



Alterations in the HLA-B*57:01 Immunopeptidome by Flucloxacillin and Immunogenicity of Drug-Haptenated Peptides

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Neoantigen formation due to the interaction of drug molecules with human leukocyte antigen (HLA)-peptide complexes can lead to severe hypersensitivity reactions. Flucloxacillin (FLX), a β -lactam antibiotic for narrow-spectrum gram-positive bacterial infections, has been associated with severe immune-mediated drug-induced liver injury caused by an influx of T-lymphocytes targeting liver cells potentially recognizing drug-haptenated peptides in the context of HLA-B*57:01. To identify immunopeptidome changes that could lead to drug-driven immunogenicity, we used mass spectrometry to characterize the proteome and immunopeptidome of B-lymphoblastoid cells solely expressing HLA-B*57:01 as MHC-I molecules. Selected drug-conjugated peptides identified in these cells were synthesized and tested for their immunogenicity in HLA-B*57:01-transgenic mice. T cell responses were evaluated *in vitro* by immune assays. The immunopeptidome of FLX-treated cells was more diverse than that of untreated cells, enriched with peptides containing carboxy-terminal tryptophan and FLX-haptenated lysine residues on peptides. Selected FLX-modified peptides with drug on P4 and P6 induced drug-specific CD8⁺ T cells *in vivo*. FLX was also found directly linked to the HLA K146 that could interfere with KIR-3DL or peptide interactions. These studies identify a novel effect of antibiotics to alter anchor residue frequencies in HLA-presented peptides which may impact drug-induced inflammation. Covalent FLX-modified lysines on peptides mapped drug-specific immunogenicity primarily at P4 and P6 suggesting these peptide sites as drivers of off-target adverse reactions mediated by FLX. FLX

modifications on HLA-B*57:01-exposed lysines may also impact interactions with KIR or TCR and subsequent NK and T cell function.

Keywords: flucloxacillin, HLA-B*57:01, drug hypersensitivity, hapten, immunogenicity, transgenic mice

INTRODUCTION

Idiosyncratic drug-induced liver injury (DILI) is a rare but potentially fatal adverse drug reaction and a major obstacle in the development of pharmaceuticals. Understanding the biochemistry and immune pathways that mediate severe adverse drug reactions is critical for identifying treatment and prevention strategies to minimize unwanted off-target drug effects. Although liver injury can result from expected toxicities based on the chemical nature of a drug, idiosyncratic reactions may also manifest in susceptible patients. These reactions are complex and many are considered to be immune-mediated based on (i) the presence of mononuclear cells, including effector CD8⁺ T cells, in liver biopsies of patients experiencing DILI (1, 2), and (ii) the reactivity of these patients' T cells to drug stimulation *in vitro*, often observed to be dependent on the expression of specific HLA Class-I and II alleles (3–5). Genome-wide association studies in patients with FLX-induced DILI found this adverse reaction to be significantly associated with the expression of HLA-B*57:01 (6) and HLA-B*57:03 (7), allelomorphs that differ by only two amino acid residues. T cell reactivity to FLX was demonstrated *in vitro* to be elicited by both soluble drug and drug-pulsed presenting cells, the latter suggested to be mediated by covalently-linked FLX on protein epitopes (4, 5, 8). FLX-stimulated peripheral blood CD8⁺ and CD4⁺ T cells from HLA-B*57:01⁺ DILI patients primarily in an antigen-processing dependent manner (4). However, T cells from HLA-B*57:01⁺ drug-naïve healthy individuals required soluble FLX in the cultures and rarely responded to drug-pulsed targets (5, 8). Of note, T cell clones from the HLA-B*57:01⁺ patients responded to soluble FLX presented by non-B*57:01 HLA alleles (8).

While there is an accepted association between the immune system and drug-mediated liver injury, the mechanisms by which drugs cause immune cell activation are still poorly understood. Proposed molecular mechanisms to explain drug hypersensitivity are based on drug-peptide interactions. T cell responses to non-covalent drug-peptide complexes are known as the pharmaceutical interaction mechanism, while antigen processing-dependent responses are thought to be driven by drug covalently linked to peptides presented by HLA molecules (9–12). In addition, we and others have described a non-covalent mechanism by which abacavir facilitates the loading of novel self-peptides with changes in the consensus C-terminal anchor residues into HLA-B*57:01, inducing autoimmune-like reactions to the drug-altered antigens (13–15). In this report, using immunoproteomics, we show that FLX treatment of antigen presenting cells expressing HLA-B*57:01 effects changes in the immunoproteome by mechanisms different from those of abacavir. Instead, without affecting the cellular proteome, FLX

increased the diversity of the peptide repertoire of sampled proteins favoring the presence of tryptophan in the F-pocket anchor motif. We also identified several peptides on the HLA-B*57:01 immunopeptidome pool with FLX-conjugated in lysine residues (FLX-peptides). These were subsequently characterized by mass spectrometry and chemically synthesized for immunogenicity evaluation in an HLA-B*57:01⁺ transgenic mouse. The immunogenic potential of the FLX-peptides was found to depend on both the peptide sequence as well as the position at which FLX was conjugated.

METHODS

Reagents

Flucloxacillin (FLX) was obtained from Apotex, UK or Sigma-Aldrich, USA. FLX-conjugated lysine residues were prepared according to the method of Scornet et al. (16) (**Figure S1** and **Supplementary Methods**) and used to synthesize FLX-modified peptides by solid-phase Fmoc chemistry.

B-Lymphoblastoid Cell Lines

The HLA-B*57:01 full-length sequence for membrane bound HLA (mHLA) was transduced using a lentiviral expression system. From here on, HLA-B*57:01-expressing cells are referred as B721-5701. The expression of soluble HLA-B*57:01 (sHLA) in the HLA class I-negative EBV-transformed B-lymphoblastoid 721.221 cell line was described elsewhere (14).

Proteomics and LC-MS/MS Analysis

Proteomic characterization of B721-5701 cells is presented in **Supplementary Methods**. Cells were grown in the absence or presence of FLX for 5 days in duplicate cultures. Cells were harvested and proteins extracted and trypsinized. After ion exchange fractionation, peptides were analyzed by LC-MS on a Q-Exactive MS with nano-LC. Data was processed with the PEAKS 8.5 software using Uniprot non-redundant human database (<https://www.uniprot.org>).

Generation, Isolation, and Identification of HLA-Bound Peptides

B721.5701 cells expressing mHLA were grown without antibiotics in G-Rex 6-Well Plates to high density for 5 days with or without 150 µg/ml FLX as described in **Supplementary Methods**. Cell lysates were immune-affinity purified using anti-HLA class-I antibody W6/32. Acid-eluted peptides were analyzed using nano-LC-MS/MS with a ThermoFisher Ultimate LC and Fusion Orbitrap MS. Soluble HLA from untreated or FLX-treated cultures was collected from continuous cultures as described in **Supplementary Methods**.

Peptides were purified from sHLA/peptide complexes by immuno-affinity purification followed by acid elution and filtration. Peptide identity was determined as detailed in **Supplementary Methods**.

Peptide Docking Modeling

Details of the protocols applied to modeling peptide interactions with drug, HLA and TCR are described in **Supplementary Methods**. Images were generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Treatment of Mice

HLA-B*57:01 transgenic/H2-K^bD^b knockout (Tg/KO) mice were generated by backcrossing HLA-B*57:01/H2-D^d Tg (17) to H2-K^bD^b KO C57BL/6 (B6.129P2-H2-K^b^{tm1}H2-D^b^{tm1} N12) mice (18) (Taconic Biosciences, Hudson, NY). Tg/KO mice were immunized with 100 µg of FLX-haptenated peptide (FLX-peptide) containing immune adjuvants as detailed in **Supplementary Methods**. At day 14, spleens were processed for *in vitro* testing.

Cell Culture

Splenocytes from Tg/KO mice were cultured for 5 days in complete RPMI supplemented with 0.5% heat-inactivated,

normal mouse serum (days 0-2) and 10% fetal bovine serum (days 3-5) in the absence or presence of 10 µg/ml of FLX-peptides or unmodified peptide. Secretion of IFN-γ was measured in cell culture supernatants by ELISA.

Flow Cytometry

Flow cytometry was used for mouse phenotyping (**Figure S2**) and to analyze immune cell subsets by intracellular and/or surface marker staining as detailed in the **Supplementary Methods**.

RESULTS

Effect of FLX Treatment on the Proteome and Immunopeptidome of B721-5701 Cells

We hypothesized that FLX-driven changes in the protein content of the cell could impact the quality and quantity of antigen processing and presentation, including FLX-haptenated epitopes. B721-5701 cells were treated with subtoxic concentrations of FLX for 5 days (**Figure S3**). On average, 3,401 proteins were identified per sample by LC-MS/MS (**Figure 1A** and **Table S1**). A high Spearman correlation (>0.943) was obtained for all samples, independent of the

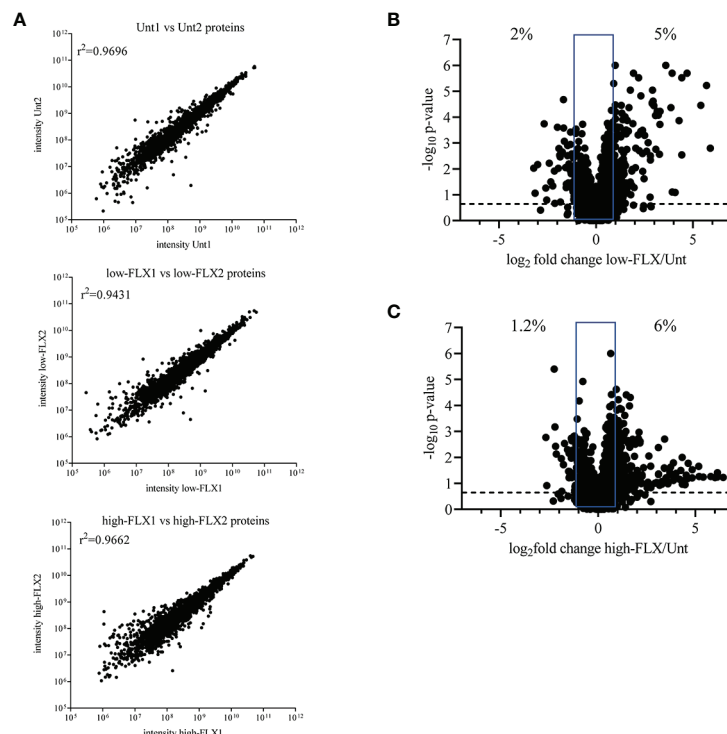


FIGURE 1 | Flucloxacillin (FLX) does not significantly alter the B721-5701 cell proteome. Protein lysates were obtained from ten million B721-5701 cells treated with FLX at 150 µg/ml (low) or 453 µg/ml (high) for 5 days, or from untreated cultures (Unt), in duplicate. Samples were processed by LC-MS/MS and analyzed by Peaks 8.5 software. **(A)** Correlation among biological replicates. Protein abundance is represented in arbitrary units of intensity. **(B, C)** Increased ($\log_2$ fold change ≥ 1) and decreased ($\log_2$ fold change ≤ -1) abundance of proteins in the proteome of cells treated with low-FLX **(B)** or high-FLX concentration **(C)** compared to Unt, considering the average abundance of the two biological replicates per treatment. Percent values indicate divergence in abundance. Statistical differences were calculated by Spearman correlation. Proteome datasets are included in **Table S1**.

treatment, indicating that overall, FLX treatment did not change the proteome of the cell. Changes in the relative counts (intensity) of <7% of proteins of the proteome of cells treated with drug vs untreated cells (**Figures 1B, C**) were not associated with any particular pathway when assessed by ingenuity pathway analysis (data not shown).

In parallel, cells were treated with FLX at 150 $\mu\text{g}/\text{ml}$ for 5 days (FLX-cells) or left untreated (Unt-cells) in two biological replicates for immunopeptidome analysis. Among all four samples, LC-MS/MS analysis identified a total of 3,610 peptides of 8–15 amino acids in length, from which 1142 were detected only in FLX-cells and 226 peptides were only found in Unt-cells. The remaining 1121 peptides were common in both treatment groups (**Figure 2A** and **Tables S2A, B**). The peptide length distribution within each one of these subgroups was similar (**Figures 2B, C**), with dominance of sequences of 9–11 amino acids ($89 \pm 4\%$), from which 50% were 9-mers. Approximately 12% of the peptides in both datasets presented

PTMs (**Figures 2D, E**). Cysteinylation peptides were twice as abundant in the FLX-cells as compared to the Unt-cells.

