The Same Major Histocompatibility Complex Polymorphism Involved in Control of HIV Influences Peptide Binding in the Mouse H-2L<sup>d</sup> System*  

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**Background:** Major histocompatibility complex (MHC) class I polymorphisms at residue 97 have been associated with HIV control and progression.

**Results:** Mouse MHC class I variant L<sup>d</sup>R<sup>97</sup> bound a broader repertoire of peptides than L<sup>d</sup>W<sup>97</sup>.

**Conclusion:** Aromatic-stacking interactions involving W<sup>97</sup> may restrict peptide-binding plasticity.

**Significance:** The homologous HLA-B allele (Arg<sup>97</sup>) likely binds a larger self-peptide repertoire than Trp<sup>97</sup>, resulting in reduced T cell diversity due to tolerance.

Single-site polymorphisms in human class I major histocompatibility complex (MHC) products (HLA-B) have recently been shown to correlate with HIV disease progression or control. An identical single-site polymorphism (at residue 97) in the mouse class I product H-2L<sup>d</sup> influences stability of the complex. To gain insight into the human polymorphisms, here we examined peptide binding, stability, and structures of the corresponding L<sup>d</sup> polymorphisms, Trp<sup>97</sup> and Arg<sup>97</sup>. Expression of L<sup>d</sup>W<sup>97</sup> and L<sup>d</sup>R<sup>97</sup> genes in a cell line that is antigen-processing competent showed that L<sup>d</sup>R<sup>97</sup> was expressed at higher levels than L<sup>d</sup>W<sup>97</sup>, consistent with enhanced stability of self-peptide-L<sup>d</sup>R<sup>97</sup> complexes. To further examine peptide-binding capacities of these two allelic variants, we used a high affinity pep-L<sup>d</sup> specific probe to quantitatively examine a collection of self- and foreign peptides that bind to L<sup>d</sup>. L<sup>d</sup>R<sup>97</sup> bound more effectively than L<sup>d</sup>W<sup>97</sup> to most peptides, although L<sup>d</sup>W<sup>97</sup> bound more effectively to two peptides. The results support the view that many self-peptides in the L<sup>d</sup> system (or the HLA-B system) would exhibit enhanced binding to Arg<sup>97</sup> alleles compared with Trp<sup>97</sup> alleles. Accordingly, the self-peptide-MHC-Arg<sup>97</sup> complexes would influence T-cell selection behavior, impacting the T-cell repertoire of these individuals, and could also impact peripheral T-cell activity through effects of self-peptide-L<sup>d</sup> interacting with TCR and/or CD8. The structures of several peptide-L<sup>d</sup>R<sup>97</sup> and peptide-L<sup>d</sup>W<sup>97</sup> complexes provided a framework of how this single polymorphism could impact peptide binding.

Human leukocyte antigens (HLA)<sup>2</sup> encoded by the major histocompatibility complex (MHC) are among the most polymorphic genes in the population (1). Class I MHC gene products display short peptides of 8–10 amino acids on the surface of all nucleated cells (2), and these complexes are recognized by the αβ T cell receptors (TCR) on the surface of T cells. TCR binding of pep-MHC molecules above a minimal threshold results in T cell activation and effector function such as target cell lysis (3–5).

The MHC locus has been shown to be associated with protection from or susceptibility to various diseases, including infectious diseases of viral origin such as HIV, as well as autoimmune conditions (6–12). Ultimately the MHC-associated control or progression of diseases is related to the presence of functional T cells that express TCRs that bind to antigenic (or self) peptide-MHC complexes. The presence of functional T cells, and the breadth of the TCR repertoire, is controlled both by thymic selection processes that occur during development of a T cell, and peripheral processes that regulate mature T cell activity (13). In the thymus, positive and negative selection operates on T cells through the αβ TCR and the CD4 and CD8 co-receptors. T cells that have TCRs with a low affinity for self-peptides presented on self-MHC molecules are positively selected, yet T cells that express TCRs with higher affinity for self-peptide-MHC complexes are clonally deleted (14–16).

It is widely hypothesized that the diversity of peripheral T cells of an individual correlates with the effectiveness of the adaptive immune system in eliminating viral infections, and that the breadth of the TCR repertoire correlates in turn with the diversity of self-peptides presented by MHC alleles of the individual during development (17). Thus, self-protein antigen processing pathways are critically linked to immune potential (16, 18–20). Importantly, the peripheral regulatory processes are also influenced by interactions of T cells with self-pep-MHC complexes, which can control functional T cell activity by promoting the deletion or anergy of particular T cells (as reviewed in Refs. 21–23).

Recent studies have implicated several positions of the human MHC locus HLA-B in the control and progression of HIV infection. Positions 67, 70, and 97 of HLA-B located in the peptide-binding groove, showed the strongest associations with differences in the frequency of HIV controllers and progressors (9). It has been hypothesized that these amino acid
positions in HLA-B could contribute to disease control or progression by affecting the ability of either foreign peptides or self-peptides to bind the different alleles; the former could operate by inducing favorable immunity, and the latter could operate by influencing thymic selection (as reviewed in Ref. 24). However, biochemical and structural explanations for these effects remain unknown, and thus a mechanistic explanation of the polymorphisms is yet to be developed.

