Fluorine substitutions in an antigenic peptide selectively modulate T-cell receptor binding in a minimally perturbing manner

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TCR (T-cell receptor) recognition of antigenic peptides bound and presented by MHC (major histocompatibility complex) molecules forms the basis of the cellular immune response to pathogens and cancer. TCRs bind peptide–MHC complexes weakly and with fast kinetics, features which have hindered detailed biophysical studies of these interactions. Modified peptides resulting in enhanced TCR binding could help overcome these challenges. Furthermore, there is considerable interest in using modified peptides with enhanced TCR binding as the basis for clinical vaccines. In the present study, we examined how fluorine substitutions in an antigenic peptide can selectively impact TCR recognition. Using a structure-guided design approach, we found that fluorination of the Tax peptide [HTLV (human T-cell lymphotropic virus)-1 Tax11-19] enhanced binding by the Tax-specific TCR A6, yet weakened binding by the Tax-specific TCR B7. The changes in affinity were consistent with crystallographic structures and fluorine chemistry, and with the A6 TCR independent of other substitutions in the interface. Peptide fluorination thus provides a means to selectively modulate TCR binding affinity without significantly perturbing peptide composition or structure. Lastly, we probed the mechanism of fluorine’s effect on TCR binding and we conclude that our results were most consistent with a ‘polar hydrophobicity’ mechanism, rather than a purely hydrophobic- or electrostatic-based mechanism. This finding should have an impact on other attempts to alter molecular recognition with fluorine.

Key words: antigenic peptide, fluorine, major histocompatibility complex (MHC), T-cell receptor (TCR), thermodynamics, vaccine design.

INTRODUCTION

TCR (T-cell receptor) recognition of peptides, bound and presented by class I or class II MHC (major histocompatibility complex) molecules, forms the basis of the cellular immune response to pathogens and cancer. Interactions between TCRs and pMHCs (peptide–MHC complexes) also shape the development and maintenance of the T-cell repertoire. Owing to the central role these interactions play in cellular immunity, there is considerable interest in the biophysical properties of TCR–pMHC interactions. Yet generally speaking, the soluble ectodomains of TCRs bind pMHCs with weak-to-moderate affinities and fast kinetics, properties which have complicated in-depth physical studies of the interactions. To help overcome this, various investigators have engineered high-affinity TCR molecules using directed molecular evolution or computational design techniques [1–3]. However, the engineered molecules all have multiple mutations in one or more of the TCR CDR (complementary-determining region) loops. This can complicate extrapolation of the results from the binding studies to the wild-type molecules.

We have been pursuing a different approach to overcome the limitation of the weak TCR binding affinity, aiming to enhance TCR–pMHC binding by generating minimally modified peptides that incorporate non-standard amino acid side chains. Our rationale is that, although peptide modifications still perturb recognition, compared with the molecular evolution approaches, more carefully controlled variations can be introduced, the consequences of which are more easily accounted for.

Peptides that specifically enhance TCR binding may also be of interest in the design of clinical vaccines based on cellular immunity. Enhancing TCR affinity via peptide modifications has been discussed as a means to help break immunological tolerance or otherwise overcome the poor antigenicity of various tumour or viral antigens [4,5]. In these cases, ideal modifications will influence TCR affinity in a selective manner, so that affinity is enhanced only against a specific receptor (or class of receptors) thus avoiding unwanted and potentially dangerous cross-reactivity. Modifications should have little or no effect on peptide conformation, and ideally concepts of both positive and negative design principles could be employed, such that affinity is raised with one set of receptors but weakened with others. In the present study we explored the use of fluorinated peptide variants as a means to alter TCR recognition in a minimally perturbing yet highly selective manner. Fluorine substitutions are often used in medicinal chemistry to enhance ligand binding affinities [6] and have recently been used to probe immune recognition [7]. We reasoned that by combining selective peptide fluorination with insight from crystallographic structures, we could target structural properties in interfaces between TCRs and their pMHC ligands, simultaneously carrying out both positive and negative design. We modified the HTLV (human T-cell lymphotropic virus)-1 Tax11-19 peptide (LLFGYPVYY; referred
to as Tax), which when presented by the class I MHC HLA-A2 [HLA (human leucocyte antigen)-A*0201] is recognized by the A6 and B7 TCRs as a strong agonist [8,9]. Despite differences in CDR loop composition and surface chemistry, the A6 and B7 TCRs bind Tax–HLA-A2 with nearly identical affinities, kinetics and structural topologies [10]. By fluorinating the central tyrosine (Tyr5) of the Tax peptide, we were able to take advantage of the differences between the A6 and B7 TCRs and generate peptides that enhance affinity with the A6 TCR, but weaken affinity with the B7 TCR. The enhancements with A6 TCR were achieved exclusively through decreases in the TCR dissociation rate and were independent of other substitutions in the interface. Overall, the results demonstrate how subtle variations in peptide composition, and fluorine substitutions in particular, can be used to selectively modulate TCR binding affinity.