A total of 1,603 different protein sequences represented by the identified 3,610 peptides (**Tables S2A, B**). Of those, 874 proteins were identified in both treatment conditions, represented by 1238 peptides of the Unt-cells and 1511 peptides of FLX-cells peptide repertoire. The remaining protein sequences were unique to each treatment (**Figure 2F**). Overall, most of the proteins were represented by a single peptide (75.7% in Unt-cells and 70.8% in FLX-cells). Only 16.5% and 18% of the proteins were represented by two peptides (Unt-cells and FLX-cells, respectively), while 7.5% and 10% were proteins with three to five peptides (Unt-cells and FLX-cells, respectively) (**Figure 2G**). Of note, FLX-cells had a larger number of proteins with higher peptide representation than the Unt-cells.

In agreement with others (19, 20), only a fraction of the identified proteins in the proteome were represented in the HLA peptidome of Unt-cells (13%) and FLX-cells (21.8%),

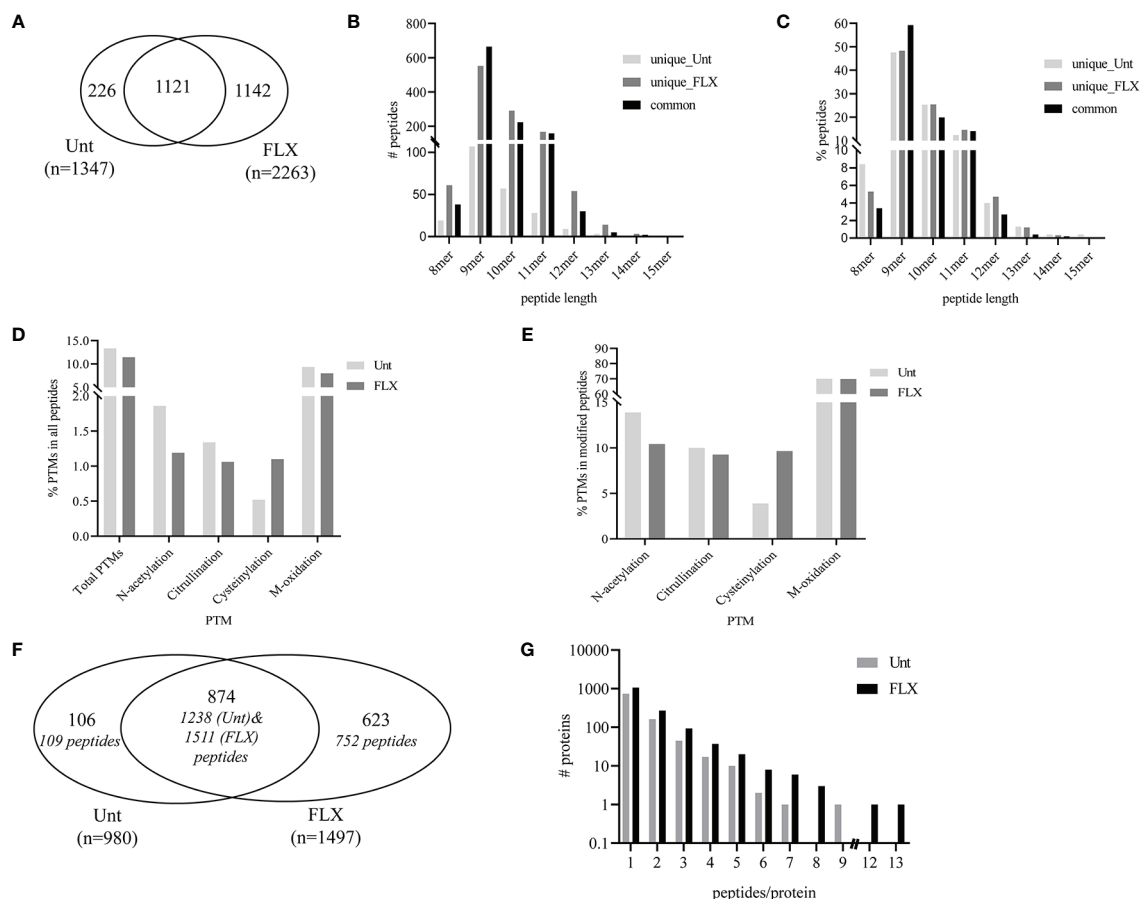


FIGURE 2 | Flucloxacillin (FLX) treatment induces diversity in the peptide repertoire of B721-5701 cells. Common and unique peptides eluted from human leukocyte antigen (HLA)/peptide complexes obtained from two independent cultures of untreated B721-5701 cells (Unt) and of cells treated with 150 $\mu\text{g}/\text{ml}$ of FLX (FLX) for 5 days were combined for the analysis (**A**). Absolute counts (**B**) or frequency (**C**) of 8–15 amino acid peptides. Frequency of peptides with post-translational modification (PTM) within the peptide repertoire (**D**) or among modified peptides (**E**). Common and unique proteins represented by immunopeptidome-contained sequences (in **Figure 2A**) (**F**). Protein distribution showing the number of peptides per protein (**G**). Data analysis was performed using Peaks 8.5 and X software. The datasets of peptides in the B721-5701 peptidomes are provided in **Table S2A, B**.

corresponding to a total of 435 and 699 proteins, respectively. As expected, longer or more abundant proteins contributed to more peptides in the immunopeptidome (**Figures 3A, B**), although the peptide number/protein was significantly higher in both sampled unique and common proteins of FLX-cells (**Figure S4** and **Table S3**). We then calculated the HLA class-I sampling density (D) as described previously (19, 20), a normalization score that takes into account the number of observed HLA peptides of a given protein as a function of its length, and used D to identify proteins that were presented at a higher rate than what was expected for their abundance (represented by D'). Most of the proteins with high over-presentation scores ($D/D' > 3$) had roles in transcription, translation or mitochondrial-related pathways (**Table S3**). Ribosomal subunits had the highest scores ($D/D' > 5$) in both treatment conditions. Interestingly, β_2 -microglobulin epitopes were found in the FLX-cell immunopeptidome but not in the Unt-cell ($D/D' > 6$). In general, the FLX-cell proteome had more over-presented proteins ($D/D' > 2$) than the Unt-cell proteome (86 vs 47, respectively) (**Figure 3C** and **Table S3**). This trend did not correlate with protein abundance ($r^2 < 0.00230$ and 0.00196 for the control and FLX-treated group, respectively) (**Figure 3C**). These results suggest a qualitative and quantitative impact of the drug in the cell immunopeptidome, which is not associated with a change of the cellular proteome but most likely to an effect on antigen processing and presentation.

FLX Treatment Increases HLA-B*57:01-Presented Peptides Containing Tryptophan in the P Ω Position

The peptide repertoire presented by HLA-B*57:01 molecules is determined by the presence of specific amino acids in the anchor positions of its binding motif. Unlike peptides identified from abacavir-treated cells (13–15), 9-mers in the immunopeptidome of FLX-cells exhibit consensus amino acids for the HLA-B*57:01 peptide anchor motif at position P2 and P9 (**Figure 4A**). However, FLX-cells presented a higher frequency of peptides with tryptophan at P Ω than those untreated, mainly in unique peptides (3.7% in Unt vs 79.8% in FLX) (**Tables S2A, B** and **Figure 4A** for 9-mers). Common peptides with C-terminal tryptophan were also more abundant in the FLX-cells (**Figure 4B**). This phenomenon was also observed in two additional independent experiments (**Figure S5**). Interestingly, peptides with C-terminal tryptophan had the highest predicted HLA-binding affinity (< 50 nM). Although arginine was dominant at P7 [a secondary anchor residue (21)] of high-affinity peptides, glutamic acid was significantly increased in peptides of FLX-cells (**Figure 4C** and **Tables S2A, B**). These findings suggest that, in contrast to the ability of abacavir to alter the anchor motif of the neoepitopes from phenylalanine, tryptophan or tyrosine to leucine or isoleucine at P Ω , FLX increases the presentation of self-epitopes with predominantly tryptophan at the C-terminal position.

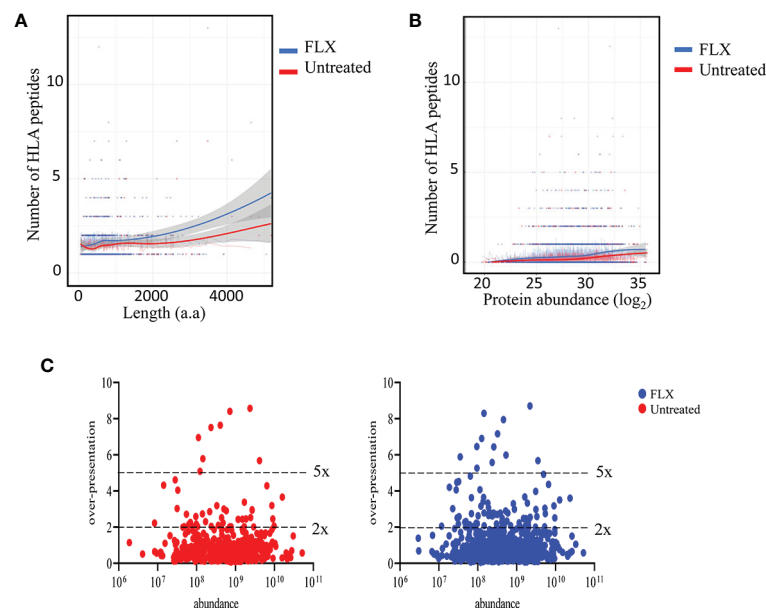
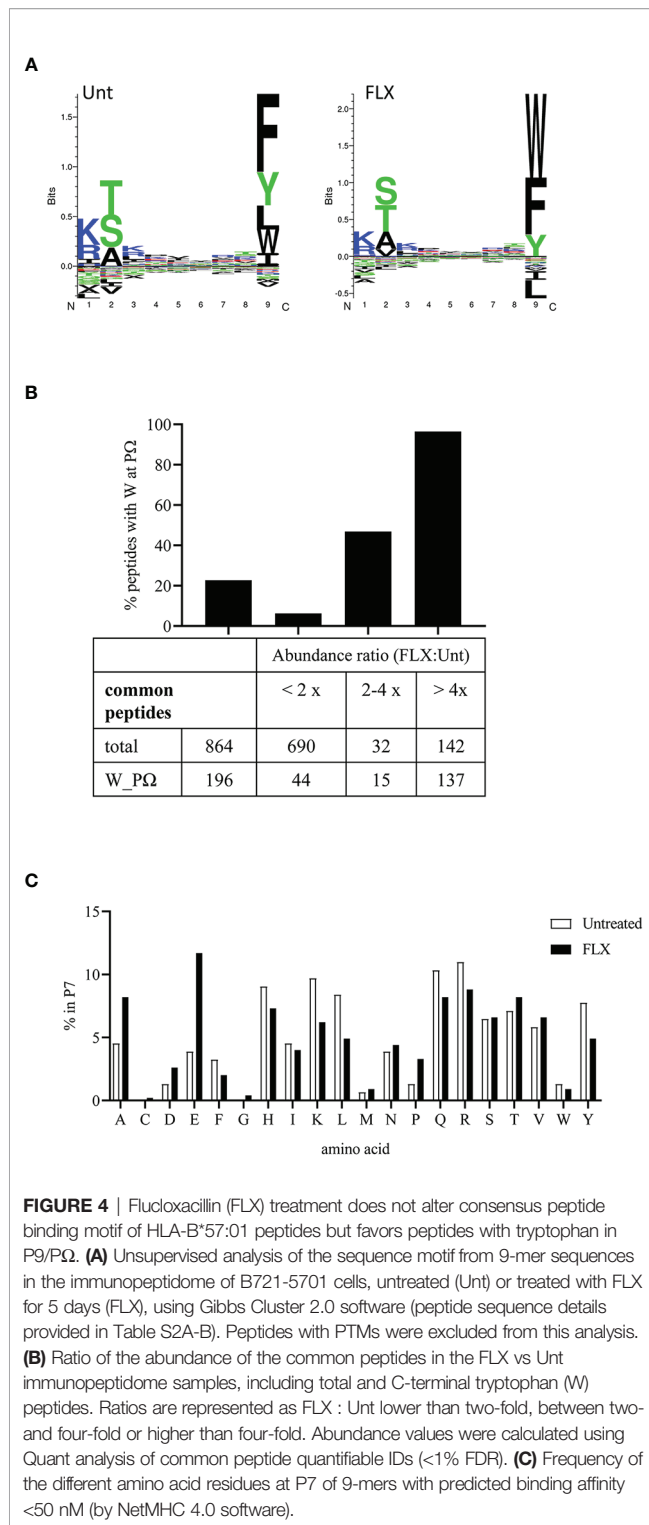


FIGURE 3 | Flucloxacillin (FLX) treatment promotes a higher peptide sampling rate of proteins, independently of the length and abundance of the polypeptide. Abundance of human leukocyte antigen (HLA) peptides from 435 and 699 proteins in the proteome of B721-5701 cells untreated or FLX-treated, respectively, as a function of the protein amino acid length (**A**) or abundance ($\log_2$) (**B**). Individual proteins and trendlines are represented in the graphs. (**C**) Protein fold over-presentation was calculated based on the ratio between observed (D) and expected (D') peptide density, and represented as function of protein abundance. The dataset of the represented proteins is provided in **Table S3**.



