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Antagonism of Antiviral and Allogeneic Activity of a Human Public CTL Clonotype by a Single Altered Peptide Ligand: Implications for Allograft Rejection

Lauren K. Ely,* Katherine J. Green,† Travis Beddoe,* Craig S. Clements,* John J. Miles,† Stephen P. Bottomley,* Danielle Zernich,‡ Lars Kjer-Nielsen,‡ Anthony W. Purcell,‡ James McCluskey,§ Jamie Rossjohn,2* and Scott R. Burrows 2†

Alloreactive T lymphocytes are central mediators of graft-versus-host disease and allograft rejection. A public CTL clonotype with specificity for the alloantigens HLA-B*4402 and B*4405 is often expanded to large numbers in healthy HLA-B*0801* individuals, driven by cross-reactive stimulation with the common, persistent herpesvirus EBV. Since such alloreactive memory CTL expansions have the potential to influence transplantation outcome, altered peptide ligands (APLs) of the target HLA-B*0801-binding EBV peptide, FLRGRAYGL, were screened as specific antagonists for this immunodominant clonotype. One APL, FLRGRFYGL, exerted powerful antagonism of a prototypic T cell clone expressing this immunodominant TCR when costimulated with target cells presenting HLA-B*0801†FLRGRAYGL. Significantly, this APL also reduced the lysis of allogeneic target cells expressing HLA-B*4402 by up to 99%. The affinities of the agonist and antagonist complexes for the public TCR, measured using solution and solid-phase assays, were 8 and 138 μM, respectively. Surprisingly, the half-life of the agonist and antagonist complexes was similar, yet the association rate for the antagonist complex was significantly slower. These observations were further supported by structural studies that suggested a large conformational hurdle was required to ligate the immunodominant TCR to the HLA-B*0801 antagonist complex. By defining an antagonist APL against an immunodominant alloreactive TCR, these findings raise the prospect of exploiting such peptides to inhibit clinical allosreactivity, particularly against clonal T cell expansions that react with alloantigens. The Journal of Immunology, 2005, 174: 5593–5601.

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cell recognition of allogeneic HLA molecules is a major barrier to successful transplantation. Although the repertoire of T cells available for use in an allosresponse against a single allo-HLA molecule is diverse, the actual repertoire used may be highly selected (1–5). The basis for this limited diversity is unclear; however, it is possible that pre-existing expansions of alloreactive T cells, which have been demonstrated in healthy individuals, could be primarily involved (6). Recent studies have characterized alloreactive T cell expansions in healthy individuals that are driven by cross-reactive stimulation with EBV, a human pathogen that persistently infects ~90% of adults (7, 8). For example, in unrelated HLA-B8* individuals, a highly dominant CTL response is generated against the nonamer EBV peptide, FLRGRAYGL, which is also alloreactive against distinct members of the HLA-B44 family. The public TCR expressed by this cross-reactive clonotype, termed LC13, is alloreactive against HLA-B*4402 and HLA-B*4405 but not HLA-B*4403. This is somewhat surprising given that there is only one amino acid difference between these alleles. These expanded alloreactive populations are often so large that such T cells dominated conventional MLRs from some HLA-mismatched individuals. Thus, a prior history of infection with an immunogenic virus such as EBV can influence an individual’s level of responsiveness to an alloantigen and such mechanisms may underlie the observed clinical association between herpesvirus exposure and graft-versus-host disease (GVHD) (9).

The limited use of the TCR repertoire in GVHD and allograft rejection (1–5) may provide the opportunity to therapeutically disrupt the allosresponse by targeting a selected T cell population for inactivation, as has been achieved in experimental animal models (10). Altered peptide ligands (APLs) represent one such strategy developed to modulate the immune response, whereby subtle alterations of the cognate peptide can potentially convert an agonist to a superagonist, weak agonist, or antagonist (for review, see Ref. 11). Despite the dramatically different biological outcomes an APL can elicit, only slight conformational readjustments are observed at

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3 Abbreviations used in this paper: GVHD, graft-versus-host disease; LCL, lymphoblastoid cell line; APL, altered peptide ligand; vdw, van der Waals; hc, H chain; β2m, β2-microglobulin; SPR, surface plasmon resonance; RU, resonance units.
the TCR/MHC interface (12, 13). With some exceptions, biological outcome is best correlated to the half-life of the TCR/MHC complexes, such that antagonists display a shorter half-life than their agonist counterparts (14, 15).