Lastly, determination of crystallographic structures of TCR–peptide–HLA-A2 ternary complexes and binding thermodynamics allowed us to probe how fluorination modulates TCR binding affinity. As recently reviewed [6], owing to fluorne’s unusual chemistry and a lack of detailed studies with fluorinated compounds, fluorine’s effects on the affinity of biomolecular interactions is poorly understood. From the structural and thermodynamic results we propose that a unique ‘polar hydrophobicity’ mechanism [11] is responsible for the modulation of the binding affinities, as neither electrostatics nor hydrophobicity alone could explain the results.

EXPERIMENTAL

Proteins and peptides

Soluble versions of the A6 and A6c134 TCRs and the HLA-A2 ectodomains were refolded from bacterially expressed inclusion bodies and purified using ion-exchange and size-exclusion chromatography as previously described [10]. Fmoc (fluoren-9-ylmethoxycarbonyl)-protected fluorinated L-phenylalanine derivatives, for incorporation at position five of the Tax peptide, were purchased from Anaspec and used without modification. Peptides were generated in-house via solid-state synthesis using an ABI 433A instrument (Applied Biosystems). Peptide identity and purity were confirmed by LC–MS. Peptide nomenclature in Figure 2 is based on standard ring numbering. The two peptides that resulted in the highest affinity with the A6 TCR contained 4-fluoro-phenylalanine at position five and 3,4-difluorophenylalanine at position five. As shown in Figure 2, these peptides are referred to as Tax-Y5F4FF and Tax-Y5F3,4FF respectively.

SPR (surface plasmon resonance) experiments

SPR experiments were performed using a Biacore 3000 instrument as previously described [10]. Briefly, the TCR was coupled to a standard CM5 sensor surface using amine coupling. All injections were double-referenced using a blank flow cell and a buffer injection. The buffer used was 10 mM Hepes, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P-20, and the experiments were performed at 25°C. Results were processed and, for dissociation rates, the fitted curves generated using the BIAevaluation 4.1 software (Biacore). Curves for steady-state experiments were fitted using custom routines in Origin 7.5 software (OriginLab). For all titrations with affinities > 10 μM, the activity of the sensor surface was determined independently using the native Tax–HLA-A2 or Tax-Y5F3,4FF–HLA-A2 ligand. This value was then fixed in the analysis of the weaker-binding ligands, increasing the accuracy of the fitted affinity. Kinetic data were collected at the maximum flow rate of 100 μl/min. Typical surface capacities were approx. 500 RU (response units). Kinetic data were fitted globally to a single exponential decay function to obtain the dissociation rate, k_{off}. The association rate, k_{on}, was determined from the ratio of k_{on}/k_{off}. Error propagation was performed using standard error propagation methods [12].

ITC (isothermal titration calorimetry)

ITC experiments were performed using a Microcal VP-ITC (GE Healthcare) as previously described [13], with 20 μM TCR in the cell and 150 μM pMHC in the syringe. The buffer used was 20 mM Hepes, pH 7.4 (room temperature pH was adjusted so that the desired value of 7.4 was maintained across the entire temperature range), containing 150 mM NaCl. Results were processed using the Microcal routines in the Origin software and analysed using in-house custom fitting routines [13]. Data analysis incorporated the use of a baseline offset, obviating the need to perform separate dilution injections.

X-ray crystallography

X-ray crystallography was performed as previously described [14]. Crystals of the TCR A6–Tax-Y5F4FF–HLA-A2 and TCR A6–Tax-Y5F3,4FF–HLA-A2 complexes were grown from 15% (v/v) PEG [poly(ethylene glycol)] 4000 and 0.2 M MgCl2 buffered with 0.1 M Tris/HCl, pH 8.5. Cryo-protection consisted of mother liquor supplemented with 25% glycerol (v/v). Diffraction data were collected at Argonne National Laboratories at the indicated beamlines. Data reduction, structure solution, refinement and structure validation were performed as previously described [14]. The search model for molecular replacement was PDB 2GJ6 [14], with the co-ordinates for the peptides and CDR loops removed. Electrostatic potentials for Figure 1 were calculated with DelPhi, as incorporated into Accelrys Discovery Studio, using default parameters. Solvent accessible surfaces were drawn with a 1.4 Å (1 Å = 0.1 nm) radius probe.