The mouse MHC (known as H-2) represented the first system to reveal the properties of polymorphism and the process of T cell recognition that required MHC "restriction" of the antigen (25). The class I H-2 molecule Ld has been well studied and is known to exhibit lower stability than many other class I products such as K\textsuperscript{b} (26–30). In addition, it appears that peptides that bind to Ld\textsuperscript{a} may be influenced by more of their individual residues than peptides that bind to K\textsuperscript{b}, which appear to have dominant anchor residues (31). Relevant to recent information about HIV and HLA-B polymorphisms are previous studies showing that two alleles called L\textsuperscript{a} and Ld\textsuperscript{d} contained the same polymorphism at residue 97 as in the HLA-B studies, and these alleles exhibited differences in stability between the ternary complexes (peptide, H-2L heavy chain, and \beta\textsubscript{2}-microglobulin) (32). In an independent approach, the W97R substitution was identified in a “needle in a haystack” experiment in which more stable Ld\textsuperscript{d} mutants were identified because they conferred higher surface levels of the protein in a yeast display system (33). Thus, completely different approaches have revealed the importance of position 97 in Ld\textsuperscript{d} stability. The crystal structures of Ld\textsuperscript{d} complexes with Trp (34, 35) and Arg (36–38) have also been solved, providing additional mechanistic insight.

To examine more fully the hypotheses regarding the impact of the position 97 polymorphisms on peptide binding, here we introduced two variants, Ld\textsuperscript{W97} and Ld\textsuperscript{R97}, into a mouse cell line and examined various aspects of peptide binding and stability of the complexes. The binding analysis was facilitated by the use of a high affinity, soluble TCR that was available against the specific peptide complex called QL9-Ld\textsuperscript{d}, allowing a quantitative assay of binding by a panel of peptides. The results showed that Ld\textsuperscript{R97}, compared with Ld\textsuperscript{W97}, conferred a greater average stability to peptide-Ld\textsuperscript{d} complexes and resulted in a broader repertoire of peptide binding from the panel of peptides. Each peptide tested showed preferential binding to one of the Ld\textsuperscript{d} variants, but most of them bound better to Ld\textsuperscript{R97}.

The structures of several different peptides bound to one or the other Ld\textsuperscript{d} variants suggested that the Arg\textsuperscript{97} residue acts not by having increased flexibility itself, but by tolerating more side chain flexibility in the vicinity of the peptide and adjacent MHC residues, compared with Trp\textsuperscript{97}. Overall, these findings are consistent with the hypothesis that many self-peptides will be presented at physiologically higher levels by an allele with Arg\textsuperscript{97}, compared with an allele with Trp\textsuperscript{97}. Thus, our results support the notion that Arg\textsuperscript{97} alleles exhibit a preference for a progres sor phenotype in HIV patients because potentially effective T cells in these individuals have been deleted by negative selection in the thymus. It is also possible that these self-peptide-Arg\textsuperscript{97} allele complexes may be capable of driving enhanced peripheral T cell anergy (as reviewed in Ref. 21).

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Ld\textsuperscript{a} Transfectants**—A derivative of BW5147, a mouse T cell thymoma (AKR mouse, H-2\textsuperscript{b}), was used for the Ld\textsuperscript{a} studies because it is readily transduced by the pMP71 retroviral vector, and it is capable of normal antigen processing of self and foreign peptides (e.g. it is TAP sufficient). The BW5147 subline called 58–/– was used to generate Ld\textsuperscript{a}-transfectants, as described below. BW5147 and the hybridoma 30-5-7 that secretes an anti-Ld\textsuperscript{a} monoclonal antibody (against the \alpha\textsubscript{2} domain) were cultured in RPMI 1640 media containing 10% fetal calf serum at 37 °C with 5% CO\textsubscript{2}.

The gene encoding the full-length murine MHC allele heavy chain Ld\textsuperscript{W97}, residues 1–338 with a 24-amino acid leader sequence, was cloned into retroviral vector pmp71 as a NotI-EcoRI fragment. The Ld\textsuperscript{W97}-pMP71 ligation product was obtained and a single-site mutation was made using the Stratagene QuikChange Lightning kit to generate a pMP71 construct containing the gene for Ld\textsuperscript{R97}. BW5147-Ld\textsuperscript{W97} and BW5147-Ld\textsuperscript{R97} cell lines were generated by retroviral transduction (38). Briefly, 20 \mu g of Ld-pMP71 DNA was incubated with 60 \mu g of Lipofectamine 2000 (Invitrogen) in 3 ml of Opti-MEM media (Invitrogen) for 20 min at room temperature. DMEM culture supernatant was removed from 3 × 10\textsuperscript{6} adherent Plat-E retroviral packaging cells and 1.5 ml of the DNA/Lipofectamine/Opti-MEM mixture was added. Plat-E cells were incubated with the DNA mixture for 3 h at 37 °C, then the DNA mixture was removed. Plat-E cells were washed with 7 ml of RPMI 1640 media, then incubated with 6.5 ml of RPMI 1640 media for 48 h at 37 °C. BW5147 cells (2 × 10\textsuperscript{6}) were transduced by centrifuging the cells in a 24-well plate (Corning costar) at 652 × g for 45 min with 3 ml of supernatant from transduced Plat-E cells. BW5147 cells were cultured overnight at 37 °C before being transferred to a T25 flask with 5 ml of RPMI 1640 media and cultured at 37 °C with 5% CO\textsubscript{2}. Ld\textsuperscript{a}-positive populations of BW5147 cells, expressing Ld\textsuperscript{W97} or Ld\textsuperscript{R97}, were enriched by staining with a saturating amount of 30-5-7 anti-Ld\textsuperscript{a} monoclonal antibody (20 \mu g/ml; purified from ascites fluid) followed by PE-conjugated goat F(ab\textsuperscript{2})\textsubscript{2} anti-mouse IgG secondary antibody (1.25 \mu g/ml; Southern Biotech) (39). After fluorescence activated cell sorting (FACS), isolated cells were cultured in RPMI 1640 at 37 °C with 5% CO\textsubscript{2}.

**Soluble High-affinity T Cell Receptor**—The soluble TCR called 2C-m6 binds to the QL9-Ld\textsuperscript{d} complex with high affinity (K\textsubscript{D} value 5 nM) (40). A single chain form of the 2C-m6 TCR (scTCR) was expressed in Escherichia coli, refolded from inclusion bodies, and purified as described previously (33). Purified 2C-m6 scTCR was biotinylated using the EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Thermo Scientific) and stored at 4 °C (41).