We have recently established the structural basis for T cell immunodominance in the CTL response to the FLRGRAYGL determinant (16). We have determined the crystal structure of the prototypical immunodominant LC13 TCR in its nonliganded state (17) and in complex with HLA-B*0801FLRGRAYGL (16, 18). The immunodominant TCR was observed to make a number of specificity-driven contacts with HLA-B*0801FLRGRAYGL, including P7-Tyr of the peptide, a pivotal residue that sat centrally within the pocket of LC13. This structure provides an important starting point from which to understand the biochemical and structural basis of APL-induced antagonism.

In the present report, we describe the inactivation via APL antagonism of the immunodominant LC13 T cell clone. The measured affinity for the LC13 TCR/HLA-B*08-agonist complex, at 8 μM, compares to a value of 138 μM for the LC13 TCR/HLA-B*08-antagonist complex. However, surprisingly the half-lives of the respective complexes were similar. Instead, the association rate for the antagonist complex was much slower than that of the authentic agonist complex, suggesting a larger hurdle for conformational change is required upon binding to the antagonist complex, which was consistent with the observed structural data. The inhibitory activity of the antagonist peptide was not dependent on binding to the allogeneic target cell since pretreatment of the cross-reactive HLA-B*0801-expressing CTLs with the peptide was sufficient to block their effector function. This raises the prospect of exploiting selected antagonist peptides to inhibit host T cell reactivity toward allografts mediated by immunodominant TCRs.

Materials and Methods

Establishment and maintenance of cell lines

Lymphoblastoid cell lines (LCLs) and PHA blasts were generated as described previously (7). LCLs were generated by exogenous transformation of peripheral B cells with EBV derived from the QIMR-Wil cell line. The LC13 CTL clone used in this study has been described previously (19, 20).

Cytotoxicity assay

Peptides were tested in duplicate for inhibition of cytotoxicity in the standard 5-h chromium release assay using an effector:target ratio of 2:1 (21). In most cases (but not the experiments presented in Fig. 2, E and F, see figure legend), 51Cr-labeled targets, CTL effectors, and the peptide remained present throughout the assay. Peptides were synthesized by Mimotopes Pty. on a 1-ng scale.

Zap70 phosphorylation

C1R.B8 APCs were loaded with 20 μg/ml of either the agonist peptide FLRGRAYGL or the antagonist peptide APL6 for 1.5 h at 37°C and then washed and centrifuged with LC13 T cells (106) before 2 min of coculture. Cells were then lysed in cold lysis buffer (50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM sodium orthovanadate, 50 mM NaCl, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40 (Sigma-Aldrich), 1 mM DTT, and 1× protease inhibitors (Complete)) for 30 min on ice. Samples were boiled in loading buffer containing lithium dodecyl sulfate (Invitrogen Life Technologies) electrophoresed in a 12% bis-Tris gel. Proteins were transferred to polyvinylidene fluoride membrane by electro blotting and the membrane was blocked in 5% skim milk in TBS (10 mM Tris-HCl, pH 7.4), 150 mM NaCl) containing 0.2% Tween 20 (Labchem). The membrane was probed with a 1/1000 dilution of Ab recognizing phosphorylated-Zap-70 (Tyr195)/Syrk (Tyr195) (Cell Signaling) in TBS containing 0.1% Tween 20/5% BSA. HRP-conjugated anti-rabbit IgG (Silenus) was used as a secondary Ab, and proteins were visualized using Renaissance chemiluminescence substrate (NEN). The stripped membrane was probed with an Ab that recognizes unphosphorylated Zap70 (Cell Signaling) to control for transfer and expression of this protein.

Expression, purification, crystallization, and structure determination

The LC13 TCR was expressed, refolded, and purified as previously described (22). The HLA-B8 antagonist complex was expressed, purified essentially as previously described (22), except that the EBV epitope was replaced by the antagonist peptide FLRGRAYGL. Briefly, the truncated forms of the HLA-B8 H chain (hc) and full-length β2-microglobulin (β2m) were expressed in Escherichia coli and each protein was purified from inclusion bodies. The complex of hc/β2m/peptide is refolded by diluting the hc and β2m inclusion body preparations into refolding buffer containing a molar excess of peptide ligand. The refolded complexes were concentrated and purified by anion exchange chromatography. The complexes were further purified by gel filtration chromatography to a high level of purity before crystallization.