RESULTS AND DISCUSSION

Fluorinated Tax peptide variants have an enhanced affinity with the A6 TCR but not the B7 TCR

A prominent feature of the A6 and B7 TCRs is a pocket formed by the hypervariable CDR3 loops of the α and β chains. In both the A6 and B7 TCR ternary complexes with Tax–HLA-A2, this pocket accommodates the central tyrosine residue (Tyr5) of the Tax peptide [9,15]. With the A6 TCR, the pocket is capped by Arg30 of CDR3β, which introduces positive-charge density and acts as a hydrogen bond donor to the Tyr5 hydroxy group (Figure 1a). With the B7 TCR, the pocket is capped by Asp30 of CDR3α, which introduces negative-charge density and acts as a hydrogen bond acceptor from the Tyr5 hydroxy group (Figure 1b). In designing peptide variants, we reasoned that owing to fluorne’s strong electronegativity, addition of fluorne atoms to the aromatic ring at position five would enhance binding by the A6 TCR yet weaken binding by the B7 TCR.

The fluorinated peptide variants examined were all commercially available derivatives of phenylalanine and included 3-fluoro-phenylalanine, 4-fluoro-phenylalanine, 3,4-difluorophenylalanine, 3,5-difluoro-phenylalanine and 2,3,4,5,6-pentafluoro-phenylalanine (Figure 2). Binding affinities with the A6 and B7 TCRs were measured at 25°C in a steady-state SPR assay. The binding results for the various fluorinated peptide variants are shown in Figure 3 and Table 1. We found
5-fluoro-phenylalanine (Y5F4F) 0.64 +− 3-fluoro-phenylalanine 1.2
3,4-difluoro-phenylalanine (Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine n.d. n.d.

The effects of substitutions at positions three and four were additive, such that substituting 3,4-difluoro-phenylalanine for tyrosine at position five in the Tax peptide resulted in an approx.

**Table 1** A6 TCR and B7 TCR binding affinities for modified Tax peptides presented by HLA-A2

ΔΔG° represents the difference in binding free energy between derivatized peptides and the native Tax peptide. n.d., no binding detected using ligand concentrations as high as 85 μM.

<table>
<thead>
<tr>
<th>Position five amino acid</th>
<th>A6 TCR</th>
<th>B7 TCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine (native Tax peptide)</td>
<td>2.1 ± 0.4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2,3,4,5,6-pentafluoro-phenylalanine</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3,5-difluoro-phenylalanine</td>
<td>2.8 ± 0.3</td>
<td>+0.2 ± 0.1</td>
</tr>
<tr>
<td>3-fluoro-phenylalanine</td>
<td>1.2 ± 0.2</td>
<td>−0.3 ± 0.1</td>
</tr>
<tr>
<td>4-fluoro-phenylalanine (Y5F4F)</td>
<td>0.64 ± 0.02</td>
<td>−0.7 ± 0.1</td>
</tr>
<tr>
<td>3,4-difluoro-phenylalanine (Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine</td>
<td>0.46 ± 0.02</td>
<td>−0.9 ± 0.1</td>
</tr>
<tr>
<td>4-methyl-phenylalanine</td>
<td>10 ± 2</td>
<td>+0.3 ± 0.2</td>
</tr>
</tbody>
</table>

**Table 2** A6 TCR binding kinetics with the native, Tax-Y5F4F and Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine variants.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>k_{on} (M^{-1} s^{-1})</th>
<th>k_{off} (s^{-1})</th>
<th>t_{1/2} (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Tax</td>
<td>(5.2 ± 0.1) × 10^4</td>
<td>0.11 ± 0.01</td>
<td>6.3 ± 0.2</td>
</tr>
<tr>
<td>Tax-Y5F4F</td>
<td>(6.0 ± 0.2) × 10^4</td>
<td>(3.85 ± 0.01) × 10^{-2}</td>
<td>18.0 ± 0.1</td>
</tr>
<tr>
<td>Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine</td>
<td>(5.1 ± 0.2) × 10^4</td>
<td>(2.40 ± 0.02) × 10^{-2}</td>
<td>28.9 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 1 The pocket that accommodates Tyr5 of the Tax peptide is oppositely charged in the A6 and B7 TCRs.

(a) A6 TCR and (b) B7 TCR electrostatic surface potentials were calculated as described in the Experimental section and are coloured red to blue, from −18 kT e + 10 kT.