**Measurement of Cell Surface Ld\textsuperscript{a} Levels**—Cell surface levels of Ld\textsuperscript{a} variants were evaluated using transduced BW5147 cells and anti-Ld\textsuperscript{a} antibody. BW5147 cells (2 × 10\textsuperscript{6}) expressing Ld\textsuperscript{W97} or Ld\textsuperscript{R97} were incubated with RPMI alone or with peptides at various concentrations in RPMI for 3 h, then washed and incubated with 20 \mu g/ml of 30-5-7 anti-Ld\textsuperscript{a} mAb followed by PE-labeled goat F(ab\textsuperscript{2})\textsubscript{2} anti-mouse IgG secondary antibody. Samples were washed, fixed with 1% paraformaldehyde in PBS, and
analyzed for PE fluorescence using an Accuri C6 flow cytometer. Data were analyzed using FCS Express.

$L^d$-binding Peptides and Recognition by 2C-m6 scTCR—The following $L^d$-binding peptides were used in this study: QLSPPFPFDL (called QL9); YPHFMPNTNL (called MCMV); TQNHRLADL (called tum−); FLSPFWFDI (called FLS), QLSDSPFMDL, SPLDSLWVWI, MPKPLSL, QPQHTVRLS, QFTTLPAGL. Peptides QLSDSPFMDL, SPLDSLWVWI, and FLSPFWFDI were identified using yeast display, as described previously (38). The remaining peptides, MPKPLSL, QPQHTVRSL, and QFTTLPAGL, were identified by mass spectrometry from HPLC fractions that contained peptides obtained from BALB/c liver tissue using an anti-Ld affinity purification scheme. The details of their isolation will be described in a separate report. Peptides were synthesized either by the University of Illinois at Urbana-Champaign Protein Sciences Facility (MCMV, SPLDSLWVWI, MPKPLSL, QPQHTVRSL, and QFTTLPAGL) or by Genscript (QL9, tum−, QLSDSPFMDL, and FLSPFWFDI) and stored in 50 mM dimethyl sulfoxide at −20 °C.

Synthetic peptides were assayed to assess binding of soluble single chain 2C-m6 to each peptide-$L^d$ complex. BW5147-$L^d$W97 or BW5147-$L^d$R97 cells (2 × 10⁶) were incubated with 100 μM peptide diluted in RPMI for 3 h at 37 °C. Cells were washed and incubated with 10 μg/ml of soluble biotinylated 2C-m6 scTCR for 40 min on ice. Cells were washed and incubated with 2.5 μg/ml of phycoerythrin-conjugated streptavidin (Streptavidin-PE, BD Pharmingen) before fixing with 1% paraformaldehyde. Samples were analyzed by flow cytometry on an Accuri C6 cytometer, and data were analyzed using FCS Express.

$L^d$-binding Assay—Because initial studies with a reference biotinylated peptide yielded very low signal to background, we developed a novel competition assay to evaluate the relative strength of each peptide in binding to $L^d$W97 and $L^d$R97. BW5147 cells (2 × 10⁶) expressing $L^d$W97 or $L^d$R97 were incubated with a mixture containing 50 nm QL9 and various concentrations of competitor peptides in RPMI (note that these competitor peptides were not detectably bound by the soluble TCR). After 2.5 h at 37 °C, cells were washed and incubated with 10 μg/ml of biotinylated 2C-m6 scTCR. After washing, cells were incubated with 2.5 μg/ml of streptavidin-PE (BD Pharmingen). Samples were fixed with 1% paraformaldehyde in phosphate-buffered saline and analyzed for PE fluorescence using an Accuri C6 flow cytometer. Data were analyzed using FCS Express. Mean fluorescence units (MFU) of samples incubated with competitor peptide and QL9 (MFUₐ) was divided by mean fluorescence units of samples incubated with QL9 alone (MFUₐ). BD₉₀ values were obtained using non-linear regression analysis (GraphPad Prism).

Soluble $L^d$R97 Stability Assay—The stabilized $L^d$m31 form (21 kDa) of MHC $L^d$ was expressed in E. coli as inclusion bodies and refolded as described previously (33, 37). Briefly, 3 μM $L^d$m31 inclusion bodies was refolded with 30, 6, or 1.2 μM dimethyl sulfoxide-dissolved peptide (MCMV, FLS, or tum−) in 25 ml of buffer containing 100 mM Tris, pH 8.0, 400 mM L-arginine, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM reduced glutathione, and 0.5 mM oxidized glutathione for 48 h at 4 °C, without refolding. Refolded mixtures were filtered through 0.22-μm vacuum filter (Millipore) and concentrated to 50 μl using a 10-kDa cut off filter (Amicon). Samples for SDS-PAGE were prepared using 8.3 μl of each concentrated protein refold, and 4−20% gradient polyacrylamide gels (Bio-Rad) were run for 45 min at 120 V, and stained with Coomassie Blue.

Scanned gel images were analyzed using the ImageJ public domain Java image processing program, first by converting the scanned image into 16-bit grayscale, and then by obtaining the area of each band and the average intensity of the band. The average intensity of each band was multiplied by the area of the band, to generate overall intensity. These values were considered proportionate to the yield of each peptide-$L^d$ complex. The overall intensity value of the band corresponding to $L^d$m31 refolded in the absence of peptide (no peptide) was set to 1, and all protein yield values relative to this were calculated.

