The α3 Domain of the Qa-2 Molecule Is Defective for CD8 Binding and Cytotoxic T Lymphocyte Activation

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Summary

Qa-2 is a nonclassical class I molecule encoded by the Q7 gene within the mouse major histocompatibility complex (MHC). Results from previous experiments on Qa-2, and on a chimeric Ld molecule (LQ3) in which the α3 domain is encoded by Q7b, suggested that the α3 domain of Qa-2 does not carry out the functions typical of the α3 domains in other classical and nonclassical class I antigens. Class I molecules that contain the Qa-2 α3 domain are poorly recognized by primary cytotoxic T lymphocytes (CTLs), and do not function normally in either positive or negative selection in vivo. By employing a cell–cell adhesion assay we demonstrate directly that the Qa-2 α3 domain in the context of the LQ3 hybrid molecule cannot bind to human CD8, although other mouse class I α3 domains bind efficiently. In addition, CD8-dependent CTL-mediated lysis of target cells, in a system which requires mouse CD8-class I α3 domain interactions, is deficient in cells that express the Qa-2 α3 domain. When combined with our earlier work on LQ3 transgenic mice, these results provide additional molecular support for the hypothesis that interaction with CD8 is required for both positive and negative selection of class I restricted T cells in the thymus. As the Qa-2 α3 domain sequence does not differ from the previously defined minimal CD8 binding sequence of other class I molecules, these results also suggest that additional amino acids in the α3 domain must be critical for CD8 binding and CTL activation.

The mouse genome contains a number of expressed nonclassical class I, class Ib, or medial class I genes encoded within the Qa, Tla, and Hmt subregions of the MHC (reviewed in reference 1). Although their function(s) remain speculative, increasing evidence suggests that some of these molecules are capable of presenting peptide antigens to CTLs. Nonclassical class I molecules have a significant degree of sequence similarity with classical class I molecules such as H-2K and H-2D, and they are also coexpressed with β2-microglobulin (1). Recent evidence indicates that the thymus leukemia antigen (TLA),1 a mouse class Ib molecule, and HLA-G, a human nonclassical class I molecule, can bind effectively to CD8 (2, 3). Furthermore, the Mta and Qa-1 class Ib molecules have been shown to require a functional peptide transporter for their surface expression and recognition by T cells (4), and T lymphocytes that recognize defined peptide antigens in the context of these two molecules have been described (5, 6).

The Q7b gene encodes a Qa-2 molecule in C57BL/10 mice (7). The results from several experiments suggest that the α3 domain of this molecule may be functionally different from other class I α3 domains. Alloreactive, Qa-2 specific CTLs can only be detected in mice that have been previously primed in vivo (8, 9). Similar results were obtained using a chimeric Ld molecule, LQ3, in which the α3 domain is encoded by Q7b and the remainder by the Ld gene (10). Primary, CD8-dependent, alloreactive or virus-specific CTLs could lyse Ld transfected cell lines but not LQ3 transfected targets. Despite this, secondary CD8-independent CTLs could lyse LQ3 transfected targets (10). The chimeric LQ3 molecule was shown to bind a viral peptide that also binds to Ld and, as is seen with Ld, surface expression of LQ3 was increased.

1 Abbreviations used in this paper: CHO, Chinese hamster ovary; LQ3, a chimeric class I molecule in which the α3 domain is encoded by the Q7b gene and the remainder by the Ld gene; TLA, thymus leukemia antigen.
lies only on the ability of mouse CD8α/β heterodimers to recognize cell targets that react to viral peptide binding (10). These results strongly suggest that the L2 peptide binding site in LQ3 is unaffected, localizing the defect in this chimeric molecule to the α3 domain.

