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Recognition of allo-peptide is governed by novel anchor imposition and limited variations in TCR contact residues

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Abstract

Immune specificity of a T cell is determined by the TCR contact residues exposed on the antigenic peptide/MHC complex. Naturally processed, biallelic epitopes from H7 minor histocompatibility (mH) antigen vary in position 7 (p7) from aspartic acid (D) to a glutamic acid (E), which differ by an additional methylene ($-\text{CH}_2$) in the side chain. Here, we show that this variation generates a strong anti-H7a or anti-H7b cytotoxic T cell responses. Further, the H7 allelic peptides use p6 asparagine as their central anchor residue and amino acid variations in either the canonical p5 or the predicted p6 anchor positions in the antigenic epitope were detrimental for TCR recognition. In addition, introduction of any other amino acids, except asparagine, in the polymorphic p7 significantly abolished the ability of anti-H7b TCR recognition. This demonstrates that only an asparagine with an amine group as a side chain instead of a charged oxygen radical could effectively stimulate the anti-H7b specific T cells. Our findings provide evidence that mH antigen-specific TCRs are highly stringent in recognizing their cognate epitopes.

Keywords

Allo peptide; Anchor residue; TCR recognition

1. Introduction

TCR recognition of antigenic epitopes bound to MHC class I and MHC class II molecules form the first critical step in T cell activation. Activation through TCR initiates signaling cascades that govern effector functions such as cytotoxicity and cytokine generation. MHC Class I complex comprised of MHC heavy chain and β -microglobulin presents 8–12 amino acid long antigenic epitopes to TCR (Falk et al., 1990). Allelic variations in MHC class I give rise to differential epitope binding due to the presence of polymorphic residues in the peptide-binding groove. Precise sites where amino acids of the peptide contact the floor of groove have been classified into six pockets, A through F (Fremont et al., 1992). Side chains from the amino acid sequence of the epitope interact with these six pockets. Chemical

composition and size of these pockets determine the 'binding-motif' of epitopes respective to the MHC class I molecule.

Conventional TCR consists of α and β chains that are held together by disulfide linkages. Each one of these chains contain four possible complementarity determining region (CDR). CDR1, CDR2 and CDR4 are germ-line encoded and the CDR3, which is highly variable, formed during thymic development by D to J and V to D gene rearrangements in the α -chain (Garcia et al., 1996). CDR3 loops of α and β chains in the TCR form the critical contact structures to the peptide/MHC complexes, whereas the CDR1, CDR2 and rarely the CDR4 interact with the 1 and 2-helices of the MHC class I heavy chain (Wilson and Garcia, 1997).

Slightest modifications in the side chains of the amino acids in the antigenic peptide can drastically perturb the structure of the peptide/MHC complex and thereby severely compromising TCR recognitions. However, the same modifications can also form the basis for 'self' versus 'non-self' discrimination by clonotypic TCRs. Epitopes derived from mH antigens (Ags) provide an excellent model system to address many issues regarding interactions between peptide/MHC complexes and the TCR. Murine mH-Ag, H7 is one such antigen used to describe MHC restriction (Bevan, 1977), immunodominance (Eden et al., 1999), and the role of a single mH-Ag in skin graft rejection and GvL (Fontaine et al., 2001). H7 is a bi-allelic mH-Ag restricted to D^b MHC class I and the antigenic epitopes have been defined as KAPDNRETL (H7a) and KAPDNRTL, (H7b) (McBride et al., 2002; Malarkannan and Pooler, 2004). Thus, the allelic peptides differ by an additional methylene in the side chain ($-\text{CH}_2$) between the glutamic acid (E) to aspartic acid (D) at position 7 (p7). This conserved amino acid variation between the allelic peptide leads to a significant reciprocal CD8 + T cell responses during graft and tumor rejections (Fontaine et al., 2001). The molecular basis for such a strong T cell response has not been understood.