Peptide-MHC Structure Analysis—Structures of the following peptide-$L^d$ complexes were analyzed QLSDSPFMDL-$L^d$R97 (PDB ID 3TFK), FLSPFWFDI-$L^d$R97 (PDB ID 3TPU), SPLDSLWVWI-$L^d$R97 (PDB ID 3TH), and peptide-$L^d$W97 structures (PDB IDs 1LD9 and 1L9). In some cases, structures were aligned and highlighted for presentation using PyMOL. Peptide-HLA-B structures examined were: RRRWRLRTL-HLA-B*14:02 (PDB ID 3BVN), VPLRPMTY-HLA-B*27:05 (PDB ID 1A1N), KGFNPVPMPM-HLA-B*57:03 (PDB ID 2HIK), GRFAAAAKAK-HLA-B*27:05 (PDB ID 1JGE), TAFTPISI-HLA-B*51:01 (PDB ID 1E28), RPHERNGFTVL-HLA-B*07:02 (PDB ID 3VCL).

HLA-B Sequence Analysis—HLA-B sequences were obtained from the International ImMunoGeneTics (IMGT) Information System, and aligned using the IMGT alignment tool. Truncated HLA-B sequences were not included in the tabulation of the amino acid identity at positions 114, 116, and 156 of the full-length protein.

RESULTS

Complexes of Self-peptide-$L^d$R97 Are Expressed at Higher Levels on Cells Than Complexes of Self-peptide-$L^d$W97—A previous study showed that the two H-2 alleles $L^d$ and $L^q$ differed at six positions, including position 97 (Trp in $L^d$ and Arg in $L^q$) (32, 42), the same single site polymorphism shown to be involved in HLA-B control of HIV (9). Experiments performed with single site mutants of $L^d$(W97R) and $L^q$(R97W) on the surface of DAP-3 cells using antibodies against assembled and empty $L^q$ or $L^d$ molecules suggested that Arg₉⁷ may contribute to greater cell surface stability, stronger binding to endogenous peptides, and higher affinity for β₂-microglobulin compared with Trp₉⁷ (32).

To determine whether this observation extended to a different antigen-processing competent cell line, we transduced the BW5147 (H-2k) cell line with wild-type $L^d$ (L₄W97) and the single site mutant $L^d$R97, using these genes inserted into the pMP71 retrovirus vector (43). To verify expression, cells were stained with the anti-$L^d$ mAb 30-5-7 and analyzed by flow cytometry (Fig. 1A). Surface levels of $L^d$R97, as judged by the MFU with anti-$L^d$ staining were consistently twice as high as surface levels of $L^d$W97 (Fig. 1A). As it is well known that the
surface levels of MHC are controlled in part by the stability of the peptide-class I heavy chain-β2m complex, this finding suggests that either there are many more endogenous self-peptides that can associate well with L^dR97, or there is a smaller subset of abundant self-peptides that bind better to L^dR97, or both.

In the L^d system, “up-regulation” assays depend on the ability of an exogenous synthetic peptide to stabilize the peptide-β2m complex and thus increase its total surface levels. The inability of the L^d (especially L^dW97) cell surface levels to be increased in the presence of some peptides is likely to be related to the expression of TAP and hence self-peptide complexes. We reasoned that perhaps L^d levels could be enhanced at lower temperatures or in the presence of β2-microglobulin (β2m). Previous studies using the RMAS cell line (45, 46) showed that lower temperatures (26 °C) allowed enhanced accumulation of empty MHC molecules at the cell surface, whereas addition of β2m during peptide loading also increased MHC surface levels on RMAS cells (47). However, neither incubation of the BW5147 cells at a lower temperature (26 °C) nor inclusion of β2m during peptide loading increased surface levels of L^dW97 or L^dR97 (data not shown).

L^dR97 Binds Some, but Not All, Peptides Better Than L^dW97—As indicated, the higher levels of self-peptide L^dR97 complexes here and in a previous study (32) could be due to a few abundant self-peptides that bind to L^dR97 better, or to a large fraction of self-peptides that bind better. The L^d up-regulation assay provides only a semi-quantitative assessment of L^d-binding ability for some peptides, we developed a competition assay that took advantage of a unique soluble TCR reagent, called 2C-m6, which detects complexes of QL9-LdW97 and QL9-LdR97. Based on peptide titrations (Fig. 2A), we estimated that the QL9 peptide binds ~2–3-fold better to L^dR97 than to L^dW97 (BD50 values of 85 nM on L^dW97 and 17 nM on L^dR97).

To examine additional L^d-binding peptides, we used a panel of peptides that had originally been isolated by one of several methods: previously characterized foreign peptides (tum and MCMV), peptides derived from a yeast-display screen involving a single chain form of L^dR97 (38), or peptides eluted from L^d as part of an anti-L^d affinity purification from BALB/c (L^dW97*) tissue. Thus, these peptides had been isolated based in part on their ability to associate with L^d. These peptides, when bound to L^dW97 or L^dR97, are not detected by the soluble 2C-m6 TCR, although weak binding was detected with peptide FLSFWDID at very high concentrations. This allowed the competitive assay to be used to determine quantitatively the binding affinity of the collection of peptides for L^dW97 and L^dR97.

In the competition assay format, cells bearing either L^dW97 or L^dR97 were incubated with synthetic peptides that were titrated in the presence of 50 nM QL9, followed by staining with the 2C-m6 TCR. Decreased fluorescent signal indicated that the competitor peptide effectively competed for binding to the anti-L^d monoclonal antibody. The level of up-regulation in response to incubation with each peptide concentration was determined relative to L^d surface levels in the absence of peptide, and the concentration of peptide resulting in 50% maximal up-regulation (SD50) was determined (Fig. 1B). Peptide MCMV induced up-regulation of L^dW97, with an SD50 value of ~0.12 μM. Peptide FLS induced modest up-regulation at only the highest concentration, 100 μM, whereas neither QL9 nor tum exhibited any detectable up-regulation. In contrast, peptides QL9, MCMV, and FLS induced significant up-regulation of L^dR97 on the BW5147 cell surface, with SD50 values in the order of MCMV < QL9 < FLS (Fig. 1B). Thus, the addition of exogenous peptides enabled the cell surface levels of L^dR97 to be increased more effectively than the levels of L^dW97.