Class I molecules rely on their α3 domains for intercellular interaction with the T cell coreceptor molecule CD8. This interaction can be demonstrated directly in binding assays using lymphoid cell lines that overexpress transfected class I genes (11). Previous assay results have shown that all mouse class I and class lb MHC antigens so far examined are capable of binding human CD8α/α homodimers (2, 11, 12). Using this assay, a minimal CD8 binding site has been located on an external facing loop of the human class I α3 domain (12). Given the CD8-independent nature of both Qa-2-specific and LQ3-specific CTLs, it is reasonable to hypothesize that the Qa-2 α3 domain does not interact with CD8. Although most of the earlier data cited above is consistent with this interpretation, there are two pieces of evidence that are inconsistent. First, it was possible in the previous studies to obtain secondary, L4-specific T cell clones that were not inhibitable by anti-CD8 mAbs, but which also could not lyse LQ3-transfected targets (10). This suggests that there could be some other defect in the LQ3, or Qa-2 α3 domain besides the inability to bind CD8, although the α3 blocking studies may not detect all CD8-dependent T lymphocytes. Second, there are no sequence differences in the defined CD8 binding site between the Qa-2 α3 domain and the α3 domains of class I molecules that are known to interact with CD8. Therefore, to determine directly if the Qa-2 α3 domain can mediate binding to human CD8, a cell-cell adhesion assay was employed. In addition, the Qa-2 α3 domain was tested in a functional assay: CTL-mediated cytolysis of cell targets that relies only on the ability of mouse CD8α/β heterodimers to interact with class I α3 domain was assessed.

Materials and Methods

Gene Constructs and Transfectants. The TLA expression construct pH3AprT18b and the chimeric L4/TLA molecule expression construct pH3AprLT18b have been previously described (2). pH3AprT18b encodes a full-length T18 cDNA while pH3AprLT18b encodes a genomic sequence containing the H-2Ld α1 and α2 domains coupled to a T18 genomic sequence encoding the α3, transmembrane, and cytoplasmic domains. Both class I gene constructs are expressed under the control of the human β-actin gene promoter (15). This promoter gives higher levels of expression of mouse class I genes in CIR B cells than do either the H-2Dq or H-2Ld promoters (2; data not shown). A chimeric gene (LQ3) encoding the H-2Ld α1, α2, transmembrane, and cytoplasmic domains along with an α3 domain supplied by Q76, expressed under the control of the β-actin gene promoter, was created as follows: A 2.9-kb BamHI fragment containing the Q76 α3 domain fused to Ld transmembrane and cytoplasmic domains was excised from the previously reported expression vector LLQL-23 (10). This fragment was cloned into the BamHI polylinker sites of the cloning vector p34E (14). The fragment was then excised from the resultant construct with Xbal digestion and ligated into place in Xbal digested pH3AprLT18b. Xbal digestion removes all T18s gene sequences. Proper orientation was determined by appropriate restriction enzyme digestion and agarose gel electrophoresis of the resultant plasmid clones. This new construct was designated pH3AprLQLL.

Gene transfer of 20 μg of each expression vector along with 10 μg of the drug selection plasmid pSV2neo (15) into human CIR B lymphoma cells was accomplished by standard electroporation procedures (Gene Transfector 300; BTX Inc., San Diego, CA; [2]). G418 (GIBCO BRL, Gaithersburg, MD) selection at 600 μg/ml active drug began 48 h after electroporation, and stable transfec-
tants were chosen 3–4 wk later as previously described (2). A CIR B cell transfectant expressing the human class I transplantation antigen HLA-A2.1 was obtained as a kind gift from Dr. D. Littman, University of California at San Francisco, San Francisco, CA (11). Detection of Class lb MHC Antigen Expression. Cell surface mAb staining of CIR transfec-
tants was performed as described previously (16). The primary mAbs used included the anti-TLA mAb TL-m4 (17), which was protein-A column purified and used at a 1:200 dilution. The TL-m4 mAb was kindly provided by Dr. S. Kimura and Dr. E. Boyse, Sloan-Kettering Memorial Cancer Institute, New York. The anti-Ld mAb 30-5-7 (18) was obtained as a kind gift from Dr. L. Sherman, Scripps Research Institute, La Jolla, CA. The secondary staining reagent employed was a 1:100 dilution of phycoerythrin-conjugated goat anti-mouse IgG (CALTAG Labs, South San Francisco, CA) that detects all three mAbs. After staining and washing, cells were analyzed in propidium iodide using a FACScan® instrument (Becton Dickinson and Co., Palo Alto, CA, and University of California at Los Angeles Flow Cytometry Core Facility, Los Angeles, CA).

