Novel CD8$^+$ T Cell Antagonists Based on β$_2$-Microglobulin*

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The CD8 coreceptor of cytotoxic T lymphocytes binds to a conserved region of major histocompatibility complex class I molecules during recognition of peptide-major histocompatibility complex (MHC) class I antigens on the surface of target cells. This event is central to the activation of cytotoxic T lymphocyte (CTL) effector functions. The contribution of the MHC complex class I light chain, β$_2$-microglobulin, to CD8ααα binding is relatively small and is mediated mainly through the lysine residue at position 58. Despite this, using molecular modeling, we predict that its mutation should have a dramatic effect on CD8ααα binding. The predictions are confirmed using surface plasmon resonance binding studies and human CTL activation assays. Surprisingly, the charge-reversing mutation, Lys$^{58}$ → Glu, enhances β$_2$m-MHC class I heavy chain interactions. This mutation significantly reduces CD8ααα binding and is a potent antagonist of CTL activation. These results suggest a novel approach to CTL-specific therapeutic immunosuppression.

The peptide-MHC$^{11}$ class I complex (pMHC) on a target cell (antigen presenting cell) is recognized by a specific T cell receptor on the surface of CD8$^+$ cytotoxic T lymphocytes (CTL). The pMHC consists of a heavy chain, which is attached to the cell membrane and contains the peptide binding site, and a light chain, β$_2$-microglobulin (β$_2$m). The CD8 molecule is a cell-surface glycoprotein present on CTL, which acts as a “co-receptor”; it is not peptide-specific, but binds to a conserved site on the pMHC molecule, which comprises several regions on the heavy chain and the small DE loop of β$_2$m consisting of residues 58–60 (Lys$^{58}$–Asp$^{59}$–Trp$^{60}$) (1).

After CTL engage pMHC, the earliest intracellular events induce specific phosphorylation of tyrosine residues in the immunoreceptor tyrosine activation motifs within the cytoplasmic tails of the TCR-associated CD8 complex. The cytoplasmic tail of the CD8 α-chain is associated with the protein tyrosine kinase p56$^{1*}$. Active p56$^{1*}$ initiates TCR signal transduction by phosphorylating the immunoreceptor tyrosine activation motifs within the CD3 complex. Inhibition of CD8 binding to pMHC therefore inhibits T cell activation (2).

Exogenous soluble β$_2$m can exchange with cell-surface-associated β$_2$m complexed to pMHC (3). Therefore, by mutating the CD8 contact site on β$_2$m, and exchanging the mutant β$_2$m into the native MHC, it should be possible to inhibit CTL activation.

EXPERIMENTAL PROCEDURES

Molecular Dynamics and Free Energy Perturbations—Initial coordinates were taken from the crystal structure of the complex between human MHC class I HLA-A2 and the T cell coreceptor CD8ααα solved at 2.65-Å resolution and deposed in the Protein Data Bank (4) under the name 1akj (1). Molecular dynamics and free energy perturbations were performed using CHARMM (version 27) (5) and the standard all-atom parameter set (6). Hydrogens were added using the HBUILD module in CHARMM. Water molecules were added to the complex by superimposing a 16-Å sphere of TIP3P water molecules centered at the β$_2$m Lys$^{58}$ N$_\text{α}$ atom.