In the competition assay format, cells bearing either L^dW97 or L^dR97 were incubated with synthetic peptides that were titrated in the presence of 50 nM QL9, followed by staining with the 2C-m6 TCR. Decreased fluorescent signal indicated that the competitor peptide effectively competed for binding to the
**MHC Polymorphisms and Peptide Binding**

**FIGURE 2.** Peptide-binding assays show variation in preference for binding to L<sup>d</sup>W97 or L<sup>d</sup>R97. A. BW5147 cells transfected with genes encoding L<sup>d</sup>W97 or L<sup>d</sup>R97 were incubated with varying concentrations of QL9 (QLSPFPFDL) peptide in RPMI, and QL9-L<sup>d</sup> surface complexes were evaluated by 2C-m6 scTCR staining. B–D, peptides QLSDVPMDL, TQNHRALDL (tum–), and MPKPLSL, respectively, in a competition assay with 50 nM QL9 for binding to cell surface L<sup>d</sup>W97 and L<sup>d</sup>R97. QL9-L<sup>d</sup> complexes on the surface were specifically determined by staining with soluble 2C-m6 scTCR. MFU<sub>0</sub> indicates mean fluorescence units of samples incubated with QL9 alone, and MFU<sub>1</sub> indicates mean fluorescence units of samples incubated with QL9 and competitor peptides at various concentrations.

MHC, thus decreasing the 2C-m6 scTCR binding and fluorescent signal. In this assay, peptide QLSDVPMDL competed with the QL9 peptide on L<sup>d</sup>W97 and L<sup>d</sup>R97 with an average difference in BD<sub>50</sub> of 14.7 (Fig. 2B). In contrast, peptide tum– (TQNHRALDL) competed with the reference peptide QL9 on L<sup>d</sup>W97 and L<sup>d</sup>R97 with an average difference in BD<sub>50</sub> of 0.11-fold (L<sup>d</sup>W97/L<sup>d</sup>R97) (Fig. 2C). Heptamer peptide MPKPLSL competed with QL9 peptide on L<sup>d</sup>W97 and L<sup>d</sup>R97 with an average difference in BD<sub>50</sub> of 0.54 (Fig. 2D).

In total, 8 peptides were evaluated for binding to L<sup>d</sup>W97 and L<sup>d</sup>R97 using the same peptide binding competition assay (Table 1). Peptide binding curves were used to calculate BD<sub>50</sub> values, and the relative peptide binding to L<sup>d</sup>R97 compared with L<sup>d</sup>W97 was determined by the ratio of BD<sub>50</sub> (L<sup>d</sup>W97)/BD<sub>50</sub> (L<sup>d</sup>R97). The results of this analysis indicated that six of the peptides bound better to L<sup>d</sup>R97 compared with L<sup>d</sup>W97, whereas the two others (tum– and MPKPLSL) bound better to L<sup>d</sup>W97 compared with L<sup>d</sup>R97 (Fig. 3). Peptide FLS (FLSPFWFDI) showed the greatest disparity in binding L<sup>d</sup>R97 versus L<sup>d</sup>W97 (Fig. 3 and Table 1). Each of the peptides had a clear preference for binding to one or the other L<sup>d</sup> position 97 variant; none of the eight tested peptides bound equally well to both L<sup>d</sup>W97 and L<sup>d</sup>R97.

Peptide Stabilization of Soluble L<sup>d</sup> Correlates with the Cell Surface L<sup>d</sup> Binding Assay—To determine whether the results of the cell-based assays extended to a cell-free system, we used a soluble form of the L<sup>d</sup>R97 molecule called L<sup>d</sup>m31 (33). L<sup>d</sup>m31 contains only the α1 and α2 domains and the W97R mutation, thus eliminating effects due to β2m association. L<sup>d</sup>m31 was originally selected by yeast display of a library of L<sup>d</sup> mutants to overcome problems with the use of full-length L<sup>d</sup>, which has been difficult to express in soluble form in E. coli.

Thus, to assess peptide stability, inclusion bodies of the L<sup>d</sup>m31 (21 kDa) molecule (3 μM) were refolded in the presence of three different concentrations of the peptides MCMV, FLS, and tum–. Samples from each refold were concentrated and analyzed by SDS-PAGE to determine amounts of refolded complexes as a measure of stability (Fig. 4A). As expected, L<sup>d</sup>m31 refolded in the absence of peptide resulted in a low yield of soluble 22-kDa protein. In contrast, L<sup>d</sup>m31 refolded in the presence of all concentrations of MCMV peptide had the largest yields of peptide-L<sup>d</sup>m31 complexes, followed by FLS-L<sup>d</sup>m31 complexes, and tum-L<sup>d</sup>m31 complexes.

The yields of peptide-L<sup>d</sup>m31 complexes, as determined by quantitative image analysis of gel bands (Fig. 4B), showed that MCMV at 1.2 μM peptide was more stable than either FLS or tum– at 30 μM, consistent with the results of the cell-based competition assay (Figs. 1C and 3, Table 1). A comparison of the yields at 1.2 μM peptide in the refolding mixtures showed that the hierarchy of stability of MCMV, FLS, and tum– as soluble L<sup>d</sup>m31 complexes (Fig. 1D) correlated directly with the BD<sub>50</sub> values of the competition assay (Fig. 1C and Table 1) and the SD<sub>50</sub> values of the up-regulation assay, each with cell surface L<sup>d</sup>R97 (Fig. 1B). Thus, we conclude that the cell-based competition assay is a valid surrogate for measuring actual stability of peptides with L<sup>d</sup>.