**Figure 2** Peptide variants used in the present study

The native Tax peptide with tyrosine at position five is shown in the top panel. The bottom panel shows the phenylalanine variants incorporated at position five.

that placing fluoride at either position three or four of the phenylalanine ring resulted in enhanced affinity with the A6 TCR. Fluorine at position four resulted in an approx. 3-fold stronger TCR binding affinity (ΔΔG° = −0.7 kcal/mol (1 kcal ≈ 4.184 kJ)), whereas fluorine at position three resulted in an approx. 2-fold stronger TCR binding affinity (ΔΔG° = −0.3 kcal/mol). The effects of substitutions at positions three and four were additive, such that substituting 3,4-difluoro-phenylalanine for tyrosine at position five in the Tax peptide resulted in an approx. 5-fold enhancement in A6 TCR binding affinity, from 2.1 μM to 0.46 μM (ΔΔG° = −0.9 kcal/mol). None of the fluorinated peptides resulted in an affinity enhancement with the B7 TCR, instead weakening binding by as much as 14-fold. No binding with either receptor was seen with the 2,3,4,5,6-pentafluoro-phenylalanine variant using peptide–HLA-A2 concentrations as high as 85 μM. Thus in accordance with the crystallographic structures and basic fluorine chemistry, fluorination at position five of the Tax peptide resulted in a selective modulation of the TCR binding affinity.

Higher affinity binding with the A6 TCR is achieved exclusively by decreases in TCR dissociation rate and cannot be attributed solely to hydrophobicity

From the initial set of peptides examined, we selected the 4-fluoro-phenylalanine and 3,4-difluoro-phenylalanine Tax variants for further study, as these resulted in the strongest binding affinity gain with the A6 TCR.

Figure 4 and Table 2 show the results of kinetic SPR experiments for A6 TCR binding to HLA-A2 presenting Tax-Y5F4F or Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine. Both peptide variants resulted in a decreased dissociation rate (or increased TCR–pMHC half-life) for the A6 TCR. Indeed, for both peptides, the enhancement in receptor binding affinity was entirely due to a decrease in the receptor off-rate; the incorporation of 4-fluoro-phenylalanine into the Tax peptide (an affinity enhancement of 3-fold) decreased the dissociation rate 3-fold (from 0.11 s^{-1} to 0.039 s^{-1}), whereas incorporation of 3,4-difluoro-phenylalanine (an affinity enhancement of 5-fold) decreased the dissociation rate 5-fold (from 0.11 s^{-1} to 0.024 s^{-1}). The Tax-Y5F4F and Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine peptides increase the half-life of the TCR–pMHC complexes from 6.3 s to 18 s and 30 s respectively.

In addition to possessing high electronegativity, fluorine is also hydrophobic. To explore the role of hydrophobicity in enhancing binding affinity with the A6 TCR, we examined the effect of substituting the position five side chain of the Tax peptide with 4-methyl-phenylalanine (i.e. the tyrosine hydroxy group is replaced with a methyl group). As seen in Table 1, the affinity of the A6 TCR for the 4-methyl-phenylalanine peptide was weakened compared with either the native peptide or 4-fluoro-phenylalanine, indicating that simply increasing hydrophobicity cannot account for the results seen with the fluorinated peptides, and that the affinity modulation achieved with the Tax-Y5F4F and Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine peptides involves fluorine’s unique chemistry.

A6 TCR recognizes fluorinated Tax variants with more favourable entropy changes and moderate heat-capacity changes

We next examined the thermodynamic basis for the A6 TCR’s enhanced affinity towards the Tax-Y5F4F and Tax-Y5F3 +− 2,3,4,5,6-pentafluoro-phenylalanine peptides using ITC. For both peptides, titrations of the A6 TCR with peptide–HLA-A2 at 25 °C yielded a very weak signal, indicative of weak binding enthalpies below the sensitivity of the calorimeter. As titrations of the A6 TCR with native Tax–HLA-A2,
Figure 3  Equilibrium-binding results for the A6 TCR and B7 TCR recognition of the position five-variant Tax peptides presented by HLA-A2

(A) A6 TCR and (B) B7 TCR equilibrium-binding results for the indicated peptide variants. Results were collected at 25°C and processed as described in the Experimental section. For all titrations with affinities > 10 μM, the activity of the sensor surface was determined independently using the native Tax–HLA-A2 or Tax–YSF<sup>1-8F</sup>–HLA-A2 ligand. The affinities are reported in Table 1. (C and D) Fitted curves from the panels in (A) and (B) plotted as a logarithmic function of pMHC concentration, illustrating the change in affinity with different peptide variants.
Fluorinated peptides modulate TCR recognition

Figure 4 Kinetic data for A6 TCR recognition of the singly and doubly fluorinated peptide variants presented by HLA-A2

A

single fluorinated Tax-Y5F4F

B

double fluorinated Tax-Y5F3,4FF

C

(a) native Tax
(b) Tax-Y5F4F
(c) Tax-Y5F3,4FF

using the same conditions and with the same concentrations, resulted in a binding enthalpy of −3.4 kcal/mol [13], the weak signal with the fluorinated peptides indicates that the A6 TCR binds the fluorinated Tax variants with a less favourable binding enthalpy and that the affinity enhancement due to fluorination is entropic in nature.