Identification of FLX-Haptenated Self-Peptides Derived From sHLA in B721-5701 Cells

Drug conjugation to self-peptides has been proposed as a molecular mechanism by which FLX leads to the generation of

neoepitopes and T cell reactivity (4, 5, 12). Thus, we focused on identifying FLX-haptenated peptides (FLX-peptides) in our peptide dataset. Initial experiments with small scale cultures did not identify FLX-peptides, suggesting that haptenated peptides were in low concentration. In an attempt to increase the amount of HLA and thus the frequency of FLX-sequences, we cultured B721-5701 producing a soluble form of HLA (sHLA) in high density biopharms as described previously (14). We identified several peptides containing FLX on lysines (K) as well as the corresponding unmodified sequences: TAAQITQRKW (TAA) from HLA, KAAKLKEKY (KAA) from the high mobility group HMGB-1 protein and RTKKVGIVGKY (RTKK) from a 60S ribosomal protein. The latter two peptides revealed multiple possible lysine-drug conjugation sites (Table 1). Collision-induced dissociation MS2 resulted in complex spectra with peptide fragments along with drug ions +160, +295, and +454 as labelled on the TAA peptide spectrum in Figure 5A, even though intact drug was not found on fragmented sequences. However, drug-modified peptides did retain diagnostic fragments containing K+294 FLX, the residual portion of FLX covalently linked to the peptides as illustrated in Figure 5A. We identified b4-K+294 in FLX-RTKK_K4 and FLX-KAA-K4, and y2-K+294 in FLX-TAA_K9. Interestingly, the modified lysine in TAA corresponded to K146 of the HLA, an interaction site with KIR-3DL immuno-regulatory receptor of NK cells. Peptides were further validated by chemical syntheses using FLX-coupled Fmoc lysines as described in Methods (Figure S6A). In addition, we synthesized two KAA peptides with FLX in lysine 6 or 8 (FLX-KAA_K6 and FLX-KAA_K8) and RTKK with drug in the lysine in position 10 (FLX-RTKK_K10), as controls for immunogenicity studies, although these were not identified in the peptide repertoire. Quality control runs of the synthesized FLX-peptides surprisingly showed RP-HPLC chromatograms with two peaks by retention time (RT) each with the same mass by LC-MS (Figures S6B, C). FLX-RTKK_K4 isomeric forms differing in RT were later confirmed in the B721-5701 immunopeptidome (Figure 5B). We hypothesized that the difference in RP-HPLC RT was secondary to conformational differences between the FLX adducts. For these reasons, the two RP-HPLC peaks of the FLX-RTKK peptides were purified separately and tested independently in *in vivo* experiments.

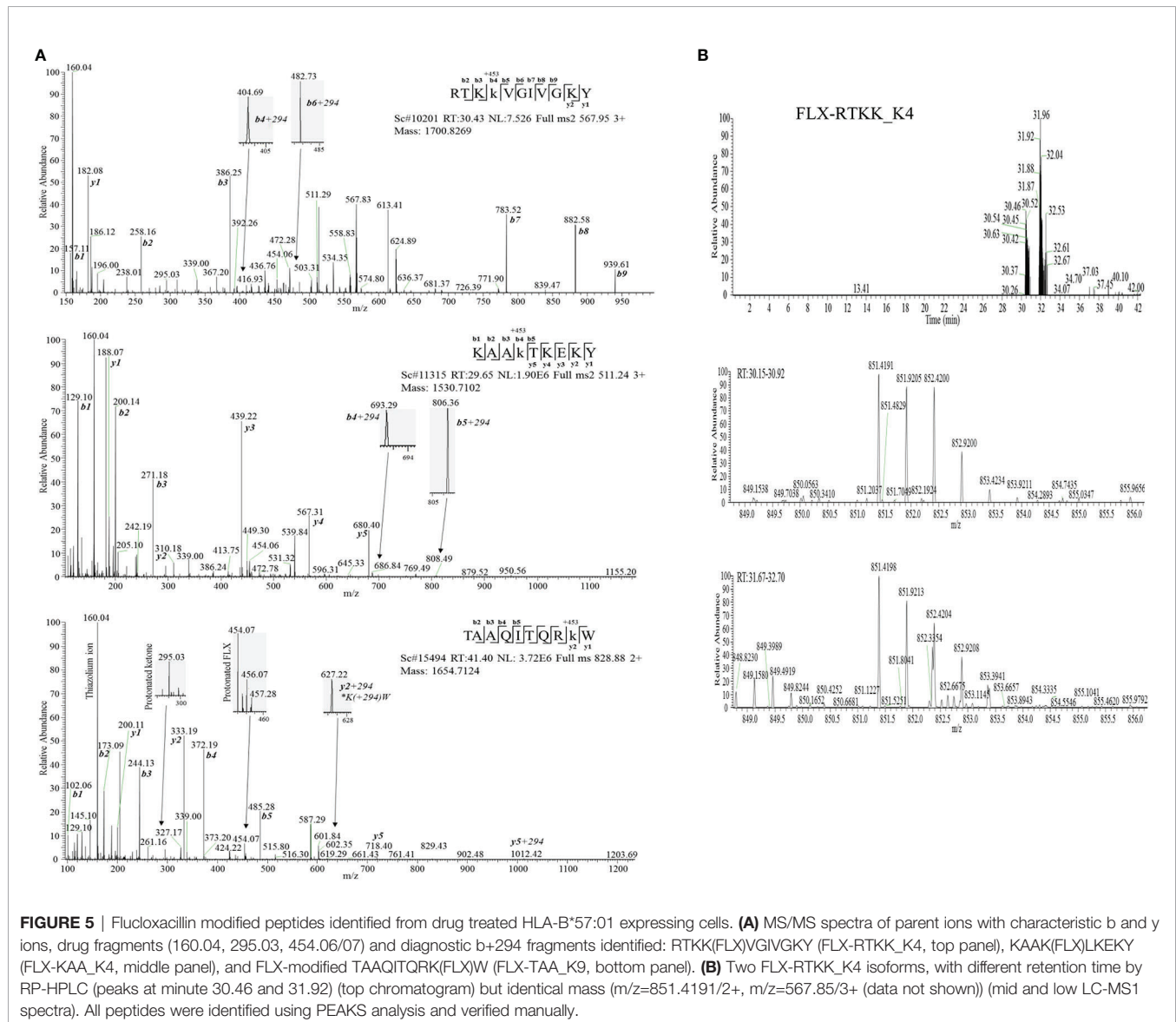
The Immunogenic Potential of FLX-Peptides Depends on the Peptide Sequence and the Drug-Conjugated Amino Acid Position

To evaluate the immunogenicity potential of the FLX-peptides, we immunized Tg mice expressing only the chimeric HLA-B*57:01/H2-D^d(α3) and not classical mouse MHC-class I (H2-K^b and H2-D^b) molecules (Tg/KO). Peptide-specific T cell responses were observed in animals treated with several FLX-peptides upon splenocyte *in vitro* restimulation (Figures 6A, B). FLX-RTKK_K4 induced higher levels of IFN-γ secretion than FLX-RTKK_K10 in cells of animals immunized with the same peptides, and did not cross-react with either the parent, unlike cells from some of the FLX-RTKK_K10-immunized mice, or the

TABLE 1 | Unmodified and flucloxacillin (FLX)-modified peptides found in B721-5701_HLA cell preparations.

Peptide	FLX-conjugated residue in the protein	Peptide Length	Mass	m/z	z	Protein Accession	Protein Description
KAALKLEKY	na	9	1,077.6545	539.8342	2	P09429 HMGB1_HUMAN	High mobility group protein B1
KAAK(+453.06)LKEKY	K150	9	1,530.7102	511.2447	3		
RTKKVGIVGKY	na	11	1,247.7714	624.897	2	P61513 RL37A_HUMAN	60S ribosomal protein L37a
RTKK(+453.06)VGIVGKY	K7	11	1,700.8269	567.9514	3		
TAAQITQRKW	na	10	1,201.6524	602.34	2	P18465 1B57_HUMAN	HLA class I histocompatibility antigen B-57 alpha chain
TAAQITQRK(+453.06)W	K145	10	1,654.7124	828.3697	2		

na, not applicable.



other modified counterpart. (**Figures 6A, C**). Both FLX-RTKK peptide isomers showed similar specificity and immunogenic potential (**Figure 6C**). The IFN- γ response was driven by CD8⁺ but not CD4⁺ T cells (**Figure 6D**). Importantly, the recognition

of FLX-peptides by CD8⁺ T cells was dependent on the HLA-B*57:01 expression, as evidenced by the effect of an anti-HLA antibody on IFN- γ secretion (**Figure 6A**). Immunizations with FLX-KAA_K4 and _K6 also generated antigen-specific and

HLA-dependent T cell responses but at lower magnitude than the FLX-RTKK_K4. Control FLX-KAA_K8 showed no T cell reactivity (**Figure 6B**). FLX-TAA_K9 also failed to induce an immune response (**Figures 6A, D**). These results suggest that peptides bearing modified lysines in specific positions are more immunogenic than others depending on their exposure to solvent and accessibility to TCR.

To visualize the spatial position of the drug on the peptide, we used computational modeling. This was achieved by docking the peptide into the HLA cleft using FlexPepDock software (22, 23), followed by addition of the drug to the appropriate lysine and energy minimization of the structure using OpenBabel (24). Differences in the location of the drug on the HLA/peptide complex were evident between the immunogenic and not immunogenic peptides (**Figure 7** and **Figure S7**). FLX in

lysines at P4 (i.e. FLX-RTKK_K4 in **Figures 7B, E**) or P6 (FLX-KAA_K6 in **Figures S7C, G**) occupies a central location on the HLA cleft, resembling the bulge of a longer peptide, whereas when conjugated in P Ω -1, (i.e. FLX-RTKK_K10 in **Figures 7C, F**) it occupies a position on the outer border of the HLA-bound peptide. Although we do not have FLX-specific T cell receptors (TCR) to model this interaction, we superposed a TCR from the HIV KAF TCR (2YPL) on FLX-RTKK_K4 and _K10 (**Figure S8**). The TCR footprint is consistent with FLX-peptide recognition at K4 interacting with the TCR V-alpha CDR3 (**Figure S8A**). This contrasts with peptide conjugated FLX at P Ω -1 that is oriented more towards TCR V-beta CDR3 (**Figure S8B**).