The solvent atoms were minimized by 500 steps of steepest descents followed by 1000 steps of conjugate gradient. At the next step, the entire system was relaxed with 500 steps of steepest descents that were switched to conjugate gradient until convergence criteria of r.m.s. gradient of the potential energy lower than 0.3 Kcal/molÅ has been achieved. A 14-Å nonbonded cutoff was employed. The dielectric constant was unity. The system was simulated using a stochastic boundary molecular dynamics (7). The reference point for partitioning the system was the β$_2$m Lys$^{58}$ N$_\text{α}$ atom. The system was divided into a 12-Å reaction region, a 4-Å buffer region, and a reservoir. The frictional coefficients for water oxygen and heavy atoms in the protein were 62 and 200 ps$^{-1}$, respectively (8). The relaxed system was equilibrated at 300 K for 150 ps with a time step of 1 fs followed by 1 ns performed for data collection with coordinates and energies saved to a disc every 1 ps. The β$_2$m Lys$^{58}$ was mutated using the Biopolymer and Homology modules in the MSI software package. For each mutation the procedure described above has been repeated. Relative binding Helmholtz free energies were calculated by the perturbation method (9) as follows: $\Delta G = \text{HLA-A2/β}_2\text{m(native)} \rightarrow \text{HLA-A2/β}_2\text{m(Lys→Glu)}$ complex and $\Delta G = \text{HLA-A2/β}_2\text{m(native)/CD8ααα} \rightarrow \text{HLA-A2/β}_2\text{m(Lys→Glu)/CD8ααα}$ complex.

Since free energy is a state function, it is path-independent, and the free energy difference: $\Delta G_{\alpha}/\Delta G_{\text{Lys}}$ is equal to the difference $\Delta G_{\text{Lys}}$ (see “Results”). Each perturbation was performed in two steps using a total number of 26 windows. At the first 16 windows the lysine His1, His1, His2, H$_2$, H$_1$, H$_2$, and H$_3$ atoms (including their charges) were deleted. C$_6$ atom type was modified to sp$^2$ carbonyl carbon. H$_2$ and C$_6$ atom types were modified to carbohydrate oxygens. At the last 10 windows, the charges of the remaining side chain were adjusted to asp side chain. Trajectories were produced by MD simulations at the same conditions to these described above with 150 ps of equilibration and 100 ps of data collection at every window.

Soluble CD8ααα Preparation—The extracellular fragment of soluble CD8ααα (residues 1–120) was expressed in Escherichia coli, refolded and...
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**Table I**

Molecular dynamics results for the interaction between the CD8αα and β₂m Lys⁸⁸ mutants

| Hydrogen bonds to the CD8αα⁺ | System | Donor | H | Acceptor | Distance D. . . A | Angle D. . . H . . A |
|-----------------------------|--------|-------|---|----------|------------------|----------------------|
| Crystal structure           | Lysαα Nι (β₃m) | Asp⁷⁵ O₂ (CD8α-1) | 2.68 |
| MD native structure         | Lysαα Nι (β₃m) | Val⁴⁰ O (CD8α-1) | 2.98 |
| MD Lysαα → Arg              | Lysαα Nι (β₃m) | Lysαα H₃ (β₃m) | 2.60 |
| MD Lysαα → Ser              | Lysαα Nι (β₃m) | Lysαα H₂ (β₃m) | 2.71 |
| MD Lysαα → Cys              | Cysαα Sι (β₃m) | Cysαα H₁ (β₃m) | 2.65 |
| MD Lysαα → Cys              | Cysαα Sι (β₃m) | H₂O₆ O (β₃m) | 2.66 |
| MD Lysαα → Cys              | Cysαα Sι (β₃m) | H₂O₆ O (β₃m) | 3.42 |

**Time averaged r.m.s. deviation of contact residues to the crystal structure**

| β₂m Lys⁸⁸ mutation | Contact residues r.m.s. |
|-------------------|------------------------|
| MD native structure | 0.65                   |
| Lysαα → Arg       | 0.66                   |
| Lysαα → Ser       | 0.70                   |
| Lysαα → Glu       | 0.71                   |
| Lysαα → Lys⁸⁸ neutral | 0.75                 |
| Lysαα → Asp       | 0.87                   |
| Lysαα → Tyr       | 0.91                   |
| Lysαα → Cys       | 0.93                   |
| Lysαα → Trp       | 0.94                   |
| Lysαα → Val       | 1.00                   |
| Lysαα → GRG       | 1.21                   |
| Lysαα → SES       | 1.78                   |