Although the weak signal at 25°C could potentially have been overcome by performing the titrations with higher protein concentrations, because of the already large sample requirements for calorimetry, to obtain better thermodynamic results we instead repeated the titrations at 4°C. Our reasoning was that the binding heat-capacity change would amplify the signal through the fundamental relationship between enthalpy, heat-capacity and temperature (ΔH = ΔH° + ΔC_p[(T2 − T1)]). Under these conditions, we obtained high-quality results that fitted well to single-site binding isotherms (Figure 5A and 5B). Again taking advantage of the heat-capacity change, we observed similarly high-quality results performing the titrations at 37°C (Figure 5C and 5D). At both temperatures, the A6 TCR recognized the fluorinated peptides with a less favourable ΔH° compared with recognition of the native peptide [13], supporting the conclusions drawn from the 25°C titrations. Entropy changes at both temperatures were more favourable than with the native peptide, and the trends in ΔH° and ΔS° were amplified as the extent of fluorination increased (Table 3).

The enhanced binding affinity of the A6 TCR to the Tax variants with fluorination at position five thus results from favourable gains in entropy, offset by unfavourable losses in enthalpy.

The availability of enthalpy measurements at 4°C and 37°C, together with the very weak binding enthalpies at 25°C, allowed us to estimate the binding heat-capacity changes as approx. −0.4 kcal/mol per K for A6 TCR recognition of both Tax-Y5F4F–HLA-A2 and Tax-Y5F3,4FF–HLA-A2. This value is identical, within error, with the value of −0.39 kcal/mol per K observed for A6 TCR recognition of the native peptide under the same conditions [13].

Structures of the A6 TCR bound to the Tax-Y5F4F and Tax-Y5F3,4FF ligands

To examine the structural consequences of recognition of the fluorinated peptides, we next crystallized and determined the structures of the A6 TCR bound to the Tax-Y5F4F–HLA-A2 and Tax-Y5F3,4FF–HLA-A2 complexes. The complexes crystallized in the same space group and with similar unit cell dimensions as other A6 TCR–peptide–HLA-A2 complexes [14–16]. The
Figure 6  For legend see facing page

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Fluorinated peptides modulate TCR recognition

Table 3  Calorimetrically determined binding thermodynamics for A6 TCR recognition of Tax-peptide variants

Results for native Tax are taken from Armstrong et al. [13]. The 4°C results were calculated from propagation of globally fitted parameters [13].

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/mol per K)</th>
<th>$K_i$ (μM)</th>
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<tbody>
<tr>
<td>Native Tax</td>
<td>4.4 ± 0.2</td>
<td>43 ± 2</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>Tax-Y5F4F</td>
<td>6.0 ± 0.1</td>
<td>51 ± 1</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>Tax-Y5F3.4F</td>
<td>7.5 ± 0.3</td>
<td>58 ± 1</td>
<td>8.7 ± 0.1</td>
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4°C

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/mol per K)</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Tax</td>
<td>7.1 ± 0.1</td>
<td>4.7 ± 0.4</td>
<td>8.59 ± 0.04</td>
</tr>
<tr>
<td>Tax-Y5F4F</td>
<td>6.4 ± 0.2</td>
<td>10 ± 1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>Tax-Y5F3.4F</td>
<td>5.4 ± 0.1</td>
<td>14 ± 1</td>
<td>9.7 ± 0.1</td>
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37°C

Table 4  X-ray data and refinement statistics

Numbers in parenthesis refer to the highest resolution shell.

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<td>224.5</td>
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<td>b (Å)</td>
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<td>Completeness (%), Min.</td>
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<td>Rfree, %, Max. no. reflections</td>
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<td>Most favoured (%)</td>
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<td>Allowed (%)</td>
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<td>Generously allowed (%)</td>
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<td>Co-ordinate error (Å)</td>
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1 Mean estimate based on maximum likelihood methods.

of 0.40 Å (superimpositions are for the backbone atoms of the two variable domains, the peptide and the peptide-binding domain). Excluding position five, all atoms of the Tax-Y5F4F and Tax-Y5F3.4F peptides superimpose on the native Tax peptide with RMSD values of 0.32 and 0.35 Å; the value for the two modified peptides is 0.22 Å.