In this study, towards understanding the biological basis of this phenomenon, we (a) quantified the number of both the allelic peptides at per cell level; (b) enumerated the frequency of antigen-specific T cells in *ex vivo*, short-term and long-term T cell cultures; and (c) analyzed the ability of T cells to mediate antigen-specific target cell lysis. Our results demonstrate that the H7a and H7b epitopes are presented at about ~300 copies per cell. Reciprocal CD8 + T cell responses were successfully generated and the frequency of antigen-specific T cells directed to H7a and H7b peptides were comparable between C57BL/6 BALB.B and BALB.B C57BL/6 immunizations. In addition, both anti-H7a and anti-H7b CTLs mediated lysis of target cells, demonstrating that the presence of closely related self-peptide did not hinder the positive selection and effector functions of T cells that recognize their allelic variants.

To further understand the basis for TCR recognition of these epitopes, we generated structural models of peptides bound to D^b MHC, which demonstrated that both H7a and H7b peptides may utilize arginine at p6 as their anchor residue instead of the canonical asparagine at p5. Our models predict that the uncharged polar side chain of this asparagine points outward the peptide groove and may form contact with TCR. In contrast, the positively charged basic side chain of arginine is buried deep inside the centrally located anchor pocket of D^b MHC, securing the H7 epitope within the peptide-binding groove. Moreover, introduction of any other amino acids except asparagine at the polymorphic p7 instead of the native aspartic acid significantly abolished the ability of anti-H7b T cell recognition. This demonstrates that only an asparagine with an amine group as a side chain instead of a charged oxygen radical of aspartic acid could effectively stimulate the anti-H7b specific T cells. Our results provide interesting insights into the molecular mechanism of 'self-non-self' discrimination by TCR to closely related allelic peptides.

2. Experimental procedures

2.1. Mice

All inbred mouse strains used were obtained from or bred at The Jackson Laboratory (Bar Harbor, ME). C57BL/6 and BALB.B mice were bred in the animal care facility, Medical College of Wisconsin, Milwaukee, WI from Jackson Laboratory stocks. All protocols used in this study were approved by the ALAAC committee of BRC, MCW, Milwaukee, WI.

2.2. Cell lines

Cells were maintained in RPMI-1640 or DMEM media (Life Technologies, Gaithersburg, MD), supplemented with 2 mM glutamine, 1 mM pyruvate, 50 μ M β -ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS (Hyclone, Ogden, UT). EL4, D^b-L cells have been described previously (Mendoza et al., 1997). COS-7 cells were obtained from ATCC. 47NPZ.1 was generated by fusing 47NP CTL with BWZ36 fusion partner as described (Malarkannan et al., 2001). To generate C57BL/6 anti-BALB.B and BALB.B anti-C57BL/6 bulk CTL lines, C57BL/6 or BALB.B animals were challenged three times with 2×10^7 splenocytes, 7 days apart. After immunization, primed responder cells from spleens were harvested and stimulated in mixed lymphocyte cultures (MLC) with 5×10^6 200-Gy-irradiated target splenocytes from respective backgrounds. All CTL lines were maintained by weekly re-stimulation with 5×10^6 200-Gy-irradiated splenocytes from respective backgrounds and 10 U/ml rIL2 (kind gift of NCI, NIH).

2.3. T cell activation assays

T cell responses specific for peptide/MHC were measured by the production of β -galactosidase (LacZ) activity in the T cell hybrids ($3-10 \times 10^4$) after overnight co-culturing with APC ($2-5 \times 10^4$) in 96-well plates. T cell responses were measured as LacZ activity using the substrate chlorophenol red β -D-galactopyranoside (CPRG). The conversion of CPRG to chlorophenol red was measured at 595 nm using 655 nm as reference wavelength in a 96-well microplate reader (Bio-Rad, Richmond, CA). Data shown are the mean absorbance of triplicate cultures and are representative of at least three independent experiments. CTL-mediated killing of peptide-loaded target cells were quantified using ⁵¹Chromium (⁵¹Cr)-labeled TAP-deficient RMA/S cells. These cells were incubated with (100 nM) of indicated peptides at 30 °C for 45 min and washed twice before use. Effector cells were added at varied Effector to Target cell (E:T) ratios. Percent specific lysis, calculated from the amount of ⁵¹Cr released into the culture supernatant is shown as the mean of triplicate cultures.