Altogether, these data indicate that FLX-peptides are immunogenic in association with HLA-B*57:01 and the

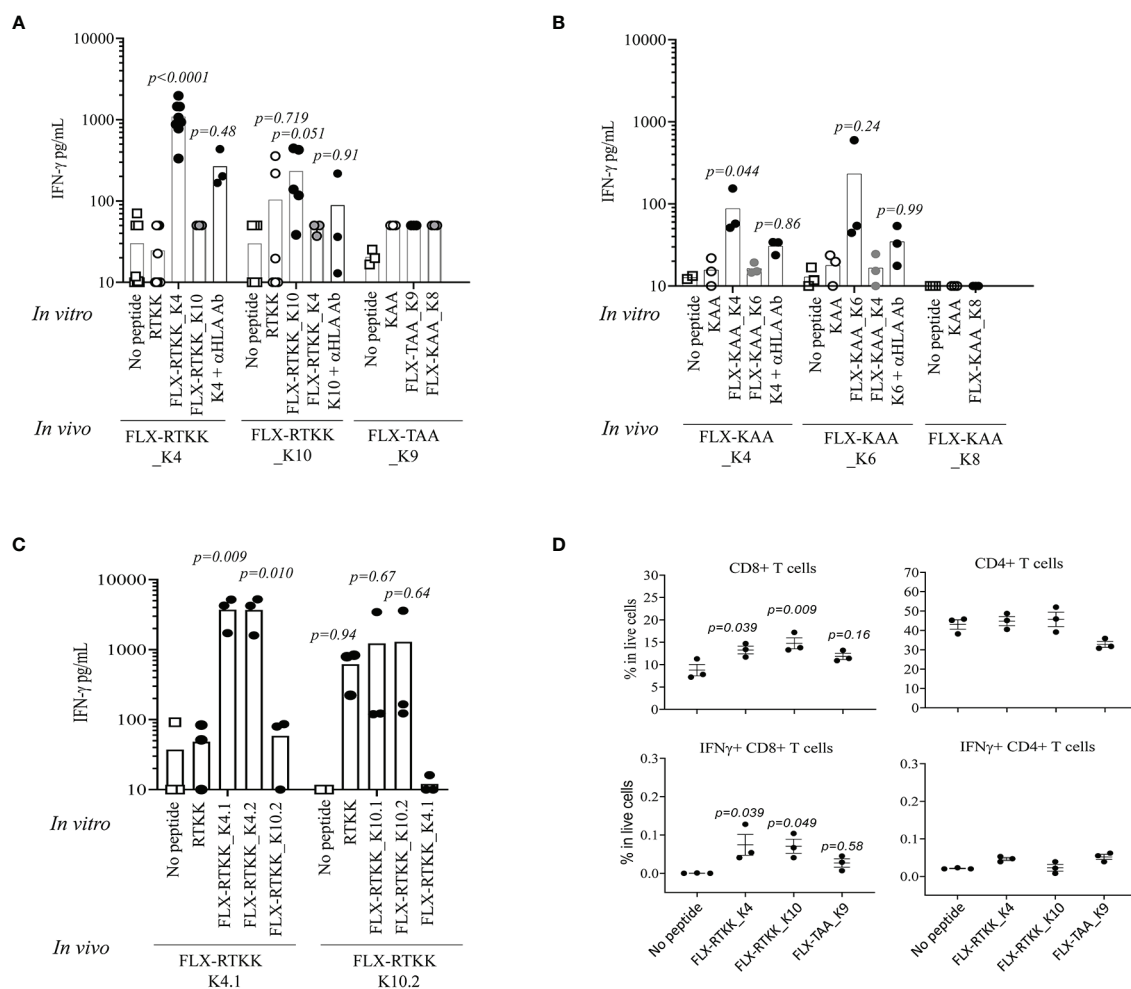


FIGURE 6 | Immunogenicity potential of flucloxacillin (FLX)-haptenated peptide is dependent on the peptide sequence, the position of the lysine to which drug is conjugated and the expression of human leukocyte antigen (HLA). (**A, B**) Tg/KO mice (n=3–9) were immunized by 2 s.c. doses of 100 µg of FLX-peptides together with 150 µg of HBV_{128–140} CD4⁺ T helper peptide with IFA. Splenocytes were subsequently stimulated *in vitro* with 10 µg/ml peptides (as indicated) for 5 days. IFN-γ was measured in the supernatant of the cultures by ELISA. *In vitro* responses to the *in vivo* immunogen were blocked by anti-HLA B/C antibody. (**C**) Immunizations and cultures were set up similarly to that described in A but with peptide isoforms of the FLX-RTKK peptides. (**D**) IFN-γ intracellular staining of day 5 splenic cell cultures from animals treated with FLX-peptides and stimulated *in vitro* with the same peptide. Graphs show results gated in CD8⁺ or CD4⁺ T cell populations (one representative of two experiments). Statistical analysis by one-way ANOVA using the no peptide condition as baseline. *p*-values are only shown if <0.999.

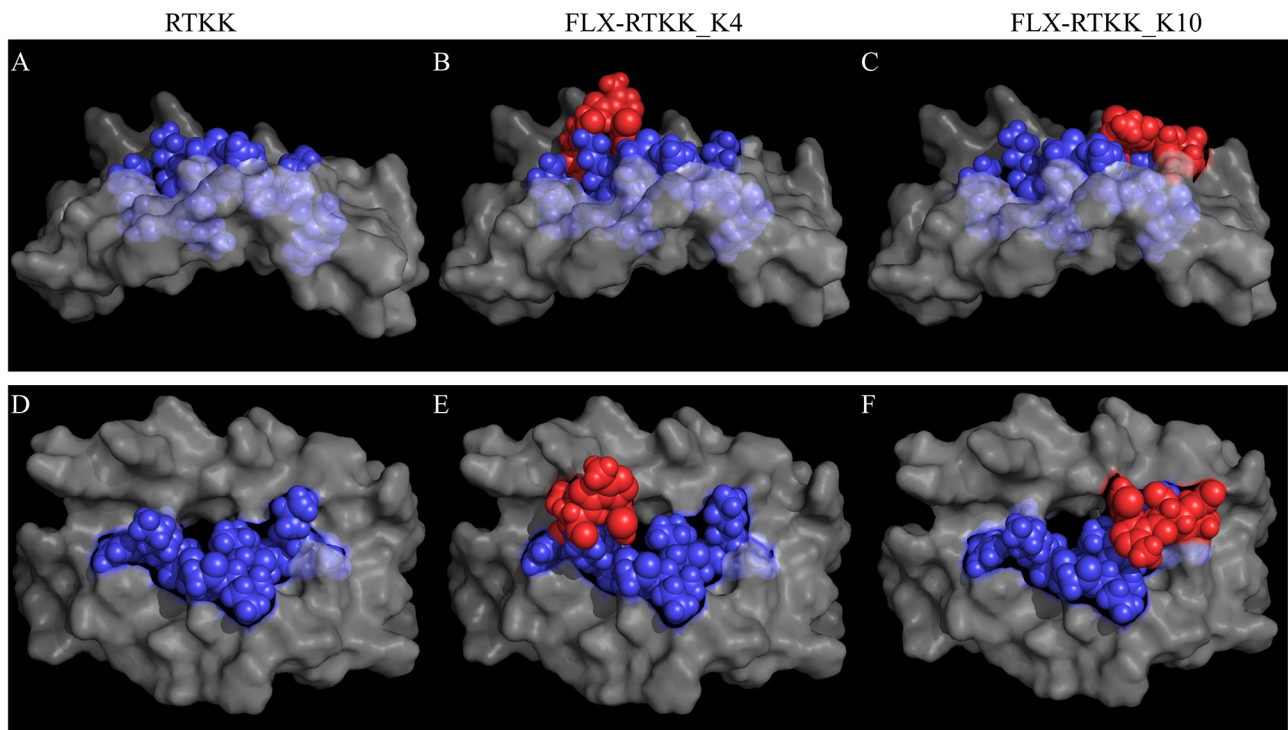


FIGURE 7 | *In silico* prediction of the interactions of parent and flucloxacillin (FLX)-RTKK peptides with HLA-B*57:01 cleft. KAF11 peptide residues (PDB ID: 2YPK) were replaced with those of the RTKK peptide. Docking the peptide into the HLA cleft was performed using FlexPepDock and addition of the drug to the appropriate lysine and energy minimization of the structure using OpenBable (v3.0.0) software. Structures were visualized in PyMOL (v2.3.4). Lateral (**A–C**) and top (**D–F**) views of the human leukocyte antigen (HLA) complex with the unmodified parent sequence of RTKK (**A, D**), FLX-RTKK_K4 (**B, E**) and FLX-RTKK_K10 (**C, F**). Peptide sequences are represented in blue spheres, drug is represented by red spheres and $\alpha 1\alpha 2$ domains of the HLA are depicted in gray color.

potential of FLX to activate a CD8+ T cell response is dependent on the position of the lysine residue in the peptide sequence.

DISCUSSION

Idiosyncratic FLX hypersensitivity reactions driven by immune cells occur at low frequency (2, 25, 26). Expression of HLA-B*57:01 and HLA-B*57:03 alleles was recognized as genetic risk factors for FLX-induced liver injury (6, 7), and thus attention has been paid to understand the molecular mechanisms by which drug can generate neoepitopes that are presented by HLA to activate T cells. To examine these mechanisms, we characterized the effect of FLX on the cell proteome and immunoproteome looking for global changes as well as appearance of drug-haptenated peptides.

Alterations of the B721-5701 cellular protein pathways by FLX could impact the abundance of self-epitopes and PTMs as well as the generation of neoepitopes, using these potential mechanisms to boost protein immunogenicity. Although no significant changes were observed in the total protein content of the cells when incubated with FLX, drug treatment induced a higher diversity in the peptide repertoire presented by the HLA which could impact antigen presentation. Increased abundance and diversity of sequences of particular proteins in MHC

complexes can be due to an enhanced processing rate of such proteins (19). Consistent with this hypothesis, we observed a larger number of proteins represented in the FLX-immunopeptidome with peptide presentation rate above those predicted considering the protein length. Alteration of the peptide processing (immunoproteasome vs proteasome) or the peptide loading (e.g. tapasin-dependent or -independent mechanisms) machineries could explain qualitative and quantitative changes on the cell immunopeptidome. Penicillinase-resistant antibiotics, including FLX can induce endoplasmic reticulum stress, which can lead to misfolding of proteins, oxidative stress and caspase-3 activation (27). Although the drug concentrations used in our study were lower than in those studies (0.3–1 mM vs >16mM) and the cells tested were different (B cells vs hepatocytes), we found that caspase-3 protein expression was elevated in our 1mM-treated FLX-cells, and thus, could indicate the occurrence of non-cytotoxic changes impacting protein processing. However, the mechanisms by which FLX may impact proteasome processing and/or peptide loading and presentation without changing the global protein content of the cell requires further study (see further discussion below).

The generation of neoepitopes by drugs inducing immune-mediated hypersensitivity reactions is believed to occur by covalent adduct formation, pharmacological interaction of the

drug with the peptide, and non-covalent interaction leading to alteration of the peptide repertoire (28) which most likely coexist *in vivo* (29). In our study, no changes were observed in the consensus anchor residues of peptides isolated from FLX-cells, either at the P2 or P Ω positions, consistently with other reports (13–15, 30, 31). A lower frequency of tryptophan at P9 was observed in some of our untreated preparations (**Figure S5**) contrasting with what others reported on HLA-B*57:01 peptide repertoires (21, 31). This variation could be attributed to differences in culture conditions such as absence of other β -lactam antibiotics in the media other than FLX or the use of different cell lines. Nevertheless, and unlike abacavir that increases the presentation of epitopes with unconventional isoleucine or leucine residues in P Ω (13–15), FLX promoted an enrichment of tryptophan at P Ω , in agreement with a trend observed in a recent report (31). We established that the overall net higher frequency of peptides with tryptophan at P Ω in the FLX-cells included both unique sequences (qualitative) and higher abundance in common peptides (quantitative). Because of its greater size, it seems unlikely that FLX can fit directly into the HLA F-pocket in a manner similar to that of abacavir. Therefore, we hypothesize that changes in the peptide repertoire may result from an effect on the peptide processing machinery. Catalytic shifts in proteasomic subunits induced by pathogenic infections but also by oxidative stress (32) increase the immunoproteasome activity of the cell resulting in an enrichment of peptides with higher content of hydrophobic amino acids at the C-terminus promoted by the chymotrypsin activity of LMP2 (33), perhaps favoring tryptophan (34). Whether this occurs indirectly or by direct interaction of FLX with one of the immunoproteasome complex subunits, such as LMP2 or LMP7, has not been addressed in our study. In our studies, peptides with tryptophan in the C-terminus have higher predicted binding affinity to HLA-B*57 (**Tables S2A, B**), consistent with other findings (21). Loading of high-affinity peptides by tapasin-dependent mechanisms could impact self-tolerance, possibly contributing to drug hypersensitivity such as in cases where tissue damage continues even after discontinuation of drug administration.