**Arg<sup>77</sup> Tolerates Greater Side Chain Flexibility of Adjacent Residues than Trp<sup>97</sup>**—The results above showed that all the peptides tested have a clear preference for binding to one particular L<sup>d</sup> variant, and more peptides have superior binding to L<sup>d</sup>R97 compared with L<sup>d</sup>W97. To explore the possible role of the position 97 residue on structural features of peptide binding, we were in the unique position of having high-resolution crystal structures of several of the peptides bound to either L<sup>d</sup>W97 (Fig. 5A) or L<sup>d</sup>R97 (Fig. 5B) (34–36, 38). Three other adjacent residues, Tyr<sup>156</sup>, Phe<sup>116</sup>, and Glu<sup>114</sup>, are also known to be polymorphic in the human HLA-B gene, although these polymorphisms appeared to be less associated with HIV immunity than position 97 polymorphisms (8, 9, 48–50). In the L<sup>d</sup>W97 complexes, Trp<sup>97</sup> formed aromatic stacking interactions with Phe<sup>116</sup> and Tyr<sup>156</sup>, and these three side chains were in similar spatial orientations regardless of the peptide sequence. Although these residues do not appear to interact directly with peptide, they could present a steric barrier that...
TABLE 1

Peptide binding to LdW97 and LdR97

| Peptide    | Origin                                                                 | BD50 W97 m | BD50 R97 m | Ref. |
|------------|------------------------------------------------------------------------|------------|------------|------|
| QLSPPFDL (QL9) | Murine 2-oxoglutarate dehydrogenase (928–936) | Reference peptide | Reference peptide | 62   |
| YPHFMPTNL (MCMV) | Murine cytomegalovirus pp89 (168–176) | 3.4 × 10⁻⁷ ± 4.5 × 10⁻⁸ | 1.1 × 10⁻⁷ ± 7.5 × 10⁻⁸ | 63, 65 |
| TQNHRLDL (turn–) | Mutated P915 protein P91A [14–22] containing R17H mutation | 1.7 × 10⁻⁵ ± 7.07 × 10⁻⁶ | 1.5 × 10⁻⁴ ± 7.6 × 10⁻⁵ | Unpublished |
| QLSVDPMDL | QL9 derivative from in vitro selection | 4.4 × 10⁻⁵ ± 3.5 × 10⁻⁵ | 3.1 × 10⁻⁶ ± 2.5 × 10⁻⁶ | 38 |
| SPLDSLLWWI | In vitro selection | >2 × 10⁻⁴ | 1.3 × 10⁻⁵ ± 1.5 × 10⁻⁶ | 38 |
| FLSFPFWFDI (FLS) | QL9 derivative from in vitro selection | >2 × 10⁻⁴ | 5.2 × 10⁻⁵ ± 2.1 × 10⁻⁷ | 38 |
| MKPKPLSL | Murine myb-related protein8 (606–612) | 4.3 × 10⁻⁵ ± 1.1 × 10⁻⁵ | 9.2 × 10⁻⁵ ± 4.9 × 10⁻⁵ | Unpublished |
| QPQHTVRLS | Murine leucine-rich repeat and calponin homology domain-containing protein2 (80–88) | 1.6 × 10⁻⁴ ± 5.5 × 10⁻⁵ | 3.7 × 10⁻⁵ ± 1.6 × 10⁻⁵ | Unpublished |
| QFTTPLAGL | Murine peripheral-type benzodiazepine receptor-associated protein1 (966–974) | >2 × 10⁻⁴ | 5.8 × 10⁻⁵ ± 2.3 × 10⁻⁵ | Unpublished |

*Concentration of peptide resulting in 50% binding (BD50) to each Ld variant. LdW97 and LdR97 BD50 values for peptides sampled in the peptide-binding assay as described in the legend to Fig. 2.

![FIGURE 3](image3.png)

Among a panel of peptides, most individual peptides bind more efficiently to LdR97 compared with LdW97. A panel of peptides was evaluated for binding to LdW97 or LdR97 using the peptide-binding competition assay described in the legend to Fig. 2. Shown is the log of the ratio of BD50 values for peptides binding to LdW97 compared with LdR97 taken from each MFU/MFU versus log [peptide] binding assay plot.

![FIGURE 4](image4.png)

The relative stability of soluble peptide LdR97 complexes correlates with cell-based assays. A, inclusion bodies of Ld m31 (Arg97) were refolded at 3 μM in the absence of peptide, or with various concentrations of peptides MCMV, FLS, and tum–. Protein refold preparations were concentrated to equal volumes and analyzed by SDS-PAGE. B, the intensity of each band shown in A was determined using ImageJ software. These values were calculated relative to the intensity of the band corresponding to Ld m31 refolded in the absence of peptide, which was set to 1.

MHC Polymorphisms and Peptide Binding

In contrast to the LdW97 structures, in the LdR97 structures Arg97 was in a position to mediate strong electrostatic interaction with Glu114 (Fig. 5B). In addition, the absence of Trp97, Tyr156 and Phe16 resides too far apart to form π-π stacking interactions with each other. Perhaps as a consequence of the absence of such interactions, in each peptide-LdR97 structure, Tyr156 was observed in a different spatial orientation, oriented toward the peptide, or away from the peptide, with various degrees of rotation in any plane.

Interestingly, in the peptide-LdR97 structures, the Arg97 side chain does not apparently use additional flexibility to accommodate different peptides in the binding groove, as has been proposed in the HLA-B system (51). Rather, the absence of the tryptophan at position 97 allowed Tyr156 to exhibit additional flexibility and thus to assume a variety of orientations. Accordingly, in LdR97 complexes, Tyr156 is free to stack or interact with aromatic residues of the peptide, such as aromatic residues at position 5 as found in both QL9 and FLSFPFWFDI. Two possible reasons for the rigidity of the Arg97 side chain are evident from existing peptide-LdR97 structures. Electrostatic interactions between side chains of Arg97 and Glu114, as well as π-π stacking interactions between Arg97 and Phe16 may contribute to holding Arg97 in the same position regardless of the peptide sequence. The corresponding plasticity of residue 156 may allow some peptides, including self-peptides, to interact more favorably with alleles that contain Arg97.

