytes from the indicated strains of mice were cultured for 48 h in the presence of 2.0 $\mu\text{g}/\text{ml}$ Con A. Viable blasts were washed, and labeled 1.5 h at 37°C with 25 μCi [^{125}I]-IUdR per 10^6 lymphoblasts. 10^4 target cells, C57BL/6-derived effector T lymphocytes at an E/T ratio of 5:1, and HA 173-190 peptide at final concentrations from 10 to 0.1 $\mu\text{g}/\text{ml}$ (results using 10 $\mu\text{g}/\text{ml}$ only are shown) were added to 96-well round-bottomed microtiter plates. Plates were centrifuged 2 min at 50 g and incubated for 3 h at 37°C in 10% CO_2 . 30 μl of 4% Triton X-100 was added to each well and mixed with a 12-channel pipette. The plates were centrifuged for 5 min at 850 g, and 0.1 ml supernatant was removed from each well and counted. The percent specific lysis was determined as described previously (12, 13). SE were <5% of the mean values in all cases and are omitted.

Peptide-reactive T Lymphocytes. HA 173-190-reactive CTL were established by a modification of a previously described method (15). Briefly, 7×10^7 naive splenocytes were cultured in the presence of 10 $\mu\text{g}/\text{ml}$ HA 173-190 (total volume, 10 ml) in upright 25-cm² tissue culture flasks. Bulk cultures were restimulated after 10 d with 10 $\mu\text{g}/\text{ml}$ HA 173-190 peptide, exogenous growth factors, and irradiated (2,000 rad) syngeneic splenocytes in 12-well tissue culture plates.

Flow Cytometry Analysis. C57BL/6-derived HA 173-190-reactive T lymphocytes were labeled with normal hamster IgG as a control or with the pan-reactive anti-TCR- α/β mAb H57-597 (10) or the pan-reactive anti-TCR- γ/δ mAb 3A10 (11), followed by staining with FITC-conjugated goat anti-hamster IgG. Flow cytometry analysis was performed on a FACS[®] 440 flow cytometer (Becton Dickinson & Co., Mountain View, CA).

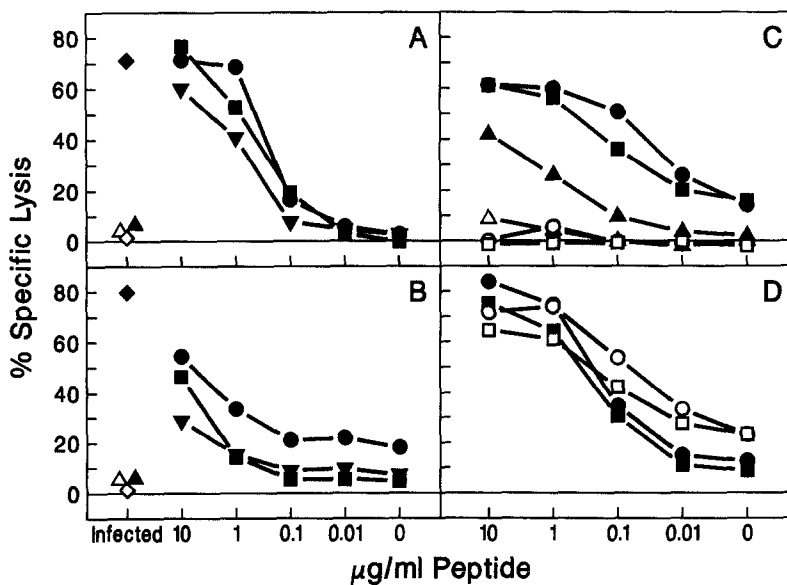


Figure 1. Recognition of target cells by HA 173-190-reactive cytolytic lymphocytes. HA 173-190 peptide-specific cytolytic lymphocytes from C57BL/6J (A) or CBA/J (B) mice were derived by in vitro stimulation of naive splenocyte precursors with peptide and tested for reactivity on P815 (H-2^d; ●), L929 (H-2^k; ▼), or EL-4 (H-2^b; ■) targets treated with the indicated HA 173-190 peptide concentrations. Lysis of uninfected and influenza A/JAPAN/57-infected P815 target cells by HA 173-90-reactive cytolytic lymphocyte (Δ , \blacktriangle) or by a K^d-restricted clone 11-1 (\diamond , \blacklozenge) directed to the immunodominant HA 202-221 site (16, 17) expressed on influenza-infected cells is included. (C) HA 173-190-reactive T cells recognizing peptide-treated target cells expressing $\beta_2\text{m}$. HA 173-190-reactive cytolytic cells of C57BL/6J (O, ●) or CBA/J (□, ■) origin were tested for lytic activity on the $\beta_2\text{m}^-$ R1.E line (open symbols) and its $\beta_2\text{m}^+$ (H-2^k haplotype) parental line R1.1 (filled symbols). Lysis by a K^k-restricted HA 252-271-reactive CTL line (18) of the $\beta_2\text{m}^-$ (Δ) and the $\beta_2\text{m}^+$ (\blacktriangle) target cells treated with the indicated concentrations of the HA 252-271 peptide is included. (D) Expression of nonconventional class I MHC molecules is sufficient to sensitize targets for recognition by HA 173-190-reactive cytolytic