Crystals of the HLA-B8-antagonist complex were obtained using the protocol previously described (22), using a reservoir buffer of 10–15% PEG 4000, 0.1 M sodium citrate (pH 5.6), 0.2 M ammonium acetate, and 10 mM cadmium chloride. The crystals belong to space group P212121, with unit cell dimensions a = 85.41, b = 90.10, and c = 125.28 Å. A 2.6 Å data set was collected using inverse φ geometry and was processed and scaled using the HKL suite of programs (23). For a summary of statistics, see Table I.

The structure was refined using the dimeric HLA-B8FLRGRAYGL complex (18) minus the peptide and the water molecules as the start model. The progress of refinement was monitored by the Rfree value (4%) of the data, with neither a σ nor a low resolution cutoff being applied to the data. The structure was refined using rigid-body fitting of the individual domains followed by the simulated-annelling protocol implemented in CNS (version 1.0) (24) using a methodology previously used (18). The electron density of the ab initio model was clearly visible for both domains, with one [19].

| Table I. Data collection statistics |
|------------------------------------|
| **Temperature** | 100 K |
| **X-ray source** | BioCARS, APS |
| **Detector** | Quantum 4 CCD |
| **Space Group** | P212121 |
| **Cell dimensions (Å) (a,b,c)** | 85.4, 90.4, 125.1 |
| **Resolution (Å)** | 2.6 |
| **Total no. of observations** | 83,403 |
| **No. of unique observations** | 28,178 |
| **Multiplicity** | 2.96 |
| **Data completeness (%)** | 94.1 (89.0) |
| **No. of data > 2σ1** | 71.5 (45.0) |
| **I/σ1** | 14.7 (4.6) |
| **Rmerge** (%) | 8.9 (33.9) |
| **Refinement statistics** | |
| **Nonhydrogen atoms** | |
| **Protein** | 6,190 |
| **Water** | 432 |
| **Cadmium** | 2 |
| **Resolution (Å)** | 50–2.6 |
| **Rmerge** (%) | 24.5 |
| **Rfree** (%) | 32.6 |

Root mean squared deviations from ideality

| Bond lengths (Å) | 0.009 |
| Bond angles (°) | 1.38 |
| Improper (°) | 0.89 |
| Dihedrals (°) | 25.02 |
| Ramachandran plot | |
| Most favored | 99.2 |
| And allowed region (%) | |

E factors (Å2)

| Average main chain | 40.6 |
| Average side chain | 42.3 |
| Average water molecule | 44.3 |
| Cadmium | 24.6 |
| rms deviation bonded Bs | 1.7 |

Values in parentheses are for the highest resolution bin (approximate interval 0.1 Å).
density for the bound antagonist peptide was very clear in both monomers. See Table I for summary of refinement statistics and model quality.

Tryptophan fluorescence measurements

Tryptophan fluorescence binding experiments were conducted at 25°C in a PerkinElmer LS50B spectrophotometer using a stirred quartz cuvette with a 1-cm path length. Samples were excited at 295 nm and the change in the emission intensity at 340 nm was recorded. Increasing concentrations of LC13 were added to 10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, and 0.005% surfactant P20 supplied by the manufacturer. The Ab W6/32 (25) was coupled in 10 mM citric acid (pH 5.0) to allow for local fitting of the binding maximum, to calculate the kinetic constants. The equilibrium data were analyzed using GraphPad Prism.

Surface plasmon resonance analysis

All surface plasmon resonance (SPR) experiments were conducted at 25°C on a Biacore 3000 instrument using HBS buffer (10 mM HEPES-HCl (pH 7.4), 150 mM NaCl, and 0.005% surfactant P20 supplied by the manufacturer). The Ab W6/32 (25) was coupled in 10 mM citric acid (pH 5.0) to 31–38% using untreated CTLs down to 0.2–5% (87–99% inhibition) with CTL effectors that were pretreated with 100 μM of the agonist peptide presented on surrounding CTLs and not the allogeneic target cells by this subdominant T cell clonotype (our unpublished data). The FLRGRFYGL antagonist peptide also failed to inhibit lysis by CTL clone LC13 of HLA-B*8001 LCLs that had been pretreated with high concentrations (>1 μg/ml) of the FLRGRAYGL agonist peptide (our unpublished data). Interestingly, this peptide could also reduce lysis mediated exclusively toward the allogeneic target Ags, HLA-B*4402 or B*4405. Thus, LCLs and PHA blast lines expressing HLA-B*4402 or B*4405, but negative for HLA-B*0801, were normally lysed efficiently by CTL clone LC13, but when peptide FLRGRAYGL was added to this culture, this lysis was reduced by up to 72% (Fig. 2D).