**purified as described previously (1). The CD8αα concentration was determined from the extinction coefficient (32,480 m⁻¹ cm⁻¹, determined by amino acid analysis), assuming 100% activity.**

**Soluble TCR Preparation**—The TCR used for the SPR experiments derives from the JM22 T cell clone (10, 11). It is specific for an HLA-A2-restricted peptide (GILGFVFTL) from the influenza matrix protein (58–66) and uses gene segments TRAVJ18/23 TRβ1/2 (13). The two fragments of soluble JM22-TCRβ (residues 1–204 for the α-chain and 1–245 for the β-chain) were expressed in E. coli, refolded and purified as described previously (12). The TCR concentration was determined from the extinction coefficient (105,500 m⁻¹ cm⁻¹, determined by amino acid analysis), assuming 100% activity.

**Soluble HLA-A2/β₂m Complex Preparation**—Soluble influenza peptide-HLA-A2/β₂m complexes were prepared by refolding HLA-A2 heavy chain carrying the biotin tag with β₂m wildtype or mutant (both expressed in E. coli) and the synthetic peptide corresponding to influenza matrix protein 58–66 GILGFVFTL (Genosys, Woodlands, TX) as described in Garboczi et al. (13). The refolded complexes were purified by both anion exchange and gel filtration before being used in SPR experiments. HLA-A2 heavy chain was enzymatically biotinylated as described (14) using N-hydroxysuccinimidyliotin (Sigma) and Biotin-1.

**Tetrameric pMHC I Complexes**—complexes containing wildtype or Lys⁸⁸ were immobilized at 12,000–12,500 Response units by injection of 5–35 µl at 40–100 µg ml⁻¹, at a flow rate of 5 µl min⁻¹. The injections of the different CD8αα (sCD8α) and JM22-TCRβ (sTCR) solutions were performed at a flow rate of 5 µl min⁻¹. Each injection of HLA-A2/β₂m (wild and mutants) complexes were immobilized at 12,000–12,500 Response units by injection of 5–35 µl at 40–100 µg ml⁻¹, at a flow rate of 5 µl min⁻¹. The injections of the different CD8αα (sCD8α) and JM22-TCRβ (sTCR) solutions were performed at a flow rate of 5 µl min⁻¹. The r.f. values obtained either by Scatchard plots or by nonlinear fitting of the Langmuir binding isotherm (A + B ↔ AB) equation (A = B + AB = K₅Jₜ + B) (where B is sCD8α (or sTCR) concentration and A is maximum sCD8α (or sTCR) binding) to the data using the Levenberg-Marquardt algorithm as implemented in the Window's Application Origin (version 6.1; Microlab Software, Northampton, MA).

**Cell Culture and CTL Activation Assays**—pBMC were isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation. CD8⁺ CTL clones were generated and maintained as described previously (15). Target cells in cytotoxicity assays were HLA-matched immortalized T cell hybridomas or Epstein-Barr virus transformed B-lymphoblastoid cell lines (B-LCL) incubated with peptide as shown, or infected with recombinant vaccinia virus (rVV), and labeled with ⁵¹Cr (Amer sham Biosciences, Amersham, UK). Peptides were synthesized using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry and were >90% pure as determined by high performance liquid chromatography (Research Genetics, Huntsville, AL). Vaccinia infections were effected at 3–5 plaque-forming units/cell for 1 h and followed by a 6-h incubation period to allow expression prior to ⁵¹Cr labeling. The rVV expressing HIV-1 Nef was constructed from a full-length proviral clone isolated from donor SC1 according to standard protocols; wild type WR rVV was used to infect control target cells (16). Lysis assays were performed in low percentage fetal calf or human serum using standard ⁵¹Cr release methodology (17). Tetrameric pMHC I complex activation assays were performed by measurement of extracellular RANTES release after 30-min exposure as described previously (18). All data points for both lysis and RANTES release assays represent the mean of triplicate readings; ± error bars show the S.D. in each case.