T-cell recognition of FLX by cells of healthy individuals carrying HLA-B*57:01 can involve processing dependent and independent pathways (4, 5, 8). However, the activation of CD8⁺ T cells from HLA-B*57:01⁺ DILI patients by FLX is peptide processing dependent and restricted to the expression of B*57:01 allele (4, 8) possibly explaining the low frequency of FLX-associated DILI and why only the expression of the HLA-B*57:01 or B*57:03 alleles has been linked to FLX-induced liver injury. Processing dependent T cell activation suggests that covalent modifications of protein epitopes generate neoantigen drug targets. To study the immunogenic potential of FLX-conjugated peptides, we used mass spectrometry analysis of HLA purified peptides and identified three FLX-peptide sequences in the B721-5701 immunopeptidome of drug-treated cells. One peptide FLX-TAA_K9 was from HLA itself suggesting that lysines on the HLA surface may also be targets for direct drug modification (31), analogous to FLX modifications of

lysines on albumin (12). The other two peptides had multiple lysine residues and were from internal proteins, KAA from a nuclear HMGB-1 DNA binding protein and RTKK from the 60S ribosomal protein, both of which are in high abundance in the cell. Both peptides were modified on the P4 lysines, suggesting a preference at this position on presented peptides. Generation of haptenated peptides could occur either on the intact protein or on free peptide after proteasome cleavage, as suggested in a recent report by Waddington et al. (31) who also identified FLX modifications on TAA and RTKK, among other peptides. In their study, the position of the modified amino acid in peptides such RTKK was ambiguous and not verified by synthetic chemistry.

Using FLX-lysine Fmoc synthetic conjugates, we synthesized both FLX-peptides identified in the cell immunopeptidome as well as other analogs with FLX on other lysine residues to confirm the identity of the sequences, the position of the drug, and to assess their immunogenic potential. Interestingly, RP-HPLC profiles of the synthesized FLX-peptides as well as FLX-RTKK_4 isolated from the cells showed two peaks with distinct retention times but with exact mass, unlike those of the unmodified sequences. We believe that these differences represented two conformational states of the open β -lactam ring of the antibiotic, since two different peaks were also observed for the Fmoc FLX-lysine conjugate after deprotection but not for the FLX-lysine with the intact β -lactam ring (data not shown). Further structural work is required on FLX-peptide conjugates to define these properties and to study further its relation to peptide immunogenicity.

To estimate the immunogenicity potential of FLX-epitope sequences, we immunized HLA-B*57:01 mice lacking the expression of the murine MHC-I molecules H-2K^b and H-2D^b with the selected FLX-peptides. Different levels of immunogenicity were observed depending on the peptide sequence and the position at which the drug was conjugated. Cells from animals primed with FLX-RTKK_K4 or FLX-KAA_K4 sequences, unlike modified TAA peptide, were quickly activated *in vitro* to produce IFN- γ in an antigen-specific and HLA-dependent manner. Interestingly, peptides not detected in the immunopeptidome like FLX-KAA_K6 and FLX-RTKK-K10 showed increased immunogenicity (the latter with less specificity), while FLX-KAA_K8 did not activate T cells. For FLX-RTKK peptides, the presumably isomeric sequences were recognized similarly by primed T cells. Failure to identify weak immunogenic peptides in the cell immunopeptidome could be explained by a disadvantage of certain lysine residues to be modified by the drug or interference in peptide loading. Both the physicochemical properties of the peptide (35) and the contact potential among TCR and peptide (36) have been postulated as critical parameters in the prediction of epitope immunogenicity. Notably, FLX conjugates were not found in anchor residues (P2 or P Ω) and the lysine side-chains of FLX-peptides were oriented exposed to solvent away from the HLA cleft. Interestingly, 3 out of the 4 immunogenic peptides tested had drug in lysines in position P4 or P6, positions that are considered critical for contact specificity and generation of

immunogenicity (35). Structural models presented here for P4 and P6 suggest that drug modifications in these positions could be seen by T cells in a manner similar to large peptides that contain bulges in central regions. Using the HLA-B*57:01 restricted HIV-KAF TCR as a model, we showed that the lysine at P4 is at a site that overlaps with the hypothetical footprint of TCR V- α CDR3 while lysines at P Ω -1 are not, suggesting that TCR chains could be differentially selected specifically based on the amino acid position of the drug linkage. FLX at P Ω -1 may be outside the footprint of the CDR3 chains or have difficulty in peptide processing/loading to HLA.

As mentioned above, FLX-TAA_K9 is derived from HLA-B*57:01 itself. Direct modification of lysines on HLA has intriguing implications in addition to being target epitopes for T cells after antigen processing. HLA-K146, corresponding to the FLX-modified lysine in FLX-TAA_K9, is positioned near the HLA F-pocket where the peptide C-terminus will interact and is highly conserved among HLA molecules. K-146 is critical for HLA binding to the inhibitory KIR-3DL1 protein on NK cells (37, 38). Direct modification of HLA-K146 by FLX as modeled in **Figure S9** could interfere with the KIR-3DL1-binding and possibly lead to NK activation. Similarly, FLX modification on lysines positioned at P Ω -1 such as on FLX-TAA_K9 could also interfere with the KIR3DL1-binding site and impact KIR function as KIR3DL1 binding was reported to depend on the nature of the amino acid at the P Ω -1 (P8 in Vivian et al. (37)). Activation of NK in addition to CD8⁺ T cells have been reported in PBMCs isolated from patients with drug hypersensitivity reactions (39) as well as in fluid of skin blisters of patients with Stevens-Johnson syndrome (40), although the exact molecular mechanism involved in such activation is yet to be determined. Further studies are required to elucidate whether K146 or other FLX-modified lysines on HLA can interfere with KIR or T cell receptor binding.

In summary, factors leading to idiosyncratic drug hypersensitivity might involve non-covalent and covalent interactions between drug and peptide. In our study, we showed that FLX treatment of B721-5701 cells can alter the presentation of self-peptides by enriching the peptide repertoire of the cell with sequences containing tryptophan at the C-terminus, a preferred amino acid for immunoproteasome processing and which confers a higher predicted binding affinity to the sequence. We also identified drug-haptenated peptides with immunogenicity potential depending on the lysine at which FLX gets conjugated. Direct modifications of the HLA by FLX at lysines that are believed to play immunoregulatory roles could also participate in the activation of innate immune pathways, contributing inflammatory events to the initiation of immune-mediated drug adverse reactions. Further animal studies will allow us to characterize the generated immune responses in more detail and whether the reactive cells can infiltrate hepatic tissue and lead to DILI. Understanding the mechanisms behind these modifications and their impact on breaking T cell tolerance, individually or as a whole, is critical to drug development and amelioration of adverse side effects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at FDA.

AUTHOR CONTRIBUTIONS

MP and MN conceived, planned, and supervised the experiments. MP, SA, RV, SP, EM, MC, SS, and GR performed the experiments. MT, SB, and GA chemically synthesized the peptides. WW performed mass spectrometry analysis. LW and ZL performed proteomics data analysis. WH generated large-scale HLA preparations. LB, KN, and DM generated the HLA-B57*01 Tg/KO mice. MP, DM, and MN edited and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.629399/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIALS and METHODS:

Reagents:

Flucloxacillin (FLX) was obtained from Apotex, UK or Sigma-Aldrich, USA. FLX-conjugated lysine residues were prepared in house following Scornet et al's method (1) and drug-conjugation was confirmed by nuclear magnetic resonance (data not shown). For details, see the section on Synthesis of FLX-Lysine and Suppl Fig 1. Drug-modified peptide sequences were synthesized by solid-phase Fmoc chemistry on a Tribute^R peptide synthesizer (Protein Technologies, Inc., Tucson, Arizona) using the FLX-conjugated lysine residues. Subsequently, peptides were HPLC purified on an XBridge BEH C18 OBD Prep column (Waters, Milford, MA), quantified and lyophilized.

B721-5701 protein lysate and LC-MS/MS analysis:

B721-5701 cells were grown in cell culture flasks for 5 days to a density of 1-2 million cells/mL in R-10 medium (RPMI (Gibco BRL) containing 10% fetal bovine serum (Atlanta Biologicals), 7.5 mM HEPES (MP Biomedicals), 2 mM L-glutamine (MP Biomedicals), and 150 μ M non-essential amino acids (Gibco BRL), in absence or presence of FLX (150 or 453 μ g/mL), in duplicate. Cells (10-12 million) were washed in 1x PBS and resuspended in RIPA Lysis Buffer containing protease inhibitors, freeze/thawed 3 times and sonicated (Branson Digital Sonifier) to obtain a cell lysate. The protein extract was clarified by spinning the sample at 14,000 $\times g$ for 15 minutes at 4 °C and its protein concentration was determined by the PierceTM bicinchoninic acid (BCA) method. Aliquots were stored at -20 °C until further use. One milligram of protein extract was treated with RapiGest surfactant (0.1%) for 30 min at 45 °C and 500 rpm. Subsequently, samples were treated with 50 μ L of 50 mM ammonium bicarbonate for 15 min at 37°C, followed by 50 μ L of 10 mM DTT at 80°C with shaking for 15 min, and followed by 50 μ L of 20 mM iodoacetamide at 37°C for 30 min, spinning in between steps to discard the liquid. Samples were further digested with trypsin at 20:1 protein:protease ratio (w/w) and incubate overnight at 37 °C. Trifluoroacetic acid was added to 0.5-1.0% (v/v) to stop the digestion by reducing the sample pH to 2 and samples were incubated at 37 °C for 10 min.

The protein digest (600 μ g) was used for strong-cation exchange pre-fractionation using PolySULFOETHYL A column 200 A x 20 cm with particle size 5 μ m (PolyLC, Columbia, MD) using the mobile phase A: 5mM KH₂PO₄, pH 2.7 and 30% ACN, and mobile phase B: 5mM

KH₂PO₄ in 350 mM KCl, pH 2.7 and 30% ACN and flow rate 200 µL/min. The gradient starts from 0% B for 10 mins, 26% B by 15 mins, 35% B by 40 mins, 60% B by 45 mins, 100% B by 49 mins. After 55 mins column comes to the initial washing conditions for 9 mins at 0% B. Each sample was collected into 9 fractions at 0-10 mins, 10-20 min, 20-22.5 min, 22.5-25 min, 25-27.5 min, 27.5-30 min, 30-40 min, 40-55 min, and 55-64 min.

SCX- fractions were lyophilized and dissolved in 20 µL of 0.1% formic acid buffer for LC-MS experiments. Thermo Q-Exactive Orbitrap mass spectrometer coupled with Eksigent nanoflex cHiPLC system was used for the proteomic experiments. Briefly, proteomic fractions were loaded on trap column and switched onto C18-analytical column at 2% B (0.1% formic acid in acetonitrile) for 2 mins. Peptides eluted linearly over 120 mins of time to reach 30% B. After, column was equilibrated with 2% B for 15 mins. The flow rate was maintained at 250 nL/min. The Q-Exactive mass spectrometry conditions are full MS spectra recorded at 70,000 resolution, scan range 400-1200 m/z, AGC target 3e6, and maximum injection time (IT) of 250 ms.

Subsequently top 10 ions were submitted for high collision-energy dissociation (HCD MS/MS) using stepped collision energy 25, 27, 30 eV at 17,500 resolution, AGC target 2e5, isolation window 2 m/z and maximum IT 120 ms with fixed first mass 100 m/z. Dynamic exclusion was set up for 15 secs. LC-MS/MS readouts were done in triplicate for each fraction.

LC-MS/MS derived .raw files were submitted for PEAKS 8.5 analysis using the Uniprot non-redundant human database. Data was analyzed at 10 ppm for MS1 analysis and 0.05 Da for MS2. We included trypsin as enzyme, carbamidomethylation as the fixed modification and variable modifications such as N-terminal acetylation of peptide, oxidation of methionine (M), deamidation of asparagine (N) and glutamine (Q). The allowable maximum missed cleavages and modifications per peptide are 2.