The FLRGRAYGL peptide was first tested as an agonist for the LC13 CTL clone with multiple analogues of the target EBV epitope. An HLA-B*0801 LCL (HLA-A1, A2, B*0801, B62) was tested for lysis by CTL clone LC13 in the presence of monosubstituted peptide analogues of FLRGRAYGL, in which every one of the 20 genetically coded amino acids was introduced in each of the nine locations within the parent sequence. The letter in parentheses represents the parent residue being replaced, and the vertical axis displays the percent inhibition of lysis relative to the level of lysis of the LCL without exogenous peptide addition (31.2%). Where peptide increased lysis of the B*0801 LCL, a value of 0% inhibition is shown. The peptide was present at 5 μmol/L (filled bars) or 0.5 μmol/L (open bars), and the E:T ratio was 2:1.

Results

APL identification

To identify APL antagonists for this immunodominant CTL clonotype that cross-recognizes the HLA-B44 allotype and the HLA-B8-binding EBV epitope FLRGRAYGL, 171 monosubstituted peptide analogues of FLRGRAYGL peptide were synthesized such that each residue was sequentially replaced with all other genetically coded amino acids. The CTL LC13, a representative clone expressing this well-characterized, cross-reactive TCR, was genetically coded amino acids. The CTL LC13, a representative clone expressing this well-characterized, cross-reactive TCR, was used as an effector in a standard 51Cr release assay against target cells expressing syngeneic HLA-B*0801 molecules and presenting endogenously processed FLRGRAYGL. CTL lysis assays were conducted in the presence of each peptide analogue at two different concentrations (5 and 0.5 μmol/L). Lysis of LCL targets expressing endogenous FLRGRAYGL in the absence of APLs was 31.2%. Data are presented in Fig. 1 as percent inhibition of lysis, relative to the lysis observed without exogenous peptide addition. One peptide analogue, FLRGRFYGL, reduced lysis of the LCLs by up to 50% in this screening assay, and this peptide was chosen for more detailed analysis.

The APL FLRGRFYGL is a potent antagonist of recognition of both cognate and allogeneic targets

The FLRGRFYGL peptide was first tested as an agonist for the LC13 CTL clone on HLA-B*0801 PHA blasts and was found to exhibit no agonist activity at peptide concentrations up to 1 μM (Fig. 2A). This peptide was then tested as an antagonist for the LC13 CTL clone over a wide range of concentrations. Lysis of the HLA-B*0801 LCL from donor RM was inhibited very efficiently (up to 69%) when the peptide was added to target cells at concentrations above 16 μmol/L (Fig. 2B). LCLs and PHA blasts coexpressing HLA-B*0801 and the allogeneic target Ag HLA-B*4402 were also tested for lysis by the LC13 CTL clone in the presence of this antagonist peptide. As shown in Fig. 2C, strong inhibition of cytotoxicity was observed when peptide FLRGRFYGL was present during the chromium release assay. As a specificity control for the experimental data shown in Fig. 2, A–C, a CTL clone that also recognizes the FLRGRAYGL epitope but that expresses a different (subdominant) TCR was also included in the assays (clone CF34, see Ref. 19). In contrast to the data shown for CTL clone LC13, addition of the FLRGRAYGL peptide had no significant effect on the lysis of the HLA-B*0801 target cells by this subdominant Tcell clonotype (our unpublished data). The FLRGRFYGL antagonist peptide also failed to inhibit lysis by CTL clone LC13 of HLA-B*8001 LCLs that had been pretreated with high concentrations (>1 μg/ml) of the FLRGRAYGL agonist peptide (our unpublished data). Interestingly, this peptide could also reduce lysis mediated exclusively toward the allogeneic target Ags, HLA-B*4402 or B*4405. Thus, LCLs and PHA blast lines expressing HLA-B*4402 or B*4405, but negative for HLA-B*0801, were normally lysed efficiently by CTL clone LC13, but when peptide FLRGRAYGL was added to this culture, this lysis was reduced by up to 72% (Fig. 2D).

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To further examine the outcome of LC13 interaction with the antagonist peptide, we studied the phosphorylation of the T cell protein tyrosine kinase, Zap70, by agonist and antagonist ligands. Previous studies have shown that “classical antagonists” induce only partial phosphorylation of Zap70 (28, 29), a kinase that is known to be critical for T cell signaling. At concentrations of FLRGRFYGL that antagonize recognition of HLA-B*0801/FLRGRAYGL, there was no detectable phosphorylation of LC13 Zap70 above background (Fig. 3). In contrast, the agonist HLA-B*0801/FLRGRAYGL ligand induced significant Zap70 phosphorylation under the same conditions. The findings provide evidence that antagonism by the FLRGRFYGL peptide is associated with unproductive TCR interaction and inadequate phosphorylation of Zap70.