2.4. HPLC analysis of naturally processed antigenic peptides

Naturally processed peptides were analyzed by extracting total acid soluble peptide from splenocytes, EL4 or D^b-L cells as described (Malarkannan et al., 1995). Briefly, cells were washed with PBS and extracted with 0.5 ml of 10% formic acid in water. Processed samples were fractionated by reverse-phase HPLC through a C18 column (Vydac, 2.1 $\times$ 250 mm, 5 μ M) in 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Fractions were collected between 26% and 32% solvent B at a flow rate of 0.35 ml/min in 96-well flat-bottom plates. After drying the plates in Speed Vac Plus (Savant), fractions were resuspended in 15% DMSO in PBS and assayed for T cell stimulating activity. Synthetic KEL9 and KDL9 peptides and mock injections were run and assayed under identical conditions. Processed peptide amounts in the cell extracts were calculated by comparison with synthetic peptide standard curves and taking into consideration of procedural loss for the given peptide.

2.5. Enumeration of antigen-specific CTLs by peptide loaded class I-DimerX complexes

Antigen-specific T cell frequency was obtained using peptide/DimerX (BD-Pharmingen, Los Angeles, CA) staining. Briefly, D^b-DimerX (4 µl) was incubated with 1.33 µl (mg/ml) of respective synthetic peptides (24 h at 37 °C followed by 24 h at 4 °C). Bulk CTL lines (4–6 × 10⁵ cells per sample) were pre-incubated with 1 µl of Fc-Block (anti-mouse CD16/CD32, BD-Pharmingen) in 100 µl of wash buffer in 96-well U-bottom plates for 1 h. After blocking, cells were washed, added with peptide/DimerX complexes and incubated for 1 h at 4 °C. In the final step, cells were washed and stained with PE conjugated anti-mouse IgG1 (BD-Pharmingen) along with FITC conjugated anti-mouse CD8 (BD-Pharmingen) antibodies for 1 h at 4 °C. Cells were washed twice and analyzed in a FACScan (BD Immuno-cytometry Systems, San Jose, CA) using CellQuest software.

3. Results

3.1. Abundance of naturally processed H7a and H7b epitopes are comparable

A single nucleotide modification from GAG to GAT in the peptide encoding region of STT3 protein resulted in the amino acid substitution of glutamic acid to aspartic acid (KAPDNRETL → KAPDNRDTL) (Fig. 1A). Thus, H7a and H7b peptides differ by a single methylene (–CH₂) side chain. However, this small change in the side chain of p7 residue has been shown to result in significant T cell responses leading to graft rejections and GvL responses (Fontaine et al., 2001). To understand the contribution of peptide abundance towards this strong T cell response, we quantified the peptide copy numbers on per cell basis. Acid soluble cell extracts were generated and fractionated in a reverse-phase HPLC. H7a peptide, KAPDNRETL (KEL9) was eluted from C57BL/6 splenocytes or EL4-thymoma and analyzed with anti-H7^a hybrid, 47NPZ.1. Similarly, H7b peptide, KAPDNRDTL (KDL9) was extracted from BALB.B splenocytes or D^b-L cell line and tested with BCZ1644 T cells. A single peak of T cell stimulating activity was detected for each of the anti-H7 specific T cells, which matched the retention time of the either synthetic KEL9 (Fig. 1B, upper panel) or KDL9 (Fig. 1B, lower panel) peptides. These results are within the limits of HPLC discrimination and confirm the identity of naturally processed peptides to synthetic KEL9 and KDL9 peptides. Using the standard curves of T cell activation generated by synthetic peptides, we enumerated the copy number of each allelic peptide in cell extracts. Known amounts of synthetic peptides were pulsed into unrelated cell extracts and analyzed to calculate the procedural losses. Using the acid soluble peptide extracts from EL4 and D^b-L cells, our results indicate that KEL9 and KDL9 peptides were present at ~300 and ~270 copies per cell, respectively. Thus, the abundance of processed H7a and H7b minimal peptides are largely comparable between each other.