In both structures, however, there are small changes in the region accommodating the position five side chain (Figure 6). In the structure with the Tax-Y5F4F peptide, these changes are limited to small movements necessary to accommodate the new chemical environment in the position five pocket (Figure 6A). These motions include a 106° rotation of the $\chi_1$ angle of Ser31 (in the CDR1 loop), which moves the serine residue’s side chain oxygen 1.6 Å further away from the fluorine atom compared to its position in the native structure. This results in a fluorine–oxygen distance of 3.8 Å (the distance would be 2.2 Å if the serine torsion had not rotated). Surprisingly, Arg95 of the CDR3β loop is translocated 1.1 Å away from its position in the native structure, placing its guanidinium group 4.7 Å away from the fluorine atom (the distance would be 3.2 Å if the arginine position had not changed). There are some small changes in the CDR3β loop, mostly stemming from $\phi/\psi$ bond rotations in the Gly-Gly-Ala motif of residues 99β–101β. A consequence of these rotations is a 3.1 Å displacement of the Gly48100 carboxy oxygen. The reason for these structural changes is unclear, as there are no steric overlaps that would otherwise necessitate them. They are probably a consequence of the new chemical environment forcing an unexpected positional change in Arg95 that propagates further down the main chain.

In the structure with the doubly fluorinated Tax-Y5F3.4F peptide, the position five side chain could be clearly refined in two positions, differing by a 185° rotation around $\chi_2$ and a 26° rotation in $\chi_1$ (Figure 6B). In this structure, there are also changes in the position of Ser31 and Arg95, similar to those observed in the structure with the singly fluorinated peptide, although the rotation of the Ser31 $\chi_1$ is smaller at only 67°. However, in the doubly fluorinated structure the $\phi/\psi$ angle changes in the CDR3β loop are larger than in the singly fluorinated structure, resulting in a 3.7 Å displacement in the Gly48103 $\alpha$-carbon. To avoid an overlap with the main chain, the solvent-exposed side chain of Arg101 occupies a different position, 2.8 Å away from the side chain of Glu134 of the HLA-A2 α2 helix (Figure 6C). As in the singly fluorinated Tax-Y5F4F structure, there is no apparent reason for this shift; this observation may reflect an intrinsic flexibility of this region of the loop, amplified owing to the altered chemistry within the position five pocket. Note that it is not unusual for the

Figure 6  Interfacial details from the structures of the A6-Tax-Y5F4F–HLA-A2 and A6-Tax-Y5F3.4F–HLA-A2 structures

(A) Cross-eyed stereo comparison of the interfaces with the Tax-Y5F4F peptide (cyan and yellow) and the native Tax peptide (pink and blue). The shifts in Arg95 of the CDR3β and Ser31 are apparent, as is the slight rearrangement of the CDR3β backbone. (B) Cross-eyed stereo comparison of the interfaces with the Tax-Y5F3.4F peptide (cyan and yellow) and the native Tax peptide (pink and blue). The dual conformations of the modified position five side chain are apparent, as is the larger shift in CDR3β. (C) Alternate view of the Tax-Y5F3.4F interface, showing the relationship between Arg101 and Glu134 of the HLA-A2 α2 helix (green).

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The affinity enhancement ($\Delta G$) due to fluorination is similar to that seen with the wild-type receptor and, as with the wild-type receptor, affinity enhancement is entropically driven and enthalpically opposed.

CDR3$\beta$ loops of TCRs, and of the A6 TCR in particular, to shift upon recognition of ligand [14,17].

The Y5F$^{3,4F}$ substitution in the Tax peptide is independent of other alterations in the interface with the A6 TCR

One use for peptide modifications that enhance TCR binding is to study the consequences of interfacial modifications that weaken binding to levels difficult to measure. Such use requires that the various modifications act independently of each other. To investigate whether the Y5F$^{3,4F}$ substitution is independent of other changes in the interface, we combined it with two other substitutions in the Tax peptide, Pro$^6$ to alanine (P6A) and Tyr$^8$ to phenylalanine (Y8F). If the Y5F$^{3,4F}$ substitution is independent of these changes, the loss in affinity with the P6A and Y8F substitutions should be the same with the doubly fluorinated phenylalanine at position five as it is with the native tyrosine at position five.