**Flow Cytometric Analysis**—Cells were stained with phycoerythrin-labeled tetrameric pMHC I complexes containing wildtype or Lys⁸⁸ → Glu forms of β₂m as described previously (19). Stained cells were analyzed using a Becton Dickinson Calibur flow cytometer with CellQuest software.
FIG. 1. The effect of various $\beta_{2m}$ Lys$^{58}$ mutations on the interaction with the CD8$\alpha$ and HLA-A2 molecules. H-bonds are shown in hashed lines, atom colors: carbon (C) = green; nitrogen (N) = blue; oxygen (O) = red; hydrogen (H) = white; sulfur (S) = yellow. $\Delta$, the importance of the positive charge on Lys$^{58}$ side chain. While the native Lys$^{58}$ forms two H-bonds to CD8$\alpha$$-1$ Asp$^{75}$ and Val$^{24}$, neutral lysine (side chain in yellow)
RESULTS AND DISCUSSION

We employed 1-ns MD simulations to study the interactions between CD8α, HLA-A2, and β2m molecules. We focused on the β2m Lys58 residue, which forms two key hydrogen bonds with CD8α-1 Asp⁷⁵ Oδ₂ and Val²⁴ O (1), to design and predict the effects of novel β2m mutants on the interaction between CD8α and pMHC. Five classes of Lys58 mutations were studied: a mutation that preserves the positive charge (Lys 58 → Arg),

FIG. 2. The affinity of CD8αa binding to HLA-A2/β2m complexes. a, CD8αa was injected at increasing concentrations for 1 min through flow cells where HLA-A2/β2m wild type (positive control), HLA-A2/β2m mutant Lys⁵⁸ → Glu were coupled via biotinylated heavy chain and through a flow cell with no protein immobilized (negative control). The amount of CD8αa that bound to the HLA-A2/β2m complexes at each concentration was calculated as the difference between the responses at equilibrium in the HLA-A2/β2m complexes and the negative control flow cells and is plotted against the CD8αa concentration. In a and b, the solid lines represent nonlinear fits of the Langmuir binding to the data. In a, the responses obtained for the HLA-A2/β2m mutant Lys⁵⁸ → Glu were too low, and it was not possible to fit the data (blue dotted line). These experiments were performed at 5 μl min⁻¹. Insets, responses obtained in the blank cell (black dotted line), in the HLA-A2/β2m wild type cell (red line), and in the HLA-A2/β2m Lys⁵⁸ → Glu for a and Lys⁵⁸ → Ser for b cell (blue line) during the CD8αa (80 μM) injection.

does not interact with the CD8α-1. In the Lys⁵⁸ → Arg mutant (side chain in purple) the H-bond to CD8α-1 Asp⁷⁵ is preserved. b, in the Lys⁵⁸ → Ser and Lys⁵⁸ → Cys mutants the interaction with the CD8αa is mediated through a water molecule: the side chain donates its hydroxyl or thiol hydrogen to a water molecule, which donates its hydrogen to CD8αa Val⁵⁴ carbonyl oxygen. c, the native structure β2m Lys⁵⁸ and CD8α-1 Asp⁷⁵ (blue) compared with steric hindrance via a bulky side chain mutants: Lys⁵⁸ → Tyr (side chain in green) and Lys⁵⁸ → Trp (side chain in purple). The bulky side chain fills the cavity between the HLA-A2/β2m CD8αa, and the interaction with the CD8αa is poor. d, comparison between the native β2m DE loop consisting of residues 58–60 (Lys⁵⁸-Asp⁵⁵-Trp⁶⁰), shown in blue, to insertions: Lys⁵⁸ → SES (green) and Lys⁵⁸ → GRG (purple). β2m residues Leu⁵⁰-Ala⁷⁹ are shown in azure. e, comparison between the native structure to a Lys⁵⁸ → Val mutation (blue). The hydrophobic valine side chain repels CD8α-1 Asp⁵⁵ carboxylate, f, mutations to a negatively charged side chain: Lys⁵⁸ → Asp (yellow), Lys⁵⁸ → Glu (purple) compared with the native lysine (gray). In both Lys⁵⁸ → Asp and Lys⁵⁸ → Glu mutations the positive charge on HLA-A2 Arg⁶ heavy chain attracts the mutant’s carboxylate. Only Lys⁵⁸ → Glu carboxylate is in close proximity to CD8α-1 Asp⁷⁵ Oδ₂.