lymphocytes. HA 173-190-reactive cytolytic lymphocytes of C57BL/6J (O, ●) or CBA/J (□, ■) origins were tested for lytic activity on the S49.35 cell line (open circle) that lacks H-2K, D/L class I MHC molecules of the H-2^d haplotype, and on the P815 target cells that express both conventional and nonconventional class I molecules.

Table 1. The Restriction Element for HA 173-190-reactive T Lymphocytes Maps to the TL Region

Exp.	Target cell [†] strain	MHC haplotype				Percent specific lysis*	
		K	D/L	Q	TL	-	+
1	BALB/c	d	d	d	d	14	92
	C3H/HeN	k	k	k	k	11	95
	A.SW	s	s	s	s	6	70
	B10.A	k	d	a	a	25	67
	B10.BR	k	k	k	a	14	79
	A.TL	s	d	d	d	9	62
	A/J	k	d	a	a	21	58
	B10.M	f	f	f	f	18	27
2	C57BL/6	b	b	b	b	7	68
	B6.AC1	f	f	b	b	6	57
	B6.Tla ^a	b	b	b	a	7	80
	A.CA	f	f	f	f	9	18
	B6.AC3	b	b	b	f	7	19

* Values are the mean percent specific release from triplicate samples. SE were <5% of mean values and are omitted. The E/T ratio is 5:1.

[†] Lymphoblasts from the indicated mouse strains were labeled with [^{125}I]-IUdR, sensitized with peptide, and used as targets in a 3-h [^{125}I]-IUdR release assay as described in Materials and Methods.

Results

MHC-unrestricted Recognition of an Influenza Hemagglutinin Site. As part of an analysis of class I MHC-restricted T cell responses directed to specific sites of the influenza A/JAPAN/57 virus HA, we screened a panel of synthetic peptides representing selected regions of the HA molecule as immunogens to induce primary HA-specific cytolytic lymphocyte responses *in vitro* (15). One peptide, HA 173–190, stimulated an *in vitro* primary CTL response from murine splenocytes of several distinct haplotypes (Fig. 1). HA 173–190-specific cytolytic lymphocytes from either C57BL/6 (H-2^b) (Fig. 1 A), CBA(H-2^k) (Fig. 1 B), or BALB/c (H-2^d) mice (data not shown) recognized peptide-treated target cells of H-2^d (P815 mastocytoma), H-2^k (L929 fibroblast), and H-2^b (EL-4 lymphoma) haplotype origin with comparable efficiency. Recognition of target cells was peptide specific as target cells treated with synthetic peptides corresponding to other sites on the influenza HA were not lysed (data not shown). Target cells derived from five independent MHC haplotypes were each lysed in a peptide-specific manner (see Table 1). Importantly, while these HA 173–190-specific cytolytic lymphocytes recognized peptide-treated target cells, the cytolytic lymphocytes did not recognize cells expressing the A/JAPAN/57 HA as a result of infection with influenza virus (Fig. 1, A and B). Influenza-infected cells were recognized by H-2K^d-restricted CD8⁺ CTL directed to an immunodominant site, HA 202–221 (16, 17), in close proximity to the HA 173–190 site on the HA molecule (Fig. 1, A and B).

Role of Serologically Defined MHC Molecules in Target Cell Recognition. To determine the role of MHC molecules in this peptide-specific, MHC-unrestricted recognition, we examined the requirement for class I MHC expression on target cells by testing the ability of HA 173–190-specific CTL to recognize the β_2m -deficient cell line R1.E. This cell is of H-2^k haplotype origin but lacks detectable surface class I MHC molecules. The β_2m^- R1.E line was not recognized by HA 173–190-specific CTL nor by an H-2K^k-restricted CTL line (specific for HA residues 252–271) in the presence of the appropriate HA 252–271 peptide (18). The corresponding β_2m^+ class I MHC expressing parental R1.1 line was recognized by both CTL populations (Fig. 1 C). The R1.E targets were, however, susceptible to lectin-dependent lysis by activated CTL populations (data not shown), indicating that the failure of R1.E to be lysed in the presence of peptide resulted from a lack of target cell recognition rather than resistance of R1.E to lysis.