To examine whether the alloreactivity of this clone could also be inhibited by other primary cells expressing HLA-B*0801, by agonist and antagonist ligands. Previous studies have shown that “classical antagonists” induce only partial phosphorylation of Zap70 (28, 29), a kinase that is known to be critical for T cell signaling. At concentrations of FLRGRFYGL that antagonize recognition of HLA-B*0801/FLRGRAYGL, there was no detectable phosphorylation of LC13 Zap70 above background (Fig. 3). In contrast, the agonist HLA-B*0801/FLRGRAYGL ligand induced significant Zap70 phosphorylation under the same conditions. The findings provide evidence that antagonism by the FLRGRFYGL peptide is associated with unproductive TCR interaction and inadequate phosphorylation of Zap70.

To examine whether the alloreactivity of this clone could also be inhibited by other primary cells expressing HLA-B*0801 and presenting the antagonist peptide, cold-target inhibition assays were performed. Unlabeled HLA-B*0801 PBMCs were pretreated with the antagonist peptide (100 or 20 μmol/L) or left untreated and tested for their ability to inhibit lysis of chromium-labeled PHA blasts expressing HLA-B*4402. As shown in Fig. 2F, at a cold:hot target ratio of just 2:1 significant inhibition of anti-B*4402 alloreactivity was observed (between 54 and 85% inhibition).

APL activity of FLRGRFYGL does not correlate with the half-life of the TCR/MHC-peptide complex

Having characterized the antagonism of the LC13 TCR, we then compared the affinities and the kinetic constants for the agonist and antagonist complexes using initially SPR experiments. The HLA-B8FLRGRAYGL complex was captured onto the chip using the MHC class I-specific mAb W6/32 (25) and increasing concentrations of the LC13 TCR were passed over the surface (Fig. 4). As depicted in Fig. 4A, a concentration-dependent increase in binding response was observed. The affinity constant was calculated to be $8.2 \times 10^5 \text{ M}^{-1}$ by fitting the binding response at equilibrium to a 1:1 binding equation (Fig. 4B). The Scatchard analysis also confirms the 1:1 binding relationship and calculates the dissociation equilibrium constant to be $8.8 \times 10^5 \text{ M}^{-1}$. The association and dissociation rate constants were calculated to be $3.58 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $0.42 \text{ s}^{-1}$, respectively, and the calculated dissociation equilibrium constant was $12.5 \mu\text{M}$.
These values are in close agreement with those reported recently (30). The affinity of the LC13/HLA-B8FLRGRFYGL interaction was also examined by SPR. The dissociation equilibrium constant was determined to be significantly weaker at $K_{\text{d}} = 138 \text{ M}$. Because the interaction between LC13 and HLA-B8 presenting the antagonist ligand is much weaker, the flow rate and surface density was optimized to measure the kinetic constants (Fig. 4, C and D). The LC13/HLA-B8\textsuperscript{FLRGRAYGL} interaction was used as a control and under the optimized flow rate conditions the affinity and kinetic constants of the LC13/HLA-B8\textsuperscript{FLRGRAYGL} interaction were not affected (data not shown). The antagonist kinetic rate constants were calculated to be $2.62 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and $0.35 \text{ s}^{-1}$ for the association and dissociation, respectively. In comparison to the authentic agonist epitope, the antagonist ligand had a significantly slower on rate (Table II); however, these ligands displayed similar dissociation kinetics with half-lives of 1.65 and 1.98 s, respectively.

The binding affinity of HLA-B8\textsuperscript{FLRGRAYGL} for the LC13 TCR was also verified in solution by measuring the change in Trp fluorescence upon complex formation (Fig. 5A). The use of Trp fluorescence to measure conformational change and binding affinities is a well-established technique (31). The majority of the Trp residues in HLA-B8 lie remote from the TCR contact site; however, Trp\textsuperscript{147} and Trp\textsuperscript{167} reside within the Ag-binding groove. Moreover, having determined the crystal structure of the LC13/HLA-B8\textsuperscript{FLRGRAYGL} complex, we were able to ascertain that Trp\textsuperscript{147} is only 3.5 Å away from the incoming CDR3\textbeta loop of LC13, and we reasoned that this Trp may act as a reporter for TCR binding. The concentration of HLA-B8\textsuperscript{FLRGRAYGL} was kept constant in the presence of increasing concentrations of LC13. These data were fitted to a 1:1 binding model and the affinity constant was calculated to be $5.5 \text{ M}$ (Fig. 5B). The Scatchard analysis of this data calculated the dissociation equilibrium constant to be $5.4 \text{ M}$ (1/slope) with a linear relationship supporting the 1:1 binding model.