3.2. High homology between KEL9 and KDL9 peptides do not hinder reciprocal T cell responses

Although T cell responses directed to H7 allelic epitopes have been described, the strength of the response and the frequency of antigen-specific T cells directed to KEL9 or KDL9 has not been determined. Since the antigenic polymorphism between the two alleles is governed by one methylene side chain, we next analyzed the efficiency of reciprocal T cell responses. Anti-BALB.B and anti-C57BL/6 bulk CTLs were used to enumerate the antigen-specific CD8⁺ T cells in *ex vivo*, short-term, or long term CTL lines. To determine the frequency of CD8⁺ T cells specific for H7 peptides, we used soluble D^b MHC molecules loaded with either KEL9 or KDL9 epitopes. The bi-directional CTL responses to H7 epitopes were evident in our analyses. In the *ex vivo* analysis, a proportion of both KEL9 (1.5%) and KDL9 (1.3%) specific T cells could be readily seen above the background levels (Fig. 2A), while the short-term cultures stimulated for 4 days demonstrated a consistent increase in both specificities (~2.9 and 3.1% of total CD8⁺ T cells, Fig. 2B). Frequency of KDL9 or

KEL9 specific T cells in long-term CTL lines, re-stimulated three times *in vitro* were 9.8 % and 7.3% in anti-BALB.B and anti-C57BL/6 CTLs, respectively, (Fig. 2C). Comparatively, the T cell frequency for a subdominant mHAg, HY, was significantly lower in C57BL/6 anti-BALB.B and BALB.B anti-C57BL/6 CTL lines. Therefore, it appears that the positive selection of KEL9 or KDL9 specific T cells is not constrained in the presence of a closely related 'self' peptide.

3.3. Anti-H7a and anti-H7b CTLs mediate comparable levels of reciprocal cytotoxic responses

To further confirm that the close structural homology between KEL9 or KDL9 peptides did not affect the effector functions by respective T cells, we tested CTLs for cytotoxicity responses. T cells obtained from the CTL cultures after three *in vitro* restimulations demonstrated lysis against RMA/S cells loaded with allogeneic KEL9 or KDL9 peptides. No lysis was detected against target cells loaded with self, K^b-binding SL8, or D^b-binding ACL9 peptides (Fig. 2D). These results demonstrate that target cells loaded with either the KEL9 or KDL9 are effectively lysed by anti-C57BL/6 and anti-BALB.B CTLs, respectively. These results demonstrate the fine specificity of TCR recognition and suggest strongly that the strength of TCR recognition is not affected by the presence of a highly homologous self-peptide in the responding mice.

3.4. Novel anchor imposition by p6 arginine instead of canonical p5 asparagine in H7 epitopes

Each MHC haplotype dictates a specific peptide-binding motif (Falk et al., 1990, 1991). Peptide-binding motif analyses helped to reveal exact locations of anchor residues in the amino acid sequence of antigenic peptides (Falk et al., 1991; Rotzschke et al., 1992). For example, peptides eluted from D^b MHC exhibit 'xxxxNxxxM/L', where 'x' is any amino acid. Both biochemical analyses and crystallographic studies provide strong evidence for the uncharged polar side chain of centrally positioned p5-asparagine within this motif to anchor the peptide inside the binding groove (Young et al., 1994). This asparagine residue forms hydrogen bonds with C-pocket residues G⁷⁰, G⁹⁷ and Y¹⁵⁶ in the -pleats that form the floor of the groove (Young et al., 1994). However, structural models of KEL9 or KDL9 peptides bound to D^b MHC generated using RasWin software and Swiss-PdbViewer, demonstrated that both these peptides may utilize arginine at p6 as their anchor residue instead of the canonical asparagine at p5 (Fig. 3). Our models predict that the uncharged polar side chain of this asparagine points outward the peptide groove and may form contact with TCR. In contrast, the positively charged basic side chain of arginine is buried deep inside the centrally located anchor pocket of D^b MHC, securing the H7 epitope within the peptide-binding groove (Fig. 3). We predict that this arginine may bind to the glycine⁹⁷ of the D^b MHC through hydrogen bonds. Thus, the H7 allelic peptides form an exception to the rule in using p6-arginine instead of p5-asparagine as central anchor residue.