Using SPR, we found with the A6 TCR that the P6A substitution in the Tax-Y5F$^{3,4F}$ peptide weakened A6 TCR binding affinity from 0.46 to 25 $\mu$M, amounting to an increase in binding free energy ($\Delta AG$) of 2.4 $\pm$ 0.1 kcal/mol. This increase was identical with that seen when the P6A substitution was introduced alone into the native Tax peptide (a change in $K_D$ from 2.1 to 120 $\mu$M, or a $\Delta AG$ of 2.4 $\pm$ 0.2 kcal/mol [18]). With the Y8F substitution in the Tax-Y5F$^{3,4F}$ peptide, we observed a drop in binding affinity from 0.46 to 10.5 $\mu$M, amounting to a $\Delta AG$ of 1.8 $\pm$ 0.1 kcal/mol. Again, this increase was identical with that seen when Y8F was introduced alone into the native Tax peptide (a change in $K_D$ from 2.1 to 42 $\mu$M, or a $\Delta AG$ of 1.7 $\pm$ 0.2 kcal/mol). Thus, the Y8F substitution is independent of the P6A and Y8F substitutions. See Supplementary Figure S2 (available at http://www.BiochemJ.org/bj/423/bj4230353add.htm) for the binding data for these additional substituted peptides.

We also examined the effect of the Y5F$^{3,4F}$ substitution on the binding of the high-affinity A6 TCR variant A6c134, which has the sequence MSAQ instead of GSSR in the CDR3$\beta$ loop [2]. Using ITC, we measured an affinity of 8 nM for A6c134 TCR recognition of native Tax and 3 nM for recognition of Tax-Y5F$^{3,4F}$ (Figure 7). The effect of the Y5F$^{3,4F}$ substitution on the binding of the A6c134 variant thus amounted to a $\Delta AG$ of $-0.7 \pm 0.3$ kcal/mol, close to the value of $-0.9 \pm 0.1$ kcal/mol measured for wild-type A6 TCR. As with the wild-type receptor, the affinity gain due to fluorination was entropically driven and enthalpically opposed. The binding of the Tax-Y5F$^{3,4F}$ peptide is thus independent of the four mutations that define the A6c134 TCR variant.

The mechanism of affinity modulation with fluorinated Tax peptides

Although fluorine is commonly used in medicinal chemistry to enhance ligand binding, the mechanisms by which fluorine influences binding affinity are poorly understood. Fluorine is hydrophobic and highly electronegative, yet it only rarely participates as a hydrogen bond acceptor due to its low polarizability [19–21]. Instead, fluorine interacts with full or partial positive charges via dipolar interactions, although the strength of these are subject to some debate [11]. A recent survey of complexes of proteins and fluorinated ligands in the PDB highlighted the propensity for fluorine to interact with the guanidinium group of arginine [6]. Indeed, such an interaction was part of our design scheme, as in the A6 TCR, the pocket which accommodates Tyr$^9$ of the peptide is capped by Arg$^{15}$ of CDR3$\beta$. The enhanced binding seen with the A6 TCR, and the corresponding reduced binding with the B7 TCR (whose pocket is capped by a negatively charged aspartic acid residue), support the assignment of the enhanced affinity to electrostatic considerations.

However, we would argue that electrostatics are not the only reason for the affinity changes with the A6 and B7 TCRs. In the X-ray structures of the A6 TCR with Tax-Y5F$^{3,4F}$ and A6 TCR with Tax-Y5F$^{3,4F}$ Arg$^{15}$ of the A6 CDR3$\beta$ loop has moved away from the fluorine atoms. Whereas it could be that this movement is necessary to optimize the distance for a favourable electrostatic interaction, the binding thermodynamics are likewise inconsistent with a purely electrostatic mechanism. For both the Tax-Y5F$^{3,4F}$ and Tax-Y5F$^{3,4F}$ peptides, the affinity gain is entropically driven and enthalpically opposed; a purely electrostatic mechanism would be expected to be enthalpically favoured. Although the binding thermodynamics must also include the energetics associated with the conformational changes seen in each structure, and these could mask the energetics associated with burial of the fluorine atoms, the enthalpic penalties and entropic gains due to fluorination of the peptide are large and additive with the degree of fluorination at both 4°C and 37°C. This suggests that the hydrophobic nature of fluorine is also a significant contributor to the affinity gain with both peptides.