**FIGURE 3.** Partial phosphorylation of T cell Zap70 by the antagonist ligand FLGRFYGL. C1R.B8 APCs were loaded with 20 μg/ml of either the agonist peptide, FLRGRAYGL (LC13/APC/FLR); the antagonist peptide FLGRFYGL (LC13/APC/AP6F) or no peptide (LC13/APC) for 1.5 h at 37°C, washed, and centrifuged along with LC13 T cells (10^6). After 2 min of coculture, the cells were immunoblotted with an Ab that specifically detects phosphorylated Syk (Tyr\textsuperscript{352}) and Zap70 (Tyr\textsuperscript{319}) (upper panel) or Zap70 alone (lower panel). The position of molecular mass markers is indicated. The membrane was stripped and reprobed with an Ab that recognizes unphosphorylated Zap70 (Cell Signaling) to control for transfer and expression of this protein (lower panel).

**FIGURE 4.** Binding kinetics monitored by SPR. A. Binding of increasing concentrations of the LC13 TCR (5–15 μM) injected over HLA-B8\textsuperscript{FLRGRAYGL}. The curve fits are shown as solid lines overlaying the data points, and the corresponding residual plot is shown below. B. Equilibrium response (RU) for increasing concentrations of the LC13 TCR (2.5–160 μM) binding to HLA-B8\textsuperscript{FLRGRAYGL}. The equilibrium dissociation constant ($K_{\text{d}}$) was calculated to be 8.2 μM by nonlinear regression. C. Kinetic data for the LC13 TCR (20–60 μM) binding to HLA-B8\textsuperscript{FLRGRAYGL}. D. Equilibrium-binding response curve for the LC13 TCR (5–60 μM) binding to HLA-B8\textsuperscript{FLRGRAYGL}; the equilibrium dissociation constant ($K_{\text{d}}$) was calculated as 138 μM. All results are shown as one representative experiment of three.
(Fig. 5C). This affinity compares favorably to the 8.8 μM calculated in solution using SPR, indicating that capturing the HLA-B8 molecule to the chip does not compromise the affinity of the TCR-binding interaction.

### Table II. SPR measurements of the agonist and antagonist complex

| Peptide         | $K_{d}$ (µM) | $K_{ad}$ ($\times 10^3$ M$^{-1}$s$^{-1}$) | $K_{diss}$ (s$^{-1}$) | $K_{calc}$ (µM) | $t_{1/2}$ (s) |
|-----------------|--------------|------------------------------------------|-----------------------|-----------------|--------------|
| FLRGRAYGL       | 8.2 ± 0.5    | 35.80 ± 5.9                              | 0.42 ± 0.02            | 12.5 ± 2.4      | 1.65         |
| FLRGRFYGL       | 138 ± 20     | 2.62 ± 0.3                               | 0.35 ± 0.04            | 132 ± 2.5       | 1.98         |

APL activity of FLRGRFYGL is associated with minor structural alteration of the HLA-B8-peptide complex

Next, we aimed to elucidate the structural basis of the observed antagonism. The crystal structure of the HLA-B8-antagonist complex was very similar to that of the HLA-B8FLRGRAYGL complex that was determined previously (18). Accordingly, the structure description will be limited to the impact the antagonist peptide has on the Ag-binding groove. The electron density for the bound antagonist peptide was unambiguous. The site of substitution (P6) represents a surface-exposed position in the FLRGRAYGL peptide that is located within the central bulge of the peptide, adjacent to the P7-Tyr residue essential for T cell activation. Accordingly, the Ala to Phe substitution, albeit a nonconservative one, neither impacts on the conformation of the bound peptide nor on the HLA-B8 conformation (18). The P6-Phe adopts a conformation such that it folds back toward the N terminus of the peptide, making van der Waals (vdw) contacts with the peptide backbone of Gly$^4$ (Fig. 6A). Accordingly, the conformation of the P7-Tyr is not affected by the conformation adopted by Phe$^6$. The conformation of the P6-Phe side chain, despite being fully solvent exposed, does not protrude prominently from the Ag-binding groove, as distinct from the prominent P-7 Tyr.