Cell-Cell Adhesion Assay. This method has been described in detail elsewhere (11, 12). Briefly, 3 d before the assay adherent human CD8α(+) or mock-transfected Chinese hamster ovary (CHO) cells at 5 × 10^5–10^6 cells per well were placed in 96-well flat-bottom microtiter plates. Optimal confluent monolayers, as judged by visual inspection, were used on the day of assay. Parent and transfec-
tant CIR B cell lines were incubated for 2 h in [3S]methionine (>12.5 mCi/10^6 cells, >600 Ci/mmol; Amersham Corporation, Arlington Heights, IL) in 1× MEM without t-methionine (Flow Laboratories Inc., McLean, VA). The labeled cells were then added to the plate wells, centrifuged to initiate contact, and incubated at 37°C for 1 h with gentle shaking. After incubation, the wells were washed 10–12 times each with assay buffer (1× PBS, pH 7.4, 10% FCS, and 0.01% NaN3) and the remaining bound cells were solubilized in 1% Triton X-100 for scintillation counting.

Cell Lysis Assay. Anti-Kr alloantigenic CTL clone lines C30 and C35 were obtained from Dr. M. Mescher (Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN). These clones were grown in HEPES-supplemented RPMI 1640 with 10% FCS and were stimulated once each week with irradiated C57Bl/6 splenocytes and twice each week with RIL-2. The CTL clones were activated by preincubation with either 10 μg/ml of fluid-phase mAb 2C11 (anti-CD3, both clones) or 10 μg/ml of mAb F23.1 (anti-V,68, clone C35 only) at room temperature for 10 min. mAb-preincubated or untreated T cell clones were then added to 96-well "V" bottom plates containing 10^4 Cr-loaded CIR transfec-
tant cells in various effector to target ratios. Assay plates were spun at 160 g for 3 min to initiate effector-to-
target contact, and lysis allowed to proceed at 37°C in 5% CO2 for 8 h. After incubation, 100 μl of supernatant from each assay
well was removed for measurement of $^{51}$Cr release and calculation of specific lysis above background.

**Results and Discussion**

The cell–cell adhesion assay measures the binding of nonadherent cells expressing transfected class I genes to monolayers of CHO cells that express high levels of human CD8α. The ability of mouse class I and class Ib molecules to bind human CD8α molecules in this assay is well established (2, 20). CIR, the recipient human B lymphoma cell line for the class I gene constructs, lacks HLA-A and HLA-B genes, and expresses only low levels of HLA-C (11, 21). Untransfected CIR cells therefore do not bind well to CD8α-positive CHO cells. CIR cells can, however, express high levels of transfected class I genes whose expression is controlled by their own or heterologous promoters. Transfected CIR cell lines will express the introduced mouse or human class I heavy chains along with human β2-microglobulin. Staining with an anti-human β2-microglobulin mAb can therefore be used to compare the level of total class I surface expression of cells transfected with the different gene constructs. Fig. 1 shows the cell surface staining profiles as analyzed in the flow cytometer of four CIR B cell lines with the anti-human β2-microglobulin mAb BBM.1. The level of expression of endogenous class I molecules in mock transfected CIR cells is very low. In contrast, the levels of β2-microglobulin are significantly higher than in the CIR parent, and roughly equivalent for transfected cell lines that express HLA-A2.1, TLA, or the LQ3 chimeric molecule. Staining with mAbs specific for the various transfected class I heavy chains confirms that the increased level of β2-microglobulin expression is due to expression of the transfected genes (data not shown). In a separate experiment, similar results were obtained using a CIR cell transfected with a gene encoding a chimeric L4/TLA gene (data not shown). Because the levels of expression of the different transfected genes are nearly equivalent (Fig. 1), the CD8 binding ability of each line can be compared.