Generation and isolation of HLA-bound peptides:

● ***Production of mHLA/peptide complexes and peptide purification:*** B721.5701 cells were grown in G-Rex 6 Well Plates (Cat. # 80240M, Wilson wolf, New Brighton, MN) seeded at 10⁷ cells per well in 30 mL of medium (RPMI containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate (MP Biomedicals)), in the presence or absence of FLX (150 µg/mL) for 5 days and grown to approximately 2x10⁸ cells. Pelleted cells were lysed with 2.5 ml of lysis buffer (consisting on 2 mM EDTA, 2 mM PMSF, Protease inhibitor cocktail (Roche), 0.5% Sodium

Deoxycholate, 2% Octyl- β -D glucopyranoside, dissolved in PBS) on ice for ten minutes. Aliquots of 0.5 mL of the cell lysate were subsequently sheared with a mechanical homogenizer and clarified by spinning it down at 4 °C at 14000 rpm for 50 minutes. Pooled lysate supernatants were immunoprecipitated with HLA I (W6/32) antibody crosslinked with Protein A overnight at 4 °C with gentle rocking. Peptide elution was performed according to Chong et al method (2) using a Waters Positive Pressure-96 Processor (Waters, Milford, MA). Briefly, 96-well single-use microplate with 3 μ m glass fiber and 10 μ m polypropylene membranes (204495-100 Agilent) were washed and equilibrated with 1 mL of 100% acetonitrile (ACN), followed by 1 mL of 0.1% TFA and lastly with 2 mL of 0.1 M Tris-hydrochloric acid (pH 8.0). The immunoprecipitated lysate was subsequently added to the plate, using four wells per sample. Samples were washed consecutively 4 times with each one of the following buffers (2 mL per wash): buffer 1 (consisting of 0.005% IGEPAL 630, 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 100 μ M PMSF and 1 μ g/mL Pepstatin A); buffer 2 (consisting of 50 mM Tris pH 8.0 and 150 mM NaCl); buffer 3 (consisting of 50 mM Tris pH 8.0 and 450 mM NaCl); buffer 4 (50 mM Tris pH 8.0). Addition of 500 μ L of 1% TFA assisted in transferring the samples onto a C18 96-well plate (ref number: 186002321, Waters), previously conditioned with 1 mL of 80% ACN in 0.1% trifluoroacetic acid (TFA) followed by 2 mL of 0.1% TFA. Upon an additional wash with 2 mL of 0.1% TFA, peptides were eluted from the HLA complexes with 500 μ L of 28% ACN in 0.1% TFA. Peptides were pooled from all 4 wells, vacuum-dried and dissolved in 25 μ L of 0.1% TFA. Samples were analyzed by LC/MS/MS using a ThermoFisher Ultimate LC and Fusion Orbitrap MS (San Jose, CA). Briefly, peptides were first loaded onto a trap cartridge (ThermoFisher PepMap, C18, 5 μ m, 0.3x5 mm), then eluted onto a reversed phase Easy-Spray column (ThermoFisher PepMap, C18, 3 μ m, 100A) using a linear 120-min gradient of acetonitrile (2-40%) containing 0.1% formic acid at 250 nL/min flowrate. The eluted peptides were sprayed into the Fusion Orbitrap. The data-dependent acquisition (DDA) mode was enabled, and each FTMS MS1 scan (60,000 resolution) was followed by FTMS MS2 scans (15,000 resolution) using top speed (acquire as many MS2 scans as possible within three second cycle time). Precursor ion (selected at 1.6 Da window) fragmentation took place at HCD cell with CE of 30. AGC targets are 2.0e5 and 5.0e4, respective, for MS1 and MS2. The spray voltage and ion transfer tube temperature were set at 1.8 kV and 250 °C, respectively.

• ***Production of sHLA/peptide complexes and peptide purification:*** sHLA from FLX-treated cultures was collected from continuous cultures as described previously (3). Briefly, B721-5701

cell transfectants were cultured in high density hollow fiber bioreactors. Milligram quantities of sHLA-peptide complexes were purified from pooled supernatants from continuous cultures using affinity chromatography W6/32 columns. Peptides were released from the HLA complex on the affinity columns by addition of 10% acetic acid, and further separated from heavy chains and β 2-microglobuline by boiling and passing the suspension through a 3-kDa cut-off membrane filter. Peptide pools from drug-treated and non-treated cells were separated by reverse phase high-pressure liquid chromatography (RP-HPLC) and fractions were collected. Lyophilized pre-fractionated samples were re-dissolved and analyzed by nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in the “B721-5701 protein lysate and LC-MS/MS analysis” section above.

Identification of HLA-bound peptides using LC–MS/MS and analysis:

Peptide identity was determined using mass tolerance of 10 ppm for precursor ion and of 0.05 Da for fragment ions subject to stringent bioinformatic parameters using PEAKS 8.5 or X software with a false discovery rate (FDR) cutoff of 5%. Post translational modifications (PTM) such as N-acetylation, methionine oxidation, cysteinylolation and citrullination were considered. The filtered data set was further analyzed manually to exclude redundant peptides and recognized contaminants. Haptenated-peptides were identified in the HLA peptide datasets by using FLX PTM of (+453.06). LC-MS/MS spectra were also manually examined for FLX-related ions with $z=1+$ charge of +454.0689 (protonated FLX) or +160.0446 or +295.0382 representing FLX fragments). FLX modified peptides also contained MS2 fragments with +294 on lysines that supported covalent linkage at specific lysine residues.

The predicted binding affinity of peptides to HLA-B*57:01 was predicted using the NetMHCcons 1.0 Server (4). The frequency of each amino acid in the peptide sequence was assessed by Gibbs cluster 2.0 software (DTU, Denmark) (5). Gene Ontology pathways were evaluated using ‘Ingenuity Pathway Analysis (IPA) (6).

Frequency of the proteome identified proteins in the HLA immunopeptidome and peptide sampling:

The intensities of proteins for which peptides were identified in the immunopeptidome, from the MaxQuant output, were averaged and used as an estimation of abundance. Proteins were cross-

referenced with their Uniprot accessions to extract their length (database downloaded May, 5, 2020) (7). Abundance data were log2-transformed and compared to the number of HLA peptides in the immunoproteome. Graphs were created in R (4.0.0) using ggplot2 (3.3.1) (8, 9). Linear trendlines for figures 3A and 3B, were fitted in quartile sections. ANCOVAs were run on each quartile using the zoo (1.8-8), reshape2 (1.4.4), and car (3.0-8) R packages (10-12).

HLA sampling density (D) was calculated according to a priory expectation as stated in Hoof et al. and Bassani-Sternberg et al. in order to account for non-unique peptide sequences (13) (14). To model the expected sampling density (D') given the abundance and length of a protein, proteins were divided into 200 bins according to their abundance. We find the average D of each bin and fit a sigmoid function to this that can be used to calculate D' for an individual protein. Fold over-presentation can then be represented as D/D'. Correlation coefficients were calculated in R.

Synthesis of FLX-modified lysine residues:

FLX-conjugated lysine residues were prepared as per Scornet et al's method (1). Specialty and common chemical reagents and solvents including FLX sodium, 4-methoxybenzyl chloride, Fmoc-Lys-OH hydrochloride *N*^α-Fmoc-L-lysine allyl ester hydrochloride (**3**), *N,N*-diisopropylethylamine (DIEA), tetrakis (triphenylphosphine)-palladium(0), triphenylphosphine, barbituric acid, anhydrous sodium sulfate, sodium bicarbonate, anhydrous *N,N*-dimethylformamide, anhydrous dichloromethane, diethyl ether, absolute ethanol, methanol, hexane, ethyl acetate and deuterated solvents (CDCl₃, DMSO-*d*₆) were all purchased from reputable commercial sources and used as received.

Flash chromatography purifications were performed on glass columns (2.5 cm or 6.0 cm I.D.) packed with silica gel 60 (230-400 mesh), whereas analytical thin-layer chromatography (TLC) analyses were conducted on 2.5 cm × 7.5 cm glass plates coated with a 0.25 mm thick layer of silica gel 60 F254.

All NMR experiments were performed using a spectrometer operating at 300.13 and 75.47 MHz for one-dimensional ¹H and ¹H-decoupled ¹³C, respectively. Samples were maintained at a temperature of 298 °K. All spectra were recorded in deuterated solvents and chemical shifts, δ, were reported in parts per million (ppm) relative to appropriate internal references. Elemental composition of new compounds is determined, under contract, by high resolution mass spectrometry (HRMS) using a Bruker Daltonics ApexQ FTICR mass spectrometer equipped

with a 12 T magnet. Electrospray ionization in positive ion mode was used to generate $[M+H]^+$ and $[M+Na]^+$ ions out of test samples (0.01 mg dissolved in 1 mL of 10 mM ammonium acetate in MeCN:H₂O (1:1 v/v)). Spectra were externally calibrated using 0.5 mg/mL solution of CsI in water, which yielded a series of peaks in the mass range used for analysis (200-2000 m/z). GC-MS experiments were carried out using PerkinElmer Autosystem XL gas chromatography system coupled to PerkinElmer Clarus 560S mass spectrometer.

4-Methoxybenzyl (2*S*,5*R*,6*R*)-6-(3-(2-chloro-6-fluorophenyl)-5-methylisoxazole-4-carboxamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylate (2). To a solution of sodium flucloxacillin **1** (4.75 g, 10.0 mmol) in anhydrous DMF (20 mL) was added 4-methoxybenzyl chloride (2 mL, 15 mmol); the solution was stirred at 60 °C over a period of 3.5 h and then poured into water (200 mL). The mixture was extracted with ethyl ether (3 × 100 mL). The combined organic extracts were collected and washed with a saturated solution of sodium bicarbonate (100 mL); the organic layer was dried over anhydrous sodium sulfate, filtered and rotoevaporated under reduced pressure to a sticky solid. The crude material was purified by silica gel chromatography using a gradient of EtOAc (0→50%) in hexane as the eluent. Fractions containing the pure product were collected and rotoevaporated under vacuum affording the pure flucloxacillin ester **2** (4.89 g, 8.5 mmol) as a white foam in a yield of 85%. ¹H NMR (300 MHz, DMSO-*d*₆) δ: 8.93 (d, 1H, *J* = 7.2 Hz), 7.59 (m, 1H), 7.48 (m, 1H), 7.42 (m, 1H), 7.35 (d, 2H, *J* = 6.7 Hz), 6.94 (d, 2H, *J* = 6.7 Hz), 5.57 (dd, 1H, *J* = 4.0 and 7.2 Hz), 5.48 (d, 1H, *J* = 4.0 Hz), 5.12 (d, 2H, *J* = 3.2 Hz), 4.39 (s, 1H), 3.75 (s, 3H), 2.68 (s, 3H), 1.53 (s, 3H), 1.32 (s, 3H). ¹³C NMR (175 MHz, DMSO-*d*₆) δ: 173.0, 171.6, 167.7, 162.2, 160.5, 159.8, 158.9, 155.0, 134.3, 133.2, 131.1, 131.0, 127.5, 126.2, 115.3, 114.3, 114.2, 113.4, 70.3, 67.8, 67.1, 64.6, 59.4, 55.5, 31.2, 26.8, 13.2. +HRMS-ESI: calcd for C₂₇H₃₅ClFN₃O₆SNa (M+Na)⁺ 596.1029, found 596.1030.