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TABLE II
Summary of affinity constants

| Immobilized complex | Soluble CD8αα binding ($K_d$ in μM) | Soluble JM22-TCRββ binding ($K_d$ in μM) |
|---------------------|-------------------------------------|-------------------------------------|
| Flu-HLA-A2/β₂m wild type | 165 ± 35 (n = 9) | 8.6 ± 2 (n = 7) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Arg | 300 (n = 1)ᵇ | 10.6 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Ser | 301 ± 2 (n = 2) | 10.5 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Cys | 558 (n = 2)ᵇ | 10.4 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Val | 1084 (n = 1) | 7.1 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Glu | NB (n = 4)ᶜ | 8.8 ± 0.8 (n = 4) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Asp | NB (n = 1)ᶜ | 7.9 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Tyr | NB (n = 2)ᵇᶜ | 8.6 ± 1 (n = 2) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → Trp | NB (n = 1)ᶜ | 4.9 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → SES | NB (n = 1)ᵇᶜ | 8.5 (n = 1) |
| Flu-HLA-A2/β₂m mutant Lys₅⁸ → GRG | NB (n = 1)ᶜ | 6.2 (n = 1) |

ᵃ Mean ± deviation from n independent determinations at 25 °C.
b Similar results were obtained with another peptide.
c No binding (response too low to determine a $K_d$).

mutations to short polar side chains (Lys₅⁸ → Ser, Lys₅⁸ → C), mutations introducing steric hindrances via bulky side chains (Lys₅⁸ → Tyr, Lys₅⁸ → Trp) or insertions (Lys₅⁸ → SES, Lys₅⁸ → GRG), a mutation to a medium length hydrophobic side chain (Lys₅⁸ → Val), mutations to negatively charged side chains (Lys₅⁸ → Asp, Lys₅⁸ → Glu).

We studied the hydrogen bond network formed between the wild type Lys₅⁸, or mutations of this residue, to CD8α-1 as shown in Table I. The perturbation to the conformation of the contact residues (CD8α-1-Arg⁴, Asp²⁷, Val⁴⁴-Val⁵⁰, β₂m-Trp⁶⁰, Lys₅⁸) (1) caused by various Lys₅⁸ mutations is presented as the root mean square (r.m.s.) deviation of these residues to that observed in the crystal structure.

Neutralizing the Lys₅⁸ positive charge in silico leads to the loss of two H-bonds to CD8α-1-Asp⁷⁵ O₂ and Val⁵⁰ O atoms, as illustrated in Fig. 1a and Table I, and formation of alternative hydrogen bonds with water molecules in the cavity of the HLA-A2/CD8αα/β₂m complex. A mutation that preserves the positive charge (Lys₅⁸ → Arg) has a less dramatic effect: the H-bond to CD8α-1-Asp⁷⁵ O₂ is preserved, and the r.m.s. value is similar to that of the wild type structure and smaller than that of the neutral Lys₅⁸ side chain. Therefore, eliminating the positive charge on the side chain is likely to reduce the binding affinity to the CD8αα.