Fig. 2 depicts representative data on the binding of the transfected CIR cell lines to CD8α-positive and control CHO cell monolayers. The LQ3 transfected lines A and D did not bind detectably above background to the CD8α-positive monolayers. By contrast, the three cell lines expressing HLA-A2.1, TLA, or L4/TLA class I molecules bound well. Although the CIR cells overexpress the transfected class I genes and the CHO cells overexpress human CD8α, it could be argued that a low but functional binding affinity is not detected by the method employed. However, in the studies of Salter et al. (12), there was complete concordance between results from the binding assay and the ability of transfectants that express class I mutants to be recognized by primary, allogeneic CTLs. The binding data shown are also consistent with our own prior functional studies, in that L4/TLA can serve as a target for primary, CD8-positive CTLs while LQ3 cannot (2, 10). Similar results were obtained in three separate experiments in which the binding to CD8 positive and negative monolayers were compared. The ratio of the percent of TLA and HLA-A2.1 positive control cells bound to CD8-positive monolayers to the percent binding of these cells to CD8-negative monolayers ranged from 2.0 to 5.1. In every case the difference in cells bound to CD8+ vs. CD8− CHO monolayers was statistically significant, with $p < 0.001$ by the Student’s $t$ test. In contrast, in two experiments, the ratio of binding of LQ3 expressing CIR cells to CD8+ and CD8− CHO monolayers was 1.0 and 1.1, respectively, and the difference in percent cells bound was not statistically significant. In the third experiment, the ratio of CD8+...
to CD8- binding by LQ3 cells was 1.9, 31.2% ± 3.0 (SE of the mean) cells bound to CD8+ CHO monolayers vs. 16.8% ± 0.6 to CD8- monolayers. However, in this experiment the negative control CIR cells also bound better to the CD8+ CHO monolayers, 21.8% ± 1.0 vs. 13.5% ± 0.7. The difference in binding to the CD8 monolayers for both LQ3 and control CIR cells is statistically significant (p <0.005) in both cases. It is therefore possible that this third experiment was for some reason unusually sensitive, enabling detection of binding by the low level of HLA-C expressed by CIR cells to human CD8. Consistent with a possible enhanced sensitivity, in this same experiment the TLA and A2.1 expressing CIR cells gave the highest percent cells bound that has been obtained in these assays, 81.8% and 67.4%, respectively. In summary, the data indicate that LQ3 cannot bind to human CD8 because in all cases the LQ3 transfectant bound much less well to the CD8+ CHO cells than did either the TLA or HLA-A2 transfectants, in all three experiments LQ3 binding was similar to that of the CIR parent negative control, and in two of three cases binding of LQ3 cells to CD8+ and CD8- CHO cells was equivalent.

Although the ζα2 ζα3 domain of LQ3 is incapable of binding human CD8α/α homodimers, it may still be capable of interacting with mouse CD8α/β heterodimers. To date, there has been complete concordance in the ability of mouse class I αα domains to interact with both human and mouse CD8 (2, 11, 12, 22). However, the data are not that extensive, particularly for nonclassical class I molecules for which only one example currently exists (2). In addition, recent work by two independent groups indicates that the presence of the mouse CD8β chain appears to broaden the range and strengthen the response of CD8-αβ domain interactions (23, 24). Therefore, in order to rule out the possibility that LQ3 could interact with mouse CD8α/β heterodimers expressed at physiologic levels, a second assay was performed.

CTLs are capable of binding and initiating a degranulation response to purified class I and class Ib MHC antigens coated on a plate when they are first suboptimally activated by preincubation with soluble anti-T cell receptor mAbs (22, 25). In the absence of mAb preincubation these functions do not occur. Furthermore, both binding and degranulation can be blocked with anti-CD8 mAbs (22). These observations suggest that, following T cell receptor activation with fluid-phase antibodies, a second signal which can be mediated through a CD8 interaction with an "irrelevant" class I molecule is required to activate the CTL. This second signal is also required for the initiation of polyphosphoinositide hydrolysis (25). In the present study, we modified this assay by using CIR target cells that express equivalent levels of transfected class I or class Ib genes (see Fig. 1) instead of purified, plate-bound MHC antigens. By using two mouse CTL clones that are Kβ-specific, no influence from bound peptides or the α1 and α2 domains of the transfected class I gene is possible (26). Additionally, use of human cell line targets should reduce the possibility of other second signals, aside from those delivered through other adhesion molecules (reviewed in reference 27), from activating the CTL. Finally, the CIR transfectant A2.1 is an ideal negative control because it has been shown that human class I αα3 domains are deficient for interactions with mouse CD8 (28, 29).