To further understand the importance of p5 and p6 residues in H7 peptides, we generated M-K(p5x)DL9 and M-K(p6x)DL9 minigene constructs with a redundant codon NNG/C at 'x' (N = A,C,G or T). These two sets of constructs encode the minimal H7b antigenic peptide (KDL9) with polymorphic amino acids at p5 (KAPD[x]RDTL) and p6 (KAPDR[x]DTL). These constructs allowed 32 different codons encoding all 20 amino acids at 'x'. Although asparagine is encoded by two codons, AAC and AAU, use of NNG/C limits the use of only AAC in this context. Therefore, the expected frequency of plasmid clones that could stimulate the BCZ1644 if the p5 is asparagine is about 1 in 32 total probabilities. To determine the critical role of asparagine at p5 and the compatibility of other amino acids at this position, a total of 360 individual plasmid clones were obtained. These plasmids were transfected into COS7 cells and tested for their ability to activate KDL9 specific BCZ1644

T cells. Results presented in Fig. 4A demonstrate a total of five plasmid DNAs having the ability to activate BCZ1644 T cells. This was statistically lower than the expected probability based on the AAC codon usage. Sequencing of these positive plasmids revealed that all these five clones were utilizing only the AAC codon encoding asparagine. We conclude that amino acid usage in p5 position is highly stringent and any other amino acids apart from asparagine at p5 are highly detrimental to either peptide binding to D^b MHC or TCR recognition by BCZ1644 T cell.

To define the role of p6-arginine as a possible anchor residue, we employed similar strategy. Redundant NNG/C use allows three codons out of 32 possibilities (AGG, CGC or CGG) to encode arginine. We tested a total of 360 individual plasmids in transfection assays and obtained 18 positive clones. If arginine is the only amino acid compatible at p6, the expected frequency for the occurrence of arginine at p6 out of 360 colonies were about 36. Sequencing these plasmid DNAs revealed that p6 position in all these 18 clones were encoded for arginine. Therefore, we conclude that p6-arginine is a critical residue that cannot be substituted with any other amino acid. These results along with the prediction from the structural model of H7 peptides demonstrate that p6-arginine plays an important role as the central anchor residue.

3.5. Anti-H7b TCR exhibits high stringency to variations at the polymorphic p7 position

KEL9 and KDL9 have remarkably identical structures except for a single methylene group. This has a profound effect in generating allo-T cell responses. Both the aspartic acid and glutamic acid are charged and have single double-bonded, uncharged oxygen except for the additional methylene group in the glutamic acid. However, this similarity did not lead to cross reactivity of T cells to respective self-peptides (Fig. 5). Anti-H7a or anti-H7b CTLs and the 47NPZ.1 or BCZ1644 hybridomas failed to recognize self-peptides even at 10,000 fold higher concentrations than the allo peptides (Fig. 2 and *data not shown*). These observations lead us to analyze the relative contribution of this methylene group in determining the stringency with which the BCZ1644 TCR recognizes the KDL9 peptide. Towards this, we introduced a degenerate codon, NNG/C at p7 of the minigene (MK[p7x]L9). Among a total of 464 individual plasmid DNAs tested 15 stimulated the BCZ1644 T cells (one of the representative assays with 159 minipreps shown in Fig. 5A). Of these 15 plasmids, six encoded aspartic acid (Fig. 5B) at p7. Apart from the expected aspartic acid, we have also found NNG/C codons in p7 specifying asparagine (KNL9) in nine other plasmid DNAs. This indicates that an asparagine with an amine group as a side chain instead of a charged oxygen radical can also effectively stimulate the BCZ1644 T cells. The naturally processed KDL9 elicited a relatively stronger BCZ1644 response compared to the KNL9 peptide (Fig. 5B). Thus, the interaction between the CDR3 loop of the TCR and the peptide/D^b complex is potentially specified through hydrogen bonding between the uncharged oxygen and the corresponding contact residues in the TCR. Based on these observations, we conclude that only an extremely limited structural variation can be recognized by the BCZ1644 TCR.