Although hydrophobicity therefore seems to be involved in the interaction, a classical hydrophobic mechanism as seen with hydrocarbons would necessitate an increase in the magnitude of the binding heat-capacity change, due to the effects of hydrocarbons on the hydrogen-bonding pattern in bulk water [22]. However, as noted above this is not observed; the binding $\Delta C_p$ for A6 TCR recognition of both the fluorinated peptides is identical, within error, with that observed with the native peptide. Importantly, however, the hydrophobicity of fluorocarbons and hydrocarbons arise from different physical mechanisms, as shown by the limited miscibility of the two classes of compounds. Indeed, classical hydrophobicity cannot account for the affinity changes, as replacing Tyr$^9$ with 4-methyl-phenylalanine resulted in weaker binding of the A6 TCR.

We therefore conclude that the A6 and B7 TCR affinity modulation with the fluorinated peptides is largely due to the
fluorine atoms, resulting in a loss of TCR binding affinity. Further gains in affinity are provided by the hydrophobic nature of fluorine, and compared with the native tyrosine, the lack of a desolvation penalty for the ring hydroxy group. The small structural differences between the complexes with native Tax and the fluorinated variants must also play a role, although given the measured binding thermodynamics and the additive effects of fluorination, we judge these effects to be secondary to the direct effects from the fluorine atoms. With the B7 TCR, fluorine’s hydrophobicity and the removal of the desolvation penalty associated with tyrosine’s hydroxy group are unable to offset the electrostatic repulsion between Asp<sup>95</sup> and the electron-rich fluorine atoms, resulting in a loss of TCR binding affinity.

Conclusion

In the present study we have demonstrated how fluorine substitutions can be used to minimally modify antigenic peptides in order to selectively modulate TCR binding. Such modifications will be useful in the design of peptides that boost TCR affinity, in turn facilitating experiments that are difficult or impossible because of the weak affinities that TCRs maintain towards their natural pMHC ligands. Such substitutions may also be useful when designing clinical vaccines based on cellular immunity, as enhanced TCR affinity can be desirable for breaking immunological tolerance or otherwise overcoming the poor antigenicity of native antigens. The ability to incorporate both positive and negative design principles with fluorine substitutions is particularly attractive for the latter case. We have demonstrated that regions of positive electrostatic potential in the TCR binding sites can be targeted to enhance affinity, whereas regions with negative electrostatic potential can be targeted to weaken affinity. Lastly, the assignment of a ‘polar hydrophobicity’ mechanism for fluorine’s affinity enhancement with the A6 TCR fills a gap in our understanding of how fluorine can be used to modulate biomolecular interactions. This should impact upon other attempts to alter molecular recognition via fluorine substitutions.

AUTHOR CONTRIBUTION

Kurt Piepenbrink, Ruth Sommese, John Clemens, Priscilla Do and Clare Desmond performed the SPR experiments and data analysis. Oleg Borbulevych performed the crystallography. John Clemens and Kathryn Armstrong performed the ITC experiments and data analysis. Kurt Piepenbrink, Oleg Borbulevych and Brian Baker designed peptides, interpreted data, wrote and edited the paper. Brian Baker conceived of and oversaw the study.

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Fluorine substitutions in an antigenic peptide selectively modulate T-cell receptor binding in a minimally perturbing manner

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Figure S1  Structural overview and electron densities

(a) Structural overview of the A6-Tax-Y5F4F–HLA-A2 (left) and A6-Tax-Y5F3,4FF/HLA-A2 complexes (right). (b) $2F_o - F_i$ electron density at 1 $\sigma$ for the peptide (left) and CDR3 loops (right) in the A6-Tax-Y5F4F–HLA-A2 structure. (c) $2F_o - F_i$ electron density at 1 $\sigma$ for the peptide (left) and CDR3 loops (right) in the A6-Tax-Y5F3,4FF/HLA-A2 structure.

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The structural co-ordinates reported will appear in the PDB under accession codes 3D39 and 3D3V.
Figure S2  Additional Biacore binding data

(a) Equilibrium binding data for A6 recognition of the doubly-substituted Tax-Y5F34FF-Y8F (green) and the Tax-Y5F34FF-P6A (blue) ligands. For comparison, data for recognition of the singly substituted Tax-Y5F34FF and Tax-Y8F ligands are also shown. Note that although saturation was not reached in all cases, the RU_{max} for all experiments was predetermined by injecting saturating concentrations of the high-affinity Tax-Y5F34FF ligand, increasing the accuracy of the low-affinity measurements (see the Experimental section in the main paper). (b) Kinetic data for dissociation of A6 recognition from the doubly-substituted Tax-Y5F34FF-P6A ligand (blue). For comparison, dissociation from the high-affinity Tax-Y5F34FF ligand is shown in red.

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