HLA-B Polymorphic Variants Have Similar Structural Features with LdW97 and LdR97—The frequency of HLA-B alleles that contained Arg97 was found to be 1.7-times greater in HIV progressors, compared with controllers (9). Conversely, the frequency of HLA-B alleles that contained Trp97 was 2 times greater in HIV controllers, compared with progressors. Trp97 and Arg97 are positioned in the floor of the HLA-B peptide binding groove (Fig. 6A), in the same position as residue 97 in Ld. Position 156 in HLA-B alleles with Trp97 (40 total alleles) are overwhelmingly skewed toward leucine (97.5%), thus preventing aromatic interactions as seen in the Ld system with Tyr156. In HLA-B alleles that contain Arg97 (1360 total alleles), there is greater diversity at position 156 (e.g., 67.5% leucine, 14.3% aspartic acid, 14% tryptophan, etc.). Based on our analysis of the Ld system, this feature alone may allow additional plasticity in the binding of peptides by the Arg97 alleles.
In HLA-B alleles with Trp\textsuperscript{97}, the majority contained asparagine at position 114 and phenylalanine at position 116 (Fig. 7A). Thus, like L\textsuperscript{4}, the Trp\textsuperscript{97} HLA-B alleles likely promote aromatic interactions within the peptide-binding groove, reducing the plasticity associated with binding of some peptides. In HLA-B alleles with Arg\textsuperscript{97}, the majority contained an aspartic acid residue at position 114 and a serine residue at position 116 (Fig. 7B). As in the L\textsuperscript{4} system, Arg\textsuperscript{97} and Asp\textsuperscript{114} likely form electrostatic interactions that accommodate more flexibility in the pocket created at residues in positions 156 and 116 (48). Thus, collectively, the Arg\textsuperscript{97} alleles are able to bind a larger collection of peptides, as observed in the L\textsuperscript{4}R97 results here.

**DISCUSSION**

The results of the recent International HIV Controllers Study suggested that polymorphic position 97 of HLA-B was the most significant residue that correlated with HIV disease control or progression. Although much has been learned about aspects of the HLA-B alleles in terms of disease association and structure, little attention has been paid to a mouse MHC system (H2-L) that has structural and genetic similarities to the HLA-B system, and could inform studies of HIV immunity. Notably, these genetic similarities include two alleles, L\textsuperscript{d} and L\textsuperscript{q}, which express Trp\textsuperscript{97} and Arg\textsuperscript{97} polymorphisms, respectively (42). Furthermore, the Arg\textsuperscript{97} substitution has been shown, by a completely independent approach, to stabilize the peptide-H\textsuperscript{2}-L\textsuperscript{d} complex (33).

Our conclusions here are that: 1) L\textsuperscript{d}R97 allows a greater repertoire of self-peptides to be bound more stably, compared with L\textsuperscript{d}W97; and 2) the structural correlate of these peptide-binding properties appears to involve increased plasticity in the class I pocket. The plasticity is enabled by arginine at position 97, but it is not due directly to flexibility of Arg\textsuperscript{97} (51), but rather that Arg\textsuperscript{97} allows adjacent residues the freedom to move in the pocket. We suggest that these same conclusions likely hold true for the HLA-B alleles, given the striking structural similarities between the two systems.

What is the structural basis for why a substantial number of the individual peptides bound better to L\textsuperscript{d}R97 than to L\textsuperscript{d}W97? Our analysis of the sequences of these peptides, and the structures of several of them in complex with L\textsuperscript{d}R97 (Fig. 5B), does not reveal any obvious residue or motif shared among them (note that for L\textsuperscript{4}, the anchor residues are thought to be a preferred proline at position 2, and a leucine or isoleucine at the C terminus). Thus, rather than pointing toward a specific structural feature of these peptides, the more general basis for their enhanced binding appears to be the nature of the pocket near residue 97.

Residue 97 is centrally located in the peptide-binding groove between the \(\alpha_1\) and \(\alpha_2\) domains of the L\textsuperscript{d} heavy chain, with the side chain directed into the groove (Fig. 6B). A bulky tryptophan at that position forces the neighboring residues, including the side chains of residues 114, 116, and 156 into relatively invariant positions. This pocket cannot accommodate peptide variability to the same extent as an allele with Arg\textsuperscript{97} at that position. This is because Arg\textsuperscript{97} allows some of these same neighboring side chains (and residue 156 in particular) the flexibility to accommodate alternate peptides (Fig. 6B). For example, the structure of peptide QLSDVPMDL showed that the hydroxyl group of the Tyr\textsuperscript{156} side chain was displaced by 9.1 Å and a 106° vertical angle compared with the complex with peptide FLSPFWFDI; among the 8 peptides, peptide QLSDVPMDL had significantly differential binding to Arg\textsuperscript{97} versus Trp\textsuperscript{97}, binding ~14-fold better to the Arg\textsuperscript{97} variant (Fig. 3). In contrast, Tyr\textsuperscript{156} showed no measurable vertical shifts within the peptide-L\textsuperscript{d}W97 structures, but the side chain was observed within a modest 36° rotational angle range; the tyrosine side chains in both structures held both their direction from the backbone and position of the hydroxyl constant. These results also suggest that Arg\textsuperscript{97} does not act directly in allowing flexibil-
ity of peptide binding, as has been suggested. Rather, the negatively charged residue at position 114 (Glu in Ld and Asp in HLA-B) creates a stable electrostatic interaction that locks Arg97 into the same position in different structures (Fig. 6), moving it out of the way of other side chains in the pocket (49). A comparison of two HLA-B complexes (B*14:02 and B*35:01) with either Trp97 or Arg97 also shows the close proximities of this same group of residues (Fig. 6), suggesting the critical importance of their interactions in peptide binding.