4. Discussion

Antigenic peptides from mH-Ags have served as useful models to understand antigen processing and presentation (Spierings et al., 2003; Engelhard et al., 2002; Malarkannan et al., 1999), immunodominance (van Els et al., 1992; Wolpert et al., 1998; Choi et al., 2002), graft rejections (Korngold and Sprent, 1983), GvL (Spierings et al., 2003; Mutis et al., 2002; Riddell et al., 2002; Nishida et al., 2004), and structural analyses of TCR-peptide/MHC interactions (Ostrov et al., 2002). H7 is one of the classical mH-Ag that was originally defined by Snell. The only difference between the allelic H7 peptides is an additional methylene (–CH₂) group in the side chain that is present in the glutamic acid in H7a

(KAPDNRETL) that is absent in the aspartic acid in H7b (KAPDNRDTL). Such a subtle difference in the antigenic epitope still evokes a strong CTL-mediated immune response leading to graft rejection and GvL effects (Fontaine et al., 2001). Our current study provides a model system to understand the molecular mechanisms by which the host-derived T cells vigorously recognize a peptide that is of exceptional structural homology to a self-peptide.

Towards understanding biological basis for strong TCR recognition of H7 epitopes, we enumerated the number of both the allelic peptides at per cell level. Since tissues such as spleen contains multiple cell types and the level of H7 expression in these cells may vary, we used cell lines to quantify peptide copy numbers. Our results demonstrate that the KAPDNRETL (KEL9) and KAPDNRDTL (KDL9) epitopes are present at about ~300 and ~270 copies per cell, respectively. To further evaluate the reciprocal CTL responses, we quantified the frequency of H7a and H7b specific T cells in *ex vivo*, short-term or long-term T cell cultures. Reciprocal CD8+T cell responses were significant and the frequency of antigen-specific T cells directed to H7a and H7b peptides were comparable between each other. Also, anti-H7a and anti-H7b T cells recognized the respective peptide-loaded targets effectively to mediate cytotoxicity, providing evidence that these CTLs are functionally competent. These results for the first time demonstrate that the presence of a self mH-Ag peptide in the host with high structural homology to an allelic peptide of donor origin does not hinder a strong CTL-mediated immune response. In this context, the allo-reactive TCR could very well be positively selected by the self-peptide than being negatively selected. Currently, we do not have evidence to show whether one or both of these H7 peptides act as a self-agonist during thymic selection of TCRs, which specifically recognize their allelic counter parts. Future experiments with targeted mutation of the H7 epitopes will provide insights on their ability to act as self-ligands for positive selection during T cell selection.