4-Methoxybenzyl (2*R*,4*S*)-2-((5*S*,12*R*)-5-((allyloxy)carbonyl)-14-(3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl)-1-(9*H*-fluoren-9-yl)-3,11,14-trioxo-2-oxa-4,10,13-triazatetradecan-12-yl)-5,5-dimethylthiazolidine-4-carboxylate (4). To the flucloxacillin ester **2** and *N*²-Fmoc-L-lysine allyl ester hydrochloride (**3**, 1.87 g, 4.00 mmol), was added anhydrous dichloromethane (16 mL). The reaction mixture was brought to a reflux; *N,N*-

diisopropylethylamine (766 μ L, 4.40 mmol) was then added to the refluxing mixture, using an automatic syringe, at the rate of 0.8 mL/h. After a total reflux time of 6 h the reaction mixture was rotoevaporated under reduced pressure to a gummy material, which was purified by silica gel chromatography. A gradient of EtOAc (33% $\rightarrow$ 50%) in hexane was used as the eluent. Fractions containing the pure product were collected and rotoevaporated under vacuum to provide the pure product **4** (2.25 g, 2.29 mmol) as a yellow solid in a yield of 57%. ^1H NMR (300 MHz, DMSO- d_6) δ : 8.20 (d, 1H, J = 8.5 Hz), 8.00 (m, 1H), 7.89 (d, 2H, J = 7.4 Hz), 7.78 (d, 1H, J = 7.7 Hz), 7.71 (d, 2H, J = 7.4 Hz), 7.53 (m, 1H), 7.44-6.95 (m, 9H), 6.92 (d, 2H, J = 8.3 Hz), 5.93-5.82 (1H, m), 5.30 (d, 1H, J = 17.2 Hz), 5.20-5.12 (m, 2H), 4.99 (d, 1H, J = 11.8 Hz), 4.79 (t, 1H, J = 8.2 Hz), 4.57 (d, 2H, J = 5.0 Hz), 4.39 (t, 1H, J = 8.8 Hz), 4.32-4.19 (m, 3H), 4.08 (m, 1H), 3.74 (s, 3H), 3.52 (d, 1H, J = 12.9 Hz), 2.99 (m, 2H), 2.64 (s, 3H), 1.64 (m, 2H), 1.43 (s, 3H), 1.32 (m, 4H), 1.02 (s, 3H). ^{13}C NMR (175 MHz, DMSO- d_6) δ : 172.6, 170.9, 170.8, 169.2, 169.1, 160.4, 159.7, 158.9, 156.6, 155.0, 144.3, 144.2, 141.1, 134.3, 132.8, 130.9, 130.8, 128.1, 127.9, 127.5, 126.0, 125.6, 120.5, 118.1, 117.1, 116.8, 115.2, 114.9, 114.2, 114.1, 72.1, 67.2, 66.5, 66.0, 65.2, 60.2, 59.2, 57.6, 55.5, 55.4, 54.3, 47.1, 38.6, 30.7, 28.7, 28.1, 27.4, 23.2, 21.2, 14.5, 12.9. +HRMS-ESI: calcd for $\text{C}_{51}\text{H}_{53}\text{ClFN}_5\text{O}_{10}\text{S}$ ($\text{M}+\text{H}$) $^+$ 982.3258, found 982.3267.

N^2 -(((9H-fluoren-9-yl)methoxy)carbonyl)- N^6 -((R)-2-(3-(2-chloro-6-fluorophenyl)-5-methylisoxazole-4-carboxamido)-2-((2R,4S)-4-(((4-methoxybenzyl)oxy)carbonyl)-5,5-dimethylthiazolidin-2-yl)acetyl)-L-lysine (5**).** To a suspension of barbituric acid (307 mg, 2.40 mmol) in absolute ethanol (6 mL) was added a solution of the L-lysine conjugate **4** (1.96 g, 2.00 mmol) in anhydrous dichloromethane (12 mL) followed by catalytic amounts of tetrakis(triphenylphosphine)-palladium(0) (69 mg, 0.06 mmol) and triphenylphosphine (8 mg, 0.03 mmol). The reaction mixture was stirred under an inert atmosphere of argon over a period of 1 h at $\sim 23^\circ\text{C}$ and then concentrated under reduced pressure to $\sim 80\%$ of its original volume. Insoluble material was filtered; the filtrate was rotoevaporated to dryness. The residue was purified by silica gel chromatography using a gradient of methanol (0% $\rightarrow$ 2%) in dichloromethane as the eluent to provide the L-lysine-conjugate **5** (1.06 g, 1.13 mmol) as a yellow foam in a yield of 56%. ^1H NMR (DMSO- d_6) δ : 12.5 (s br, 1H), 8.18 (d, 1H, J = 8.7 Hz), 8.00 (t, 1H, J = 5.5 Hz), 7.89 (d, 2H, J = 7.4 Hz), 7.72 (d, 2H, J = 7.4 Hz), 7.65-7.50 (m, 3H), 7.44-7.28 (m, 8H), 6.92 (d, 2H, J = 8.7 Hz), 5.17 (d, 1H, J = 11.8 Hz), 5.00 (d, 1H, J = 11.8 Hz), 4.79 (t, 1H, J = 8.0 Hz), 4.40 (t, 1H, J = 8.8

Hz), 4.28-4.19 (m, 3H), 4.08 (m, 1H), 3.89 (m, 1H), 3.74 (s, 3H), 3.52 (d, 1H, $J = 12.5$ Hz), 2.99 (d br, 1H, $J = 5.1$ Hz), 2.65 (s, 3H), 1.67 (m, 1H), 1.58 (m, 1H), 1.43 (s, 3H), 1.33 (m, 4H), 1.23 (s, 3H). ^{13}C NMR (DMSO- d_6) δ : 174.5, 170.9, 162.2, 160.4, 159.7, 158.9, 156.6, 155.0, 144.3, 144.2, 141.1, 134.5, 134.4, 133.0, 132.8, 132.0, 130.9, 130.8, 128.1, 127.9, 127.5, 126.0, 125.7, 120.5, 117.1, 116.8, 115.2, 114.9, 114.2, 114.1, 72.1, 67.2, 66.5, 66.0, 59.2, 57.6, 55.5, 55.4, 55.3, 54.2, 47.1, 38.7, 30.8, 28.8, 28.1, 27.4, 23.3, 12.9. +HRMS-ESI: calcd for $\text{C}_{48}\text{H}_{49}\text{ClFN}_5\text{O}_{10}\text{S}$ (M+H) $^+$ 942.2945, found 942.2951.

The flucloxacillin-lysine conjugate is then used in the solid-phase peptide synthesis to provide the flucloxacillin peptide conjugate used in the proposed study.

Peptide docking modeling:

Coot (v0.8.9.2) (15) was used to replace the KAF11 peptide residues (PDB ID: 2YPK) with those of the RTKK peptide, LF9 peptide residues (PDB:2RFX) with those of KAA peptide and TW10 peptide residues (PDB:5V5M) with the TAA amino acids. The peptide structure was then docked with FlexPepDock (16, 17) using 100 low-and-high resolution structures. FLX (CID 21319) was fused to the resulting lowest energy peptide conformation at the corresponding lysine position using PyMOL (The PyMOL Molecular Graphics System v2.3.4, Schrödinger, LLC), and the resulting structures were energy minimized using OpenBabel (v3.0.0) (18) with the MMFF94 forcefield and steepest descent for a maximum of 2500 steps. Images were generated using PyMOL. For receptor modeling we used HIV KAF TcR (PDB ID: 2YPL) and KIR-3DL (PDB ID: 3VH8).

Mice and *in vivo* treatment:

HLA-B*57:01 Tg (19) and $H2\text{-}K^bD^b$ knockout C57BL/6 (B6.129P2- $H2\text{-}Kb^{tm1}H2\text{-}Db^{tm1}$ N12) (obtained from Taconic Biosciences, Hudson, NY) strains were backcrossed to generate that HLA-B*57:01 Tg / mouse MHC-I KO strain (Tg/KO) exclusively expressing HLA-B*57:01 as the MHC-I allele. Animals were phenotyped by flow cytometry as detailed below (also see Suppl Fig 2). Experimental mice were above 12 weeks of age. All mice were bred and housed under specific pathogen-free conditions in the animal facility of the NIAID, NIH (Rockville, MD) or in the AAALAC accredited animal facility of the U.S. FDA's Division of Veterinary Medicine

(Silver Spring, MD) in compliance with the Institutional Animal Care and Use Committee regulations of the U.S. FDA. Tg/KO mice were injected subcutaneously at the base of the tail on day 0 and day 7 with a mixture of 100 µg of FLX-haptenated peptide (FLX-peptide), 150 µg of HBV-co-peptide, a CD4⁺ helper T cell peptide, and IFA at a ratio of 1:1:2 (100 µL total volume). Control mice were treated with equal volume of Vehicle (WFI) lacking FLX-peptide. On day 14, mice were euthanized, and spleens were collected in sterile conditions for *in vitro* expansion.

In vitro cell cultures:

In vitro T-cell responses to FLX-peptides were measured in cultures of total splenocytes of Tg/KO mice. Splenocyte suspensions were prepared by macerating the spleen through 70-µm cell strainers and lysis of red blood cells with Ammonium-Chloride-Potassium (ACK) lysing buffer (GIBCO). Cells were cultured for up to 5 days in complete RPMI (cRPMI) consisting of RPMI 1640 supplemented with 2 mM L-Glutamine, 100 U/mL Penicillin, and 100 µg/mL Streptomycin (all from GIBCO). During the first 2 days of culture, cRPMI was supplemented with 0.5% heat-inactivated, normal mouse serum (Equitech-Bio, Inc.) to reduce the background due to *in vivo* cell activation. At day 2, half of the media volume was replaced by fresh cRPMI containing 10% heat-inactivated, low-endotoxin FBS (Premium Select FBS, Atlanta Biologicals). Cells were seeded in 48-well tissue culture treated plates (Cat# 3548, Corning) at 3×10^6 cells per well in 1 mL or in 96-well tissue culture treated plates (Cat# 3596, Corning) at 0.9×10^6 cells per well in 0.3 mL and incubated at 37 °C and 5% CO₂. Cells were left untreated or were cultured with 10 µg/mL of FLX-peptides or unmodified peptide (parent). Cultures without drug (None) served as a negative control. FLX-peptides were dissolved at 10 mg/mL in Water For Injection (WFI) for Cell Culture (GIBCO) at RT, aliquoted, and stored at -20°C. At day 5 of the *in vitro* stimulation, secretion of IFN-γ was measured in cell culture supernatants by ELISA using commercial kits for mouse IFN-γ (eBiosciences). Production of IFN-γ and expression of PD-1 in T-lymphocytes were analyzed at the same time point by flow cytometry as detailed in the methods section below.

Flow cytometry:

Flow cytometry was used to phenotype the transgenic mice (Suppl Fig 2) and analyze immune cell subsets by intracellular and/or surface marker staining. Expression of the chimeric HLA molecule in T-lymphocytes, and B cells was measured by cell surface marker staining of blood. The blood was collected in MiniCollect 0.8 mL LH Lithium Heparin Sep. tubes (Cat# 450479, Greiner Bio-one GmbH) and transferred into 96-well sterile V-bottom plates (BRAND plates Cat# 781661, BRAND) for flow cytometry staining. Prior to staining, red blood cells were lysed with ACK lysing buffer as follows: one incubation with 75 μ L of ACK /sample at RT for 5 min followed by a centrifugation (300 xg) at 4°C for 3 min and subsequently by a second incubation with 150 μ L of ACK /sample at RT for 5 min. Neutralization of the ACK was accomplished by washing leukocytes twice in cold PBS (pH 7.4) without calcium chloride and magnesium chloride (GIBCO) supplemented with 5% heat-inactivated FBS. To prevent non-specific antibody (Ab) binding, cells were pre-incubated at 4°C for at least 15 min in 100 μ L of Stain Buffer (BD Biosciences)/sample containing 10 μ g/mL of anti-CD16/CD32 mAb (mouse Fc Block, clone 2.4G2, BD Biosciences). After blocking, samples were stained at 4°C for 30 min in the dark with 100 μ L of a mixture constituted by 50% of Stain Buffer, 50% of Brilliant Stain Buffer (BD Horizon), and the appropriate diluted antibodies for cell surface markers. Once labeled, cells were washed by centrifugation at 4°C for 5 min with ACK Lysing buffer (150 μ L per sample) and cold PBS supplemented with 5% heat-inactivated FBS (250 μ L per sample) and finally resuspended in cold Stain Buffer prior to flow cytometry acquisition.