In short polar side chain mutants, Lys₅⁸ → Ser and Lys₅⁸ → Cys, the interaction with CD8αα is mediated through a water molecule as shown in Fig. 1b and Table I. The side chain donates its hydroxyl or thiol hydrogen to a water molecule, which donates its hydrogen to CD8αα Val⁵⁰⁴. Unlike Lys₅⁸ → Ser, where the H-bond network is stable, the H-bond interaction with the thiol moiety and the water molecule in the Lys₅⁸ → Cys mutant fluctuates during the simulation. This has an impact on the r.m.s. values shown in Table I, which increase from 0.70 for Lys₅⁸ → Ser to 0.93 for Lys₅⁸ → Cys. These results suggest that removing the H-donor group from the side chain at position 58 should reduce the binding affinity to CD8αα.

Mutations introducing steric hindrance via a bulky side chain, Lys₅⁸ → Tyr and Lys₅⁸ → Trp, are illustrated in Fig. 1c. The bulky side chain fills the cavity between HLA-A2/β₂m and CD8αα, and the interaction with CD8αα is impaired by the lack of H-bonding, resulting in high r.m.s. values (Table I). Lys₅⁸ → GRG and Lys₅⁸ → SES insertions (Fig. 1d) perturb the tertiary structure and lead to increased r.m.s. values of 1.21 and 1.78, respectively. The higher r.m.s. value of the Lys₅⁸ → SES insertion is due to reversal of the positive charge and the fact that serine has a side chain that contributes to the overall steric hindrance.

The Lys₅⁸ → Val mutation yielded a higher r.m.s. value than all other single substitution mutations. In bulkier mutations, such as Lys₅⁸ → Trp, the side chain can orient itself toward the HLA-A2/β₂m-CD8αα cavity, whereas the valine side chain is not long enough to have this effect. As a result, the hydrophobic side chain repels the polypeptide CD8α-1 Asp⁷⁵ (Fig. 1e).

Fig. 1f shows mutations to a negatively charged side chain (Lys₅⁸ → Asp, Lys₅⁸ → Glu). Repulsion between the carboxylates leads to an average distance of 6.27 Å between the CD8α-1 Asp⁷⁵ O₂ and Lys₅⁸ O atoms. Strikingly, the distance between CD8α-1 Asp⁷⁵ O₂ and Glu⁵⁸ O₁ oxygens in the Lys₅⁸ → Glu is only 4.09 Å. Both Lys₅⁸ → Asp and Lys₅⁸ → Glu are attracted to the positive charge on HLA-A2 Arg⁶ heavy chain: in 52.9% of the frames taken from the MD simulation, the distance between the mutated Glu⁵⁸ O₂ and HLA-A2 Arg⁶ N₁ atoms was lower than 5 Å, fluctuating to a minimum of 3.18 Å. This interaction stabilizes the HLA-A2/β₂m complex and orients the Lys₅⁸ → Glu side chain toward CD8α-1 Asp⁷⁵. HLA-A2 Arg⁶ heavy chain attracts the Lys₅⁸ → Asp carboxylate as well; however since Lys₅⁸ → Asp side chain is shorter, it is distant from Asp⁷⁵ O₂.

We defined the free energies for the association reactions as following: $ΔG₃ = HLA-A2/β₂m-native complex + CD8αα → HLA-A2/β₂m-native/CD8αα complex + ΔG₃ = HLA-A2/β₂m-(Lys₅⁸ → Glu) complex + CD8αα → HLA-A2/β₂m-(Lys₅⁸ → Glu)/CD8αα complex. We employed free energy perturbations to calculate the relative association free energy: $ΔG₃ = ΔG₃ = +14.95$ Kcal/mol. These results show that the complex containing β₂m(Lys₅⁸ → Glu) has a considerable lower affinity for CD8αα than the wild type complex.

The theoretical results show that, although the contribution of the β₂m Lys₅⁸ to CD8αα binding is relatively small (1), its mutation should have a marked effect upon CD8αα binding.