MHC peptide-binding motifs played critical role in the identification of multiple human and murine antigenic epitopes. These motifs are also extensively used in vaccine designs (Rammensee et al., 2002). Earlier studies have demonstrated that obligate contacts between a central anchoring amino acid to the -pleat of MHC either through direct contacts or by water-mediated hydrogen bonding are important to secure the peptide to the groove (Fremont et al., 1995). Apart from this, a hydrophobic amino acid located at the COOH-terminal of the peptide (p) acts as a primary anchor helping to stabilize the peptide with MHC. Since H7 is an immunodominant mH-Ag, understanding how its peptides are anchored to D^b MHC is of tremendous clinical relevance. Both KEL9 and KDL9 have leucine at their COOH-terminal, which helps in the primary anchoring. Models of peptides bound to D^b MHC demonstrated that both H7a and H7b peptides may utilize arginine at p6 as their anchor residue instead of the canonical asparagine at p5. Our model predicts that the uncharged polar side chain of this asparagine points outward the peptide groove and may form contact with TCR. In contrast, the positively charged basic side chain of arginine is buried deep inside the centrally located anchor pocket of D^b MHC, securing KEL9 or KDL9 epitopes within the peptide-binding groove. Absence of the centrally located anchor motifs has been previously reported for two mH-Ag peptides, H13 (Mendoza et al., 1997) and H47 (Mendoza et al., 2001). The H13 peptide that is also presented on D^b MHC contains a glycine at p5 instead of the canonical asparagine or arginine (SSVVGVWYL). In this case, the peptide binding occurs with the help of a water molecule, which stabilizes the peptide and the MHC in the absence of either direct or hydrogen bonding (Ostrov et al., 2002). Similarly, H47-derived peptide, SCILLYIVI, lacks a defined central anchor residue; however the mechanism that helps in its binding to D^b MHC is yet to be elucidated. Compared to H13 and H47 epitopes, H7 peptides may use yet another exclusive mechanism to bind to D^b MHC, in that they use the p6-arginine residue to anchor rather than the p5-asparagine.

The topologies of KEL9 and KDL9 peptides as deduced through structural models are remarkably similar with the most prominent difference being the extension of p7 side chain by a single $-\text{CH}_2$ group in KEL9 peptide (H7a). Presence of one methylene side chain can trigger a number of van der Waals contacts between the CDR3 loop of the TCR and the p7-glutamic acid, which could form the basis for the KEL9/D^b (H7a) recognition. In contrast, how a buried aspartic acid in KDL9/D^b (H7b) creates contact with the TCR is not yet clear. It is likely that p7 is solvent-accessible as shown in the crystal structures of H13 peptides/D^b complexes (Ostrov et al., 2002). Crystallographic structures in association with peptide/MHC complexes have provided evidence that TCR binding can distort the peptide confirmation on the MHC (Garboczi et al., 1996). Thus, it is possible that TCRs that recognize KDL9 peptide may also alter the peptide confirmation to generate unique structural features that are recognized by anti-H7b TCRs.

Introduction of any other amino acids except asparagine at the polymorphic p7 instead of the native aspartic acid significantly abolished the ability of anti-H7b T cell recognition. This demonstrates that only an asparagine with an amine group as a side chain instead of a charged oxygen radical in aspartic acid could effectively stimulate the anti-H7b specific T cells. However, the overall response of BCZ1644 T cells directed to asparagine at p7 was several folds weaker and needed a higher concentration of plasmid DNA to detect a comparable level of T cell activation. Consequently, the inability of any other amino acid to replace aspartic acid at p7 in KDL9 peptide indicates that the anti-H7b TCR of BCZ1644 T cell is extremely constrained in recognizing any modifications at p7 position.

Our results provide intriguing insights into how slight modifications in the peptide structure contribute to allo-TCR recognitions. Our results also provide evidence for the possible anchor imposition by a centrally located p6 anchor residue for D^b MHC rather than the canonical p5 position. Further, mutations at the polymorphic p7 residue in KDL9 peptide demonstrated that the anti-mH-Ag specific TCRs are probably extremely constrained. These observations indicate that additional considerations should be placed in utilizing published motif rules and in future vaccine designs.

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(A) H7 Allelic differences

H7 ^a :	AAA	GCA	CCT	GAC	AAC	AGG	GAG	ACA	CTA
	K	A	P	D	N	R	E	T	L
H7 ^b :	AAA	GCA	CCT	GAC	AAC	AGG	GAT	ACA	CTA
	K	A	P	D	N	R	D	T	L

(B) Naturally processed H7 peptides in cell extracts

Fig. 1.