Having recently determined the structure of the LC13/HLA-B8-agonist complex (16), we were able to address, in part, how this antagonist peptide impacts on the mode of LC13 binding. A conserved diagonal footprint on the agonist (Fig. 6B) and antagonist complexes (Fig. 6C) was considered most probable. Accordingly, the LC13/HLA-B8FLRGRAYGL complex was superposed onto the HLA-B8FLRGRFYGL complex. Visual inspection of the docked LC13/HLA-B8FLRGRFYGL complex revealed that the Phe$^6$ side chain of the antagonist did not stericly clash with any residues from the CDR loops of LC13. Thus, it is viewed most likely that the conformation of the CDR loops in the docked TCR/HLA-B8FLRGRFYGL complex will be very similar to the observed conformation of the CDR loops in the LC13/HLA-B8FLRGRAYGL crystal structure. Definitive experimental proof of this hypothesis will require the crystal structure determination of the LC13/HLA-B8FLRGRFYGL complex. As can be seen from this docked model (Fig. 6C), the bulky Phe$^6$ side chain nests into a groove formed by the CDR3$\alpha$ loop, making vdw contacts with Leu$^{94}$ and Gly$^{97}$ of the TCR, whereas in the crystal structure of the agonist complex, this pocket is occupied by a water molecule.

### Discussion

Previous studies have shown that APLs of immunogenic peptides may profoundly reduce the magnitude of the response to the wild-type epitope (32–39). In this report, we have identified and characterized an antagonist APL for an immunodominant antiviral and alloreactive human TCR. The CTL response to EBV in unrelated HLA-B8$^+$ individuals is dominated by reactivity toward the RAKFKQQL peptide from the lytic Ag BZLF1 and the FLRGRAYGL peptide from the latent EBNA-3A Ag. Moreover, the CTL response to this latter determinant is characterized by highly restricted TCR usage, such that in unrelated EBV$^+$ HLA-B8$^+$ individuals, the αβ TCR sequence is virtually identical (20). Fine epitope mapping of the FLRGRAYGL epitope revealed that the prototypic immunodominant TCR, termed
We recently provided a structural basis for this immunodominant LC13 TCR/HLA-B8FLRGRAYGL interaction, where the P7-Tyr was highly sensitive to substitutions at the P6 position within the FL-GRAYGL determinant, thereby suggesting the need for a small side chain at P6 to permit TCR ligation (16). Therefore, we sought the conformational changes that the CDR3 loop of the LC13 TCR undertakes upon complexation to HLA-B*0801FLRGRAYGL. Magenta, Nonliganded CDR3α loop; red, liganded conformation. Conformation of the loops are based on crystallographic data. C, Molecular model of the LC13/HLA-B*0801FLRGRAYGL complex. The bulky P6-Phe group can be seen to be readily accommodated in this complex, making favorable vdw contacts with the CDR3α loop (red). Interactions involving the P6 position are shown as dashed lines.

FIGURE 6. Structural basis of the FLRGRAYGL antagonism. A, Superposition of the crystal structure of the authentic agonist (green) and the crystal structure of the antagonist (yellow) peptides within the HLA-B8 groove. For clarity, the front helix is removed from this figure. B, View of the conformational changes that the CDR3α loop of the LC13 TCR undertakes upon complexation to HLA-B*0801FLRGRAYGL. Magenta, Nonliganded CDR3α loop; red, liganded conformation. Conformation of the loops are based on crystallographic data. C, Molecular model of the LC13/HLA-B*0801FLRGRAYGL complex. The bulky P6-Phe group can be seen to be readily accommodated in this complex, making favorable vdw contacts with the CDR3α loop (red). Interactions involving the P6 position are shown as dashed lines.

LC13, was exquisitely sensitive to substitutions at the P7-Tyr position, as well as the small flanking residues of P6-Ala and P8-Gly (19). We recently provided a structural basis for this immunodominant LC13 TCR/HLA-B*0801FLRGRAYGL interaction, where the P7-Tyr was observed to sit centrally within the TCR pocket, while the small P6-Ala and P8-Gly side chains enabled the tyrosine to protrude deeply into the pocket (16).