To test these predictions, we used SPR to measure the binding of soluble CD8αα (sCD8) and soluble JM22 T cell receptor (sTCR) to HLA-A2-influenza matrix peptide complex, containing wild type or mutant β₂m (Fig. 2). The affinity of sTCR for pMHC was similar for all of the complexes studied (Table II), indicating that the active material on the chip surface was correctly folded. However, the relative measurements of response varied markedly between the different complexes, indicating varying proportions of active material on the chip surface. This implies that some mutant β₂m complexes are more stable than others. In particular, HLA complexes containing Lys₅⁸ → Tyr, Trp, SES, and GRG mutations show reduced stability (data not shown). These observations correlate with refolding efficiency, with yields of Lys₅⁸ → Arg and Lys₅⁸ → Glu complexes being several times higher than those for Lys₅⁸ → SES and Lys₅⁸ → GRG complexes (data not shown). This difference agrees with the MD simulations, which predict
that the mutant Lys58 → Arg complex structure is closest to that of the wild type (Table I), and that the complex containing the βm mutant Lys58 → Glu mutant is stabilized by the interaction with HLA-A2 heavy chain R6.

There is a strong correlation between the measured sCD8 binding (Table II) and the predicted H-bonds network formed between Lys58 and Asp75 or Thr52O (Table I). The wild type structure forms two hydrogen bonds and shows the highest affinity for sCD8. Complexes containing βm mutants Lys58 → Arg, Ser, or Cys are predicted to form only a single H-bond (mediated through a water molecule for Lys58 → Ser and Lys58 → Cys) and show decreased binding affinity for sCD8 (Fig. 2, Table II). In complexes containing other βm mutants (Lys58 mutations to Val, Asp, Tyr, Trp, GRG, SES, and Glu), where no H-bond was predicted, the sCD8 binding was negligible (Ki > 1 mM). Similarly, mutations of Asp59 and Trp60 also greatly reduced sCD8 binding (data not shown). The BIAcore binding data showed that sCD8 binding is undetectable in HLA complexes containing βm in which Lys 58 is mutated to Glu (Fig. 2, Table II), yet this complex behaves identically to complexes containing wildtype βm in terms of sTCR binding, refolding yield, and stability.
The strong correlation between the predictions of the molecular dynamics simulations, free energy calculations, and biophysical measurements of sCD8 binding led us to conclude that the $\beta_2m$ mutant containing a glutamate residue at position 58 ($\text{Lys}^{58} \rightarrow \text{Glu}$) was the most promising candidate for further investigation.

The impact of mutating $\beta_2m$ on the interaction between soluble antigen and TCR expressed on the cell surface was investigated using tetrameric pMHC I complexes. Fluorescence-labeled tetramers containing either wild type or $\text{Lys}^{58} \rightarrow \text{Glu}$ forms of $\beta_2m$ stabilized pMHC I at equivalent levels across a range of concentrations (Fig. 3a). However, tetrameric complexes containing the $\text{Lys}^{58} \rightarrow \text{Glu}$ form of $\beta_2m$ were substantially impaired in their ability to activate CTL. This effect was titratable and most apparent at lower concentrations (Fig. 3b). These data indicate that, at similar levels of interaction with cell surface TCR, the impaired ability of complexes containing the $\text{Lys}^{58} \rightarrow \text{Glu}$ form of $\beta_2m$ to engage the CD8 coreceptor translates into a biologically significant effect. This is consistent with previous work demonstrating that the CD8 coreceptor translates into a biologically significant effect.

The Lys58 → Glu mutant was found to inhibit this fresh PBMC lytic response to endogenously presented pMHC class I antigen in comparison to equimolar levels of wild type $\beta_2m$ (Fig. 3d).

In conclusion, we have developed stable $\beta_2m$ mutants that inhibit CD8 coreceptor binding to pMHC I and exert an inhibitory effect on CTL activation. These data suggest that such mutant forms of $\beta_2m$ could be used to selectively modulate the CD8$^+$ cellular immune response, a principle that could be applied therapeutically (21).

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