Anti-Kβ CTL lines C30 and C35 were made competent for killing by preincubation with fluid-phase mAb 2C11 just before use. Target cells loaded with 51Cr were incubated with effectors at various ratios for 8 h and the amount released, due to specific lysis above background, was measured. When either of the CTL clone lines were not made competent by mAb preincubation, or when line C30 was preincubated with the irrelevant mAb F23.1, the resultant 51Cr release was very low in all repeats of the assay (maximum 0.8% at an effector to target ratio of 5:1, data not shown). However, specific lysis of CIR transfectants expressing the Lα/Lβ TLA chimeric molecule is significantly above the background seen with the negative control transfectant A2.1 (Fig. 3). Background 51Cr release is consistently ~10% in all 8-h assay repeats using effector-to-target ratios of 20:1 down to 1.25:1. Previously it was shown that the Lα/Lβ TLA chimeric molecule could support a CD8-dependent alloantigenic response and this reactivity could be blocked with anti-CD8 mAbs (2). The Lα/Lβ TLA-mediated cytolysis is blocked by 21% and 32% at effector-to-target ratios of 20:1 and 5:1, respectively, with a 10-μg preincubation of anti-CD8α mAb (M. Teitell, unpublished data, not shown). Also, the low level of background lysis due to CIR A2.1 transfectant targets was not affected by effector clone preincubation with anti-CD8α mAb (data not shown). This result further supports the proposed mechanism of CTL activation through CD8 interactions. Several other CIR transfectants that express different class I molecules are effective in this assay, including one molecule that did not function in the cell–cell adhesion assay, and this cytolysis also was partially blocked with anti-CD8α mAb preincubation (M. Teitell, manuscript in preparation). In contrast, Fig. 3 demonstrates that the chimeric LQ3 molecule is unable to activate a CTL response through CD8 interactions, as the amount of 51Cr released from loaded targets is equivalent to that released from control CIR A2.1 targets.

Figure 3. CD8-mediated lysis of transfected CIR target cells. The percent cytolysis is calculated as (specific release – spontaneous release)/total release × 100 and depicted as a function of the effector-to-target ratio. The figure shows the cytolysis of cell lines expressing equivalent levels of HLA-A2.1 (A2.1), LQ3, and Lα/Lβ TLA (L/Lα) transfected genes by mAb 2C11 preincubated CTL clone C30. These results are identical to those obtained with CTL clone C35 and are reproducible for effector-to-target ratios up to 20:1. Error bars represent the SD of three measurements.
The LQ domain might not bind human CD8 or permit mouse CTL activation because the Ld α1 and α2 domains could potentially distort or interfere with the normal conformation or accessibility of the Qa-2 α3 domain. We consider these possibilities unlikely because the Ld and Qa-2 α3 domains are highly similar in sequence. Furthermore, Qa-2-specific CTLs also appear to be CD8 independent. In addition, results depicted in Fig. 3 and those of previous experiments on the chimeric Ld molecule Ld/TLA support the notion that α3 domain functions are not disturbed in chimeric molecules by the α1 and α2 domains (2). It is also possible that the α3 domain in LQ is distorted by its association with human β2-microglobulin in the CIR transfectants and also, presumably, by bovine β2-microglobulin obtained by exchange in the tissue culture medium. Although not formally excluded, we consider these explanations unlikely for the reasons outlined here. First, the behavior of intact Qa-2 itself is at least consistent with an inability to interact with CD8. Second, distortion of the LQ domain in the presence of heterologous β2-microglobulin could not also apply to Ld or Ld/TLA molecules despite the high degree of sequence conservation. It should be noted that based upon the structures of both human and mouse class I molecules (30-32), all of the amino acids likely to be important for interacting with β2-microglobulin in Kb, TLA, and Qa-2 are conserved (Fig. 4). We conclude therefore that the most likely explanation is that Qa-2 does not interact with either mouse or human CD8 and that those secondary CTLs that recognize Ld but not LQ, and which are not inhibitable with anti-CD8 mAbs (10), probably still require a CD8 interaction in order to become activated. It is still formally possible, however, that there is a second defect in the Qa-2 α3 domain in the LQ hybrid.