For intracellular detection of IFN- γ , cultures of 3×10^6 splenocytes were left untreated or incubated with 10 μ g/mL of FLX-peptide for approximately 5 days as detailed in the method section “*In vitro* culture assays”. During the last 8h of culture, cells were treated with Brefeldin A Solution (eBioscience) at a final dilution of 1/1000 and then tested. Viability was assessed by LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) following manufacturer’s directions while non-specific Ab binding was blocked with mouse Fc Block. After blocking, cells were stained for cell surface markers as indicated above, fixed, permeabilized using the BD Cytofix/Cytoperm Plus kit (BD Biosciences) as per manufacturer’s instructions, and finally stained at 4°C for 30 min in the dark with anti-mouse IFN- γ Ab. Once labeled, cells were washed twice with cold 1x BD Perm/Wash buffer (250 μ L per sample) and finally resuspended in cold Stain Buffer prior to flow cytometry acquisition.

Antibodies used for staining were: anti-HLA B/C PE (clone B1.23.2, eBioscience), anti-H-2D[d] FITC (clone 34-2-12, BD Pharmingen), anti-CD3 APC/Cy7 (clone: 17A2, Biolegend), anti-CD4 BV605 (clone: RM4-5, BD Horizon), anti-CD8a BV711 (clone: 53-6.7, BD Horizon), anti-CD19 Alexa Fluor 700 (clone: eBio1D3 (1D3), eBioscience), anti-CD279 (PD-1) BV785 (clone: 29F.1A12, Biolegend), anti-IFN- γ APC (clone: XMG1.2, eBioscience). All antibodies were titrated and tested alongside their specific isotype controls and fluorescence minus one control samples in preliminary experiments.

Flow cytometry data acquisition and analysis were performed using LSR Fortessa X20-SORP and Diva 6.2/8.0.2 (BD Biosciences). Daily calibration and QC were performed using CS&T Beads (Cat# 642412 and 655051, BD Biosciences). CD4⁺ and CD8⁺ T-lymphocytes were pre-gated on live singlets CD3⁺ cells; B cells were pre-gated on live singlets CD3⁻ cells and identified as B220⁺CD19⁺.

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Supplementary Figures

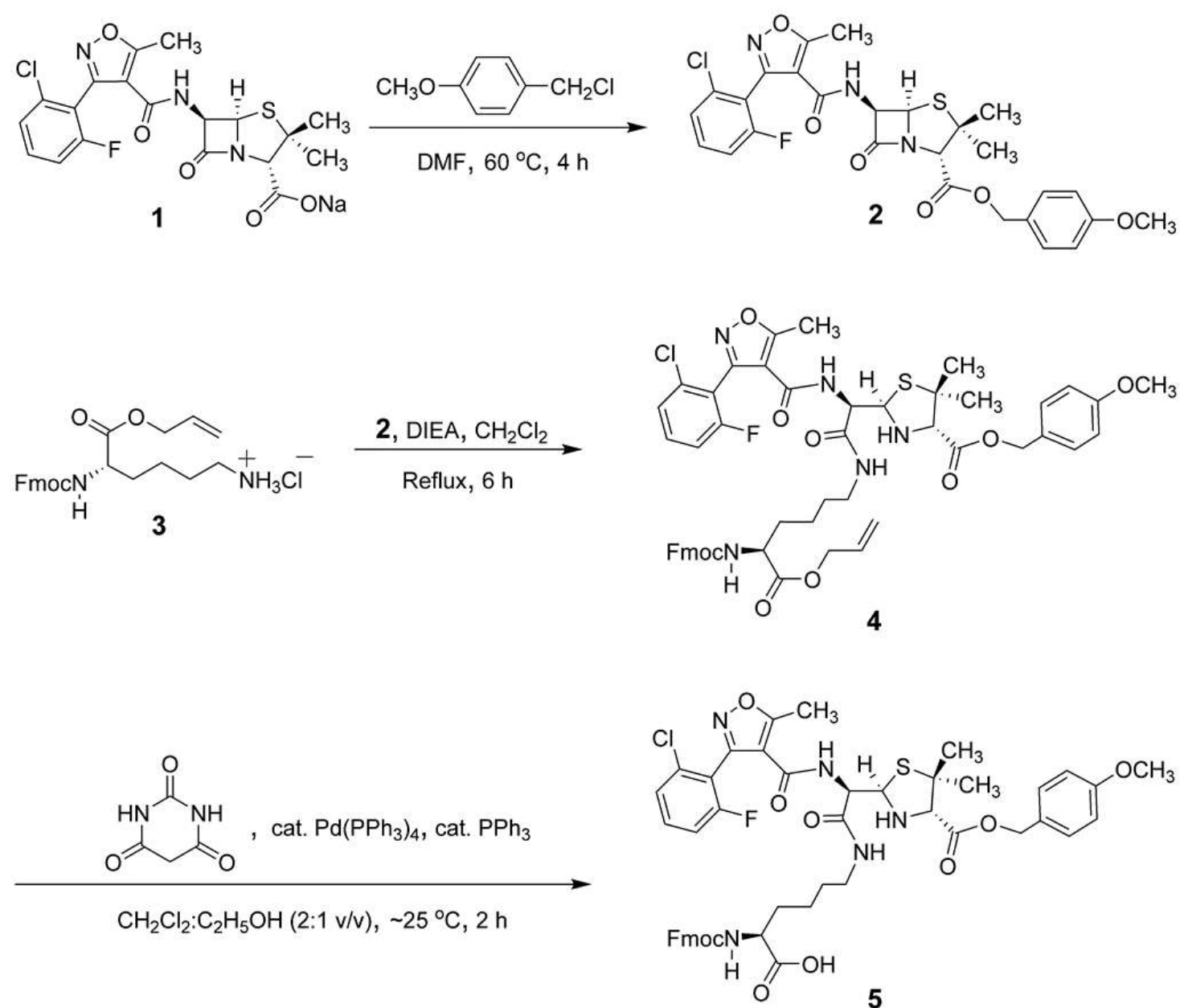


Figure S1. Steps in the chemical synthesis of FLX-conjugated lysine residues as per Scornet, Delarue-Cochin et al.'s method (2016). Fmoc: 9-Fluorenylmethoxycarbonyl; DIEA: N,N-diisopropylethylamine

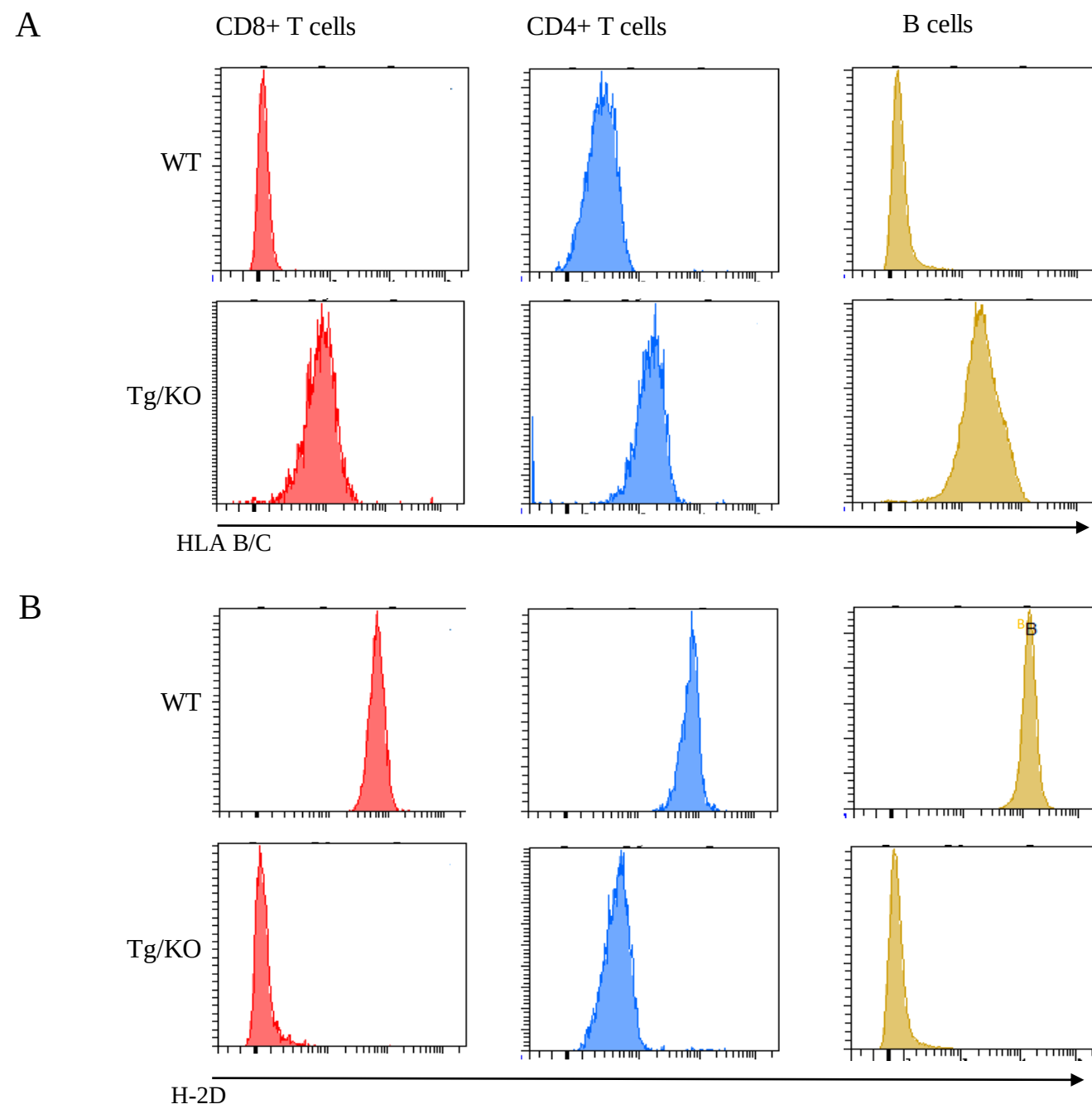


Figure S2. Phenotypic analysis of the HLA-B*57:01 Tg/ H2-K^bD^b KO mice by flow cytometry –Tg/KO mice express HLA-B*57:01 **(A)** but not mouse MHC-I (H-2D) **(B)** in gated blood CD8+ T cells (CD8+CD3+), CD4+ T cells (CD4+CD3+) and B cells (CD19+CD3-). Wild type mice (WT) with the same background as the Tg/KO, C57BL/6, were used as controls.

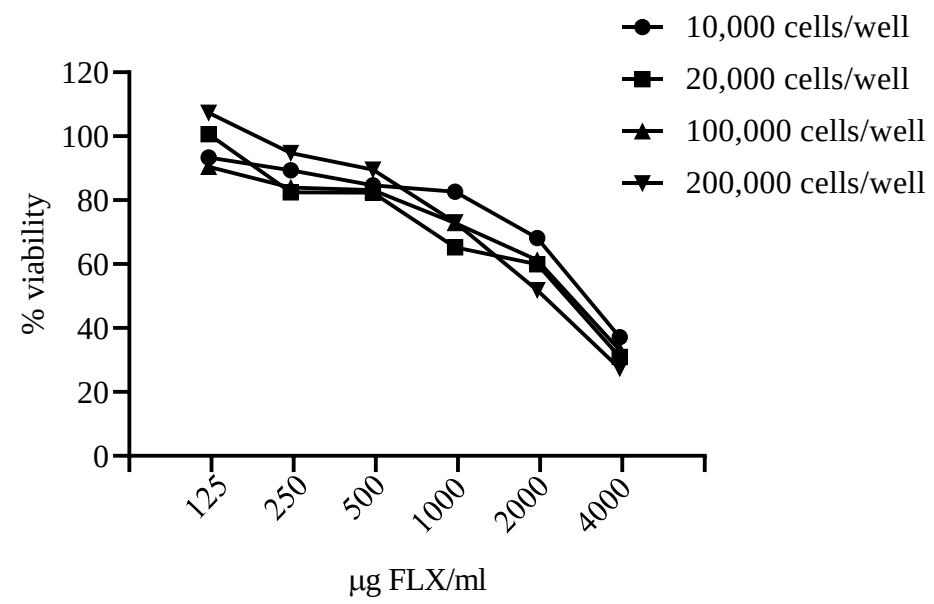


Figure S3. FLX toxicity on B721-5701 cell viability is concentration dependent – B721-5701 cells were seeded into 96-well plates at the concentrations shown in the legend and treated with 2-fold dilution of FLX. Cell viability was measured after 24 hours of incubation using the MTT assay, as per manufacturer instructions (Life Technologies).