Since this antiviral CTL response is cross-reactive with several alloantigens and could therefore influence allograft rejection and GVHD, it was of interest to ascertain whether antagonist APLs could be identified. From an exhaustive screen, whereby each amino acid of the nonamer was substituted with the remaining 19 aa acids, one candidate antagonist was found. This antagonist peptide, FLRGRAYGL, was shown to be very effective in reducing lysis, as judged by the Cr release assay. The FLRGRAYGL peptide displayed no agonist activity and moreover antagonist activity that could not be explained by simple competition for binding to HLA-B*0801. Thus, prepping the LC13 CTL clone with peptide, followed by washing away the free APL, led to inhibition of LC13 responses toward both the cognate HLA-B*0801FLRGRAYGL agonist as well as the alloantigens HLA-B*4402 and HLA-B*4405. This observation is consistent with the current view that CTL antagonism is through competition by antagonist MHC peptide complexes for TCR binding at the expense of agonist MHC peptides. Importantly, antagonist activity was also observed when the FLRGRAYGL peptide was present throughout the culture with agonist-bearing APC, a desirable property of any potential therapeutic antagonist.

To further characterize the molecular basis for the antagonism of this public CTL clonotype, we then determined the respective affinities and kinetic constants of the immunodominant TCR for the HLA-B*0801FLRGRAYGL and HLA-B*0801FLRGRFYGL complexes. Using SPR, the measured affinity for the agonist complex, $K_a = 8 \mu M$, was in good agreement with the solution-phase experiments, where a $K_d = 5.5 \mu M$ was obtained using a Trp fluorescence reporter assay. This suggests that Trp fluorescence measurements may provide an alternative for the cell-based assays, ligand-labeling studies, analytical ultracentrifugation, or calorimetry techniques (reviewed in Ref. 40) that have previously provided the main alternative to SPR measurements in defining the affinity of a TCR/MHC interaction. A $K_d$ of 8 $\mu M$ falls within the range of the measured affinities of previous TCR-class I interactions; however, by comparison the association and dissociation rates for the agonist complex were faster.

In other studies the functional outcome of TCR ligation with APLs has correlated best with TCR affinities, most notably the dissociation rate with MHC-APL complexes. However, a number of exceptions to this correlate have been noted (14, 40–42). As determined using Biacore, the affinity for the agonist complex (138 $\mu M$) is 17-fold lower than the agonist (8 $\mu M$). Surprisingly however, the association rate of the antagonist complex is significantly slower (2.6 vs 35.8 $\times 10^4 M^{-1} s^{-1}$), yet the half-life is actually marginally slower than the agonist complex (1.98 vs 1.65 s). Accordingly, factors other than binding kinetics govern the biological outcome from engagement of this immunodominant TCR.

The antagonist activity induced by the Ala–Phe substitution at P6 was surprising, given our earlier observation that LC13 is highly sensitive to substitutions at the P6 position within the FLRGRAYGL determinant, thereby suggesting the need for a small side chain at P6 to permit TCR ligation (16). Therefore, we sought the structural basis for this observed phenomenon. Previously APLs against HLA-B8-HIV epitopes were observed to cause small but significant Cα backbone shifts of the helices of the Ag-binding cleft (43). In addition, the crystal structures and models of a number of TCR-ligated APL complexes have suggested that only subtle conformational changes are required within the hot spot of the TCR/MHC-peptide interface to accommodate the APLs (12, 13, 44). The P6-Phe residue is solvent exposed and was observed not
to impact on the conformation of the peptide nor the conformation of the HLA-B8 when compared with the binary agonist complex. As yet, we have not been able to obtain crystal of the LC13/HLA-B8\textsuperscript{FLRGRAYGL} complex, presumably due to the low affinity of the interaction. Nevertheless, molecular modeling suggests that the bulky Phe\textsuperscript{6} group is accommodated readily in the docked LC13/HLA-B8-antagonist complex. This suggests that the conformation of the CDR loops in the docked antagonist complex will be virtually identical to the conformation of the CDR loops observed in the crystal structure of the LC13/HLA-B8-agonist complex (16). How do these structural observations relate to the observed slower rate on for the antagonist complex in comparison to the agonist counterpart? Upon LC13 binding to HLA-B8\textsuperscript{FLRGRAYGL}, large movements in a number of the CDR loops were observed (16). The small side chain of P6-Ala reduced the steric hindrance of the movements in a number of the CDR loops were observed (16). The identified of an APL antagonist for this immunodominant TCR is of particular interest because this Ag receptor is alloreactive with HLA-B\textsuperscript{4402} and HLA-B\textsuperscript{4405}, and such alloreactivity could exacerbate T cell-mediated transplant rejection (7, 8). Furthermore, this public TCR is expressed by an expanded program for assistance.

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