To assess the role of serologically defined H-2K, D, or L locus class I molecules in the recognition of the HA 173–190 site, we tested the ability of these CTL to recognize the H-2^d lymphoma S49.35, which expresses nonconventional class I MHC antigens but not H-2K or D/L molecules (19). HA 173–190-specific CTL of both C57BL/6 and CBA/J origin lysed HA 173–190-sensitized S49.35 and P815 cells (Fig. 1 D). The H-2K^d-restricted CTL clone 11-1 (16, 17) lysed P815 but not S49.35 targets in the presence of the appropriate peptide (i.e., HA 202–221), consistent with a lack of K^d expression on the surface of the S49.35 cells (data not

shown). Our findings with the R1.E and the S49.35 line implied that the cytolytic lymphocytes directed to HA 173–190 were restricted to a β_2m -dependent nonpolymorphic gene product, presumably a nonconventional class I MHC molecule mapping telomeric to the H-2 D/L region.

Genetic Mapping of the Restriction Element to the TL Region. In an attempt to more precisely map the restricting element for the HA 173–190 response to the Q or TL regions, we examined peptide-dependent recognition of lymphoblast target cells from mouse strains with deletions and/or serological polymorphisms in the Q or TL regions. Target cells of most Q/TL haplotypes were lysed in a peptide-dependent fashion with comparable efficiency (Table 1, Exp. 1). In contrast, target cells of the TL^f haplotype (B10.M strain) were not (Table 1, Exp. 1). To further link unresponsiveness to the TL^f haplotype and to exclude the possibility of recognition of a Q region product encoded in a region deleted from the B10.M genome (20), we analyzed the recognition of lymphoblast targets from recombinant congenic mouse strains. Target cells from B6.AC1 (Q^b TL^b) and B6.Tla^a (Q^b TL^a) mice, but not B6.AC3 (Q^b TL^f), were recognized in a peptide-dependent fashion by HA 173–190-specific CTL.

Table 2. Recognition of a Nested Series of Peptides by HA 173–190-reactive T Cells

Synthetic peptide [§]	Percent specific ⁵¹ CR release*	
	HA 173–190-reactive T cells [†]	CBA/J C57BL/6
173 176 180 184 188 192 198		
VAKGSYNNTSGEQMLIIWGVHHPIDET		
VAKGSYNNTSGEQMLIIW	68	73
VAKGSYNNTSGE	0	ND
KGSYNNTSGEQM	0	ND
SYNNTSGEQMLI	0	7
NNTSGEQMLIIW	76	70
TSGEQMLIIWGV	70	73
GEQMLIIWGVHH	77	76
QMLIIWGVHHPI	71	72
MLIIWGVHHPID	52	79
LIIWGVHHPIDE	1	7
IIWGVHHPIDET	0	7
No peptide	0	9

* As in Table 1.

† Indicates strain of origin of HA 173–199-reactive T cells.

§ Indicates sequence of the synthetic peptide used to treat the target cells. The amino acid sequence corresponding to A/JAP/305/57 HA residue 173–199 is given for reference.

Thus, HA 173–190 reactivity maps to a restriction element shared by strains of the TL a, b, d, k, or s haplotypes, but absent from TL^f mice.

Although these data definitively map the restriction element distal to the Q region, the distal border of the TL region is not precisely defined and thus we cannot exclude the possibility that the restriction element maps telomeric to the TL region. This possibility seems less likely, given the highly conserved nature of the relatively few class I-like molecules that have been mapped to this telomeric region.

TL-restricted Recognition Is Residue Specific. TL region-linked recognition by HA 173–190-specific T cells required a critical core of residues within the HA 173–190 site. Table 2 illustrates an analysis with a set of nested 12 mer peptides spanning residues 173–199. Peptides containing the hydrophobic residues 186–190 (MLIIW) were recognized by HA 173–190-specific T cells from both C57BL/6 and CBA/J mice. This result suggests that a single common epitope may be recognized in association with the TL region product by HA 173–190-reactive T cells from several distinct haplotypes.

Cell Surface Phenotype of TL-restricted T Lymphocytes. The phenotype of HA 173–190-specific cytolytic cells from BALB/c × C57BL/6 F₁, CBA/J, and C57BL/6 mice, as determined by flow cytometry, was predominantly (>75%) Thy-1⁺, CD3⁺, CD8⁺ (data not shown). After primary stimulation of splenocyte precursors, a subset of Thy-1⁺, CD3⁺, CD4⁺ cells initially present was lost upon subsequent stimulation of the cells with HA 173–190 peptide, and the resulting popu-

lation was exclusively CD8⁺. Bulk lines of HA 173–190-specific CD8⁺ cytolytic cells also stained with a mAb specific for a framework determinant on TCR- α/β (10) indicating that these TL region-restricted CD8⁺ CTL used TCR- α/β (Fig. 2 A). The HA 173–190-reactive population did not stain with a γ/δ framework reagent (11). Cytolysis of HA 173–190-sensitized targets was blocked by anti-CD8 antibody but not by anti-CD4 antibody (Fig. 2 B), suggesting a functional interaction between the CD8 molecules and the TL region-encoded class I MHC molecules.

Discussion

The function of Q and TL region MHC molecules is unclear. The predicted structures of Q and TL molecules derived from examination of a number of cloned genes suggests these molecules should fold into structures capable of forming a peptide binding groove (21). In this report, we demonstrate that CD8⁺, TCR- α/β ⁺ CTL from several independent haplotypes can recognize a site on a viral antigen in association with a TL region-encoded class I MHC product. These results along with structural evidence of sequence homology with conventional class I MHC molecules support the concept that Q and TL region molecules are capable of binding processed antigen fragments (21). The demonstration of peptide sequence-dependent sensitization of target cells by the HA peptide as well as the demonstration of a nonresponder haplotype (i.e., TL^f) also suggest that there is

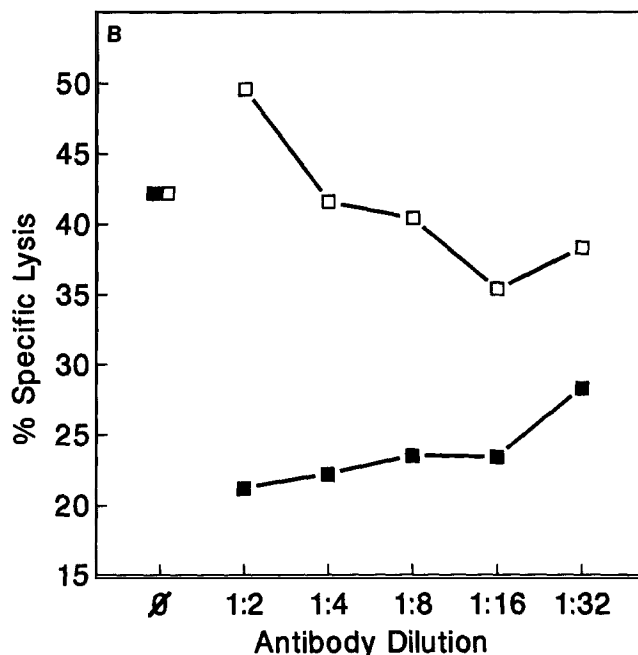
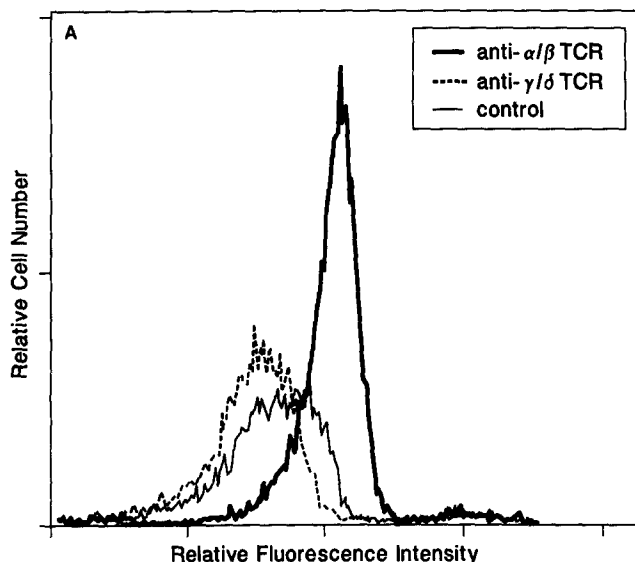


Figure 2. TCR chains used by HA 173–190-reactive T lymphocytes. (A) C57BL/6-derived HA 173–190-reactive T lymphocytes were labeled with normal hamster IgG as a control or with the pan-reactive anti-TCR- α/β mAb H57-597 (10) or the pan-reactive anti-TCR- γ/δ mAb 3A10 (11), followed by staining with FITC-conjugated goat anti-hamster IgG. Flow cytometry analysis was performed on a FACS[®] 440 flow cytometer (Becton Dickinson & Co.). (B) CBA/J-derived HA 173–190-reactive lymphocytes were incubated with anti-CD8 (■) or anti-CD4 (□) antibody-containing supernatants at the indicated dilutions for 30 min at 37°C before the addition of P815 targets and HA 173–190 at a final concentration of 10 μ g/ml. Percent specific lysis for a 6-h ⁵¹Cr release assay was calculated as for Fig. 1.

a selective interaction between specific residues in the peptide and critical contact residues in a TL region-encoded MHC molecule. Since processed antigen can selectively bind to TL region molecules and since there are reactive CD8⁺ T cells in mice of several distinct haplotypes, the inability of HA 173–190-specific T cells to recognize virus-infected cells (Fig. 1, *A* and *B*) suggests that complexes of processed antigen and TL region molecules may not be formed or formed efficiently during viral infection.

For an antigen to be recognized by T cells, processed antigen fragments must have the capacity to bind to MHC molecules, and T cells capable of stimulation by the relevant peptide MHC complex must be resident in the responding individual. The finding that the HA 173–190 peptide can stimulate CD8⁺ CTL that recognize HA 173–190-treated cells in a TL-restricted haplotype-dependent fashion suggests that in several different mouse strains potentially reactive T cells are present and TL product–peptide complexes can be formed. The inability of HA 173–190-specific CD8⁺ CTL to recognize virus-infected cells (Fig. 1, *A* and *B*) and our failure to detect HA 173–190-reactive TL-restricted CD8⁺ CTL during influenza infection (22) implies that complexes between processed HA and TL region molecules may not be efficiently formed (or formed at all) during influenza infection of target cells in vitro or APC in vivo.

It is possible that the HA 173–190 site is not generated during HA processing in influenza-infected cells because of inaccessibility of this region of the molecule to proteolytic cleavage. This remains a formal possibility but seems unlikely since HA 173–190 is in close proximity to an immunodominant site (HA 202–221), which is readily generated during infection with influenza virus (17, 22). Furthermore, we have expressed the preprocessed HA 173–190 site into target cells in the form of a minigene (18, 23) encoding only HA residues 173–190. Target cells expressing this minigene product are not recognized by the HA 173–190-reactive CD8⁺ CTL (G. N. Milligan, unpublished observations). It is also formally possible that the residues in HA 173–190 site critical for TL binding are selectively destroyed during processing of HA in the infected cell. While we cannot exclude this possibility in the case of HA 173–190 recognition in association with TL, it seems unlikely that such a mechanism could account for the general failure of processed antigen from the

large number of viruses studied to date to be recognized in association with nonconventional class I MHC antigens.

One attractive explanation for the failure of influenza and other environmental antigens to stimulate CD8⁺ CTL restricted to nonconventional class I MHC products is that nonconventional class I molecules are charged by antigen fragments processed via a pathway distinct from the processing pathway used in the presentation of environmental antigens in association with conventional class I MHC molecules. Thus, as first suggested from the analysis of CTL recognition of the maternally transmitted antigen(s), processed environmental antigens like the influenza HA may not be available to charge TL-encoded class I molecules. These Q/TL products may only be charged by a unique subset of antigen fragments originating from self proteins within the cell. While this hypothesis is intriguing, it is equally likely that the kinetics of synthesis and turnover of the critical Q/TL molecules that serve as restricting elements, their steady state level of expression, or their rate of transit through particular intracellular compartments may limit their ability to bind processed viral antigens. Accordingly, efficient presentation of foreign antigenic peptides in association with Q/TL molecules may not occur when the antigen is transiently expressed in the cell, e.g., during viral infection. Furthermore, the tissue specificity and relatively low level of expression of most nonconventional class I antigens probably also limits their capacity to serve as restricting elements in the presentation of viral antigens to CD8⁺ CTL. Experiments are currently underway to assess the effect of the magnitude and duration of viral gene expression on the recognition of the HA by TL restricted CD8⁺ CTL.

In conclusion, we have demonstrated that a nonconventional MHC class I locus allele mapping to the TL region presents an influenza HA fragment to CD8⁺ CTL. Thus, TL region products appear to have the capacity to present an environmental antigen to CD8⁺ CTL but fail to do so during viral infection. The mechanistic basis for the defect in the presentation of environmental pathogens in association with nonconventional class I gene products remains to be elucidated. Understanding the nature of this presentation defect should provide additional insight into the cell biology of antigen processing.

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