To further explore the possible structural parallels with our findings involving Ld, and the HLA-B system, we examined in more detail the co-expressed polymorphisms in residues 97, 114, and 116 among the position 97 alleles that operated either as “HIV controllers” (Fig. 7A) or “HIV progressors” (Fig. 7B). We also examined a structural representative of each position 97 allele to gain further insight into the positions of these key residues, and the bound peptides. The analysis included four HLA-B alleles associated with HIV control (HLA-B*14:02 (Trp97), HLA-B*57:03 (Val97), HLA-B*27:05 (Asn97), HLA-B*51:01 (Thr97)) and two alleles associated with HIV progression (HLA-B*35:01 (Arg97) and HLA-B*07:02 (Ser97)) (Fig. 7).

The frequency analysis of all position 97 alleles (Val97, 82 alleles; Asn97, 118 alleles; Thr97, 491 alleles; and Ser97, 549 alleles), in addition to Trp97 and Arg97 alleles described above, revealed a clear predominance of commonly associated residue pairs at 114/116. For example, the majority of alleles with valine at position 97, which exhibited the highest allele frequency ratio (5.5) of controllers to progressors, contained an Asp at 114 and a Ser at 116. These exact two amino acids were also the predominant residues found in the Arg97 alleles, which exhibited the

![Figure 7. Polymorphic residues at positions 97, 114, and 116 of HLA-B alleles, and how they may affect interactions in the peptide-binding groove.](image-url)
HIV progressor phenotype. Thus, it is reasonable to predict that the combination of residues at these positions impact immune status, presumably by affecting the ability to associate with different numbers or types of self-peptides.

Inspection of the positioning and structures of the various peptides from complexes of the controller or progressor alleles did not provide evidence for a common motif. Although it is interesting that both progressor alleles (HLA-B*35:01 with Arg97 and HLA-B*07:02 with Ser97) have an unusual position 2 anchor (proline), it remains to be seen if this provides some biochemical feature that enables such peptides to bind more effectively to these alleles. In this regard, it has been suggested previously that there are fewer self-peptides in the human proteome with binding motifs of the controller alleles HLA-B*57:01 (Val97) and HLA-B*27:06 (Asn97) than there are self-peptides with binding motifs of the progressor alleles HLA-B*35:01 (Arg97) and HLA-B*07:02 (Ser97) (17). It is clear that the size of the self-peptide repertoire for an allele might not only impact CD8+ T cell capacity (e.g., through negative selection in the thymus), but could of course be related to the association of some alleles with autoimmunity.

Although observations showed an association between position 97 alleles of HLA-B and HIV immunity, the lack of a strict correlation implies that other factors also impact immunity. In this regard, a more recent study revealed disparate effector functions of T cells from controllers versus progressors that all expressed the “control-associated” allele HLA-B*27 (52). T cells from the controller population were more potent and cross-reacted with variants of the HIV gag epitope. However, there was no evidence presented on the binding affinities and kinetics of the TCRs from the corresponding T cells. We speculate that the TCRs from the controllers exhibited higher affinities/longer off-rates, which would explain both their potency and cross-reactivity (40). If so, the question remains why the HLA-B*27-positive progressors did not have T cells with TCRs of similar functional capacities? We speculate that the answer could be that either these individuals had some polymorphic self-peptides that deleted these T cells, or these individuals had been exposed to foreign peptides (infectious or normal flora) that regulated the peripheral activity of these T cells. A mouse system, for example, Lδ/L8 or HLA-B transgenic mice, could provide models with more limited variables (e.g., same repertoire of self-peptides) to examine some of these issues.

Our findings within the Lδ system do not support the view that Trp97 alleles confer greater immunity because they result in a more robust response against some HIV peptide-HLA-B complexes. In this scenario, particular HIV antigenic peptides would bind better to Trp97 allele products than Arg97 allele products. Contrary to this notion, our data suggest that a substantial fraction of peptides actually bind better to LδR97, whereas fewer peptides bind preferentially to LδW97. Of course, we cannot definitively rule out that there is not some unique peptide from HIV, not yet mimicked in our H-2L study, that binds better to a Trp97 allele and mediates an enhanced anti-HIV immune response.

Based on our results, we favor the possibility that a subset of self-peptides bind better to Arg97 alleles, and that these complexes operate in a tolerance mechanism to eliminate or suppress potential HIV-reactive T cells (8, 49, 50, 53, 54). These self-peptides may be predominately those that have very weak binding to the Trp97 alleles, and thus one might speculate that this increase in binding ability for Arg97 sufficiently stabilizes the complexes such that they can operate in either a central or peripheral selection processes. In principle, the repertoire of self-peptides with enhanced binding to the Arg97 alleles includes among them some that could have interacted with CD8+ T cells during thymic development, resulting in deletion. Furthermore, this premise would require that some of these T cells would have been effective against HIV epitopes.

It is possible that these self-peptide-R97 complexes function mechanistically not by inducing T cell deletion in the thymus but by stimulating T regulatory cells, which then suppress the HIV response. This mechanism cannot be ruled out. Alternatively, self-peptides can act as antagonists in T cell function (55), and can induce TCR-mediated signaling, which results in peripheral T cell tolerance (see Ref. 17 and reviewed in Ref. 56). Thus, the self-peptides associated with binding to Arg97 alleles could act to tolerize a peripheral population of potential HIV-reactive T cells. Whether there are dominant self-peptides, for example, with HIV peptide structural homologies, that operate in this capacity remains to be seen (57–61).

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