The Qb α3 domain differs from the Ld α3 domain, which does bind to CD8 (data not shown), by only six amino acid substitutions and a three amino acid insertion at the carboxy terminus (33, 34; Fig. 4). Although it is not possible to reach a definitive conclusion as to which of the changes are critical for interfering with CD8 binding, the carboxy-terminal three amino acid insertion at positions 275-277 is perhaps responsible for the lack of CD8 interaction. The H-2Dα3 domain has a carboxy-terminal insertion of similar length (35), and it is presumed not to be altered in its ability to interact with CD8. However, the Qb α3 domain sequence contains a bulky tryptophan group at position 277, which is not present in the H-2Dα3 domain, and this substitution could prevent α3 domain–CD8 binding. Qa-2 molecules that span the plasma membrane, and which therefore are not phosphatidylinositol linked (36), could contain this tryptophan group up to 10-15 amino acids external to the plasma membrane. The K to Y and E to A substitutions, at positions 196 and 198, respectively, may also affect α3 domain–CD8 interactions. Extrapolation of data from the crystal structure suggests that these changes are found on an external facing loop between the α3 domain β-strands 1 and 2 (30-32), and they replace two charged amino acids with two neutral ones. However, positive, negative, and in some cases even neutral amino acids can occupy these positions in various class I molecules that are presumed capable of CD8 binding. Among the remaining substitutions that distinguish the Qb and Ld α3 domains, the E to D and S to P alterations at positions 183 and 184 and the R to H change at position 260 are not likely to have an important effect on CD8 binding. These changes replace Ld amino acids with those found in both TLA and the human consensus α3 domain sequences. Finally, the Y to N substitution at position 262 is potentially important, but the human consensus α3 domain sequence contains a glutamine at this position which is a rather conservative change from the asparagine in Qb. Whichever substitutions turn out to be critical, the data suggest that a larger than expected area of the α3 domain may be involved directly in CD8 interactions. Alternatively, the substituted amino acids might disrupt the conformation or accessibility of the previously defined minimal CD8 binding domain.

Analysis of transgenic mice that express LQ has shown that this class I molecule does not function normally in either positive or negative selection of the T cell repertoire (37). The data presented here provide strong evidence that the defect in LQ is indeed due to the loss of binding to CD8, and therefore that effective CD8 interactions are required for positive and negative selection in the thymus. Our data on LQ is consistent with recent experiments showing that mutant class I molecules, bearing α3 domain substitutions that disrupt CD8 binding, cannot carry out either positive or nega-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Consensus sequence of the human class I α3 domain compared to Ld, TLA (encoded by T18), and Qa-2 (encoded by Q76) α3 domains. Boxed areas in the consensus sequence represent human class I α3 domain polymorphic amino acids as described in Parham et al. (45). The Ld sequence was obtained from Link et al. (33), the TLA sequence was obtained from Fisher et al. (46), and the Qa-2 sequence was obtained from Devlin et al. (34). Amino acids that differ between Ld, TLA, and Qa-2 and the consensus sequence are indicated. The six amino acids that differ between Ld and Qa-2 are numbered, as is the three amino acid insert near the carboxy terminus of Qa-2. Arrows above the consensus sequence indicate the position of Qa-2 variant residues, which are due to encoding of this class I molecule by different genes with the Qa locus (34). Underlined portions of the sequence have been implicated in binding to CD8 (12).
tive selection (38, 39). They are also consistent with studies demonstrating that human HLA class I molecules do not carry out positive selection in transgenic mice (28), probably because human class I molecules do not bind to mouse CD8 (28, 29).

The Q7-encoded Qa-2 class I molecule is unusual in several respects. It is attached to the plasma membrane by a phosphatidylinositol linkage (36, 40), its expression as a cell-bound molecule is controlled in part by alternate splicing mechanisms (41), and it can transmit an intracellular activation signal when cross-linked by specific cell surface antibodies (42, 43). Although, like LQ3, Qa-2 is probably incapable of binding CD8, this does not imply that this molecule has no significant role in antigen presentation to T lymphocytes and it does not exclude a possibility that it participates in negative or positive selection. Some or all of the previously characterized anti-Qa-2 allogeneic CTL may have arisen as a result of positive selection of these T cells not on Qa-2, but on another class I molecule(s), most likely with CD8 binding capabilities. Recently, however, Qa-2 has been shown to bind endogenously derived nonamer peptides (44; Joyce, S., P. Tabaczewski, R. H. Angeletti, S. G. Nathenson, and I. Stroynowski. Manuscript submitted for publication). Thus, it remains possible that anti-Qa-2 CTL, which are directly selected on Qa-2-peptide complexes, originate from a novel and thus far not identified developmental pathway that does not depend on CD8 coreceptor.

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