(A) A single nucleotide modification from *G* to *T* results a conserved amino acid change from glutamic acid (H7^a) to aspartic acid (H7^b) at p7 and defines the polymorphism of D^b restricted H7 mH-Ag. (B) Quantification of H7 peptide copy numbers in cells. Cell pellets were subjected to 10% formic acid and the total acid soluble peptide was extracted and passed through 10 kDa cutoff filters before injected into a reverse-phase HPLC fitted with narrow-bore C18 column. Fractions were collected in 96-well plates, dried, resuspended in 15% DMSO in PBS and used in T cell activation assays as described. Splenocyte extracts from C57BL/6 and BALB.B or from cell lines EL4 and D^b-L were tested for H7 peptides. Synthetic peptides and their mock runs were shown to confirm the molecular identity of the

naturally processed H7 peptides through the matching retention times. Total copy numbers of H7a or H7b peptides per cell were calculated from EL4 and D^b-L cells, respectively. Copy numbers shown are averages from four independent experiments.

(A) Ex vivo

(B) 4-days in vitro

(C) Long-term-antigen specificity

(D) Long-term

Fig. 2.

Flow cytometric analysis of KEL9 (H7a), KDL9 (H7b), and WI9 (HY) specific CD8⁺ T cell frequencies using peptide-loaded D^b MHC dimers. Data presented were obtained with either anti-C57BL/6 (B6) or anti-BALB.B bulk CTL lines and analyzed as (A), *ex vivo*; (B), short term (4 days after one *in vitro* restimulation) or (C), long-term (after 4 weekly restimulations). Results presented are a representative three independent experiments. (D), Cytotoxic potential of anti-H7a and anti-H7b CTLs were analyzed by KEL9 or KDL9-loaded target cell lysis. K^b MHC binding ovalbumin-derived, SIINFEKL (SL8) and D^b MHC binding H3-derived, ASPCNSSTVL (ACL9) were used as negative controls. Data shown are the average of triplicate wells and a representative experiment out of three.

Fig. 3.

RasWin-generated models of H7a and H7b peptides indicate that presence of an additional methylene (–CH₂) side chain modifies the structure of TCR contact residue. (A) Single additional methylene side chain defines the variation between the conserved glutamic and aspartic acids. (B) RasMol molecular graphics program from RasWin software package was used to upload the D^b MHC structure along with the indicated KEL9 or KDL9 peptide sequence. Stick and ball diagram of peptides and D^b MHC shown. Presence of an additional –CH₂ in the side chain significantly extends the p7 TCR contact residue in KEL9 compared to the KDL9 peptide. (C), Wire-frame structures of KEL9 or KDL9 (red) in the peptide-binding groove of D^b backbone (blue). Arrow heads indicate the following novel features: p5-asparagine exposed outside the groove as a TCR contact residue rather than acting as an anchor; p6-arginine buried inside the groove as an anchor residue; and p7 polymorphic residues generate the structural differences that form the H7 allelic peptide discrimination. Numbers indicate the location of each of the nine amino acids in H7 peptides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4.

H7 epitopes contain a novel anchor imposition and no amino acid variations are allowed at p5 and p6 positions by anti-H7b TCR. Minigene constructs that encode the KDL9 peptide with a degenerate codon (NNG/C) for p5 (A) or p6 (B) amino acid position were analyzed for their ability to activate anti-H7b T cells, BCZ1644. NNG/C encodes all 20 amino acids through 32 possible codons. 'X' in the sequence denotes any amino acid. Horizontal cross line denotes the cut-off for the background in the T cell activation assay. A total of 360 individual plasmid clones were tested for either p5x or p6x constructs.

Fig. 5. Analyses of amino acid variations that are allowed for the anti-H7^b TCR recognition. (A), Individual minigene constructs encoding MK[p7X]L9 with a degenerate codon (NNG/C) for p7 position were analyzed for the ability to activate anti-H7^b T cell, BCZ1644. NNG/C encodes all 20 amino acids through 32 possible codons. 'X' in the sequence denotes any amino acid. Horizontal line indicates the background cutoff. Data presented were representation of 159 plasmids from a total of 464 individual clones analyzed. Right-side panel shows non-mutated minigene construct coding for MKDL9 as positive and empty vector as negative controls. (B), Titration concentrations of large scale DNA preparations of positive plasmid clones were tested for their ability to activate BCZ1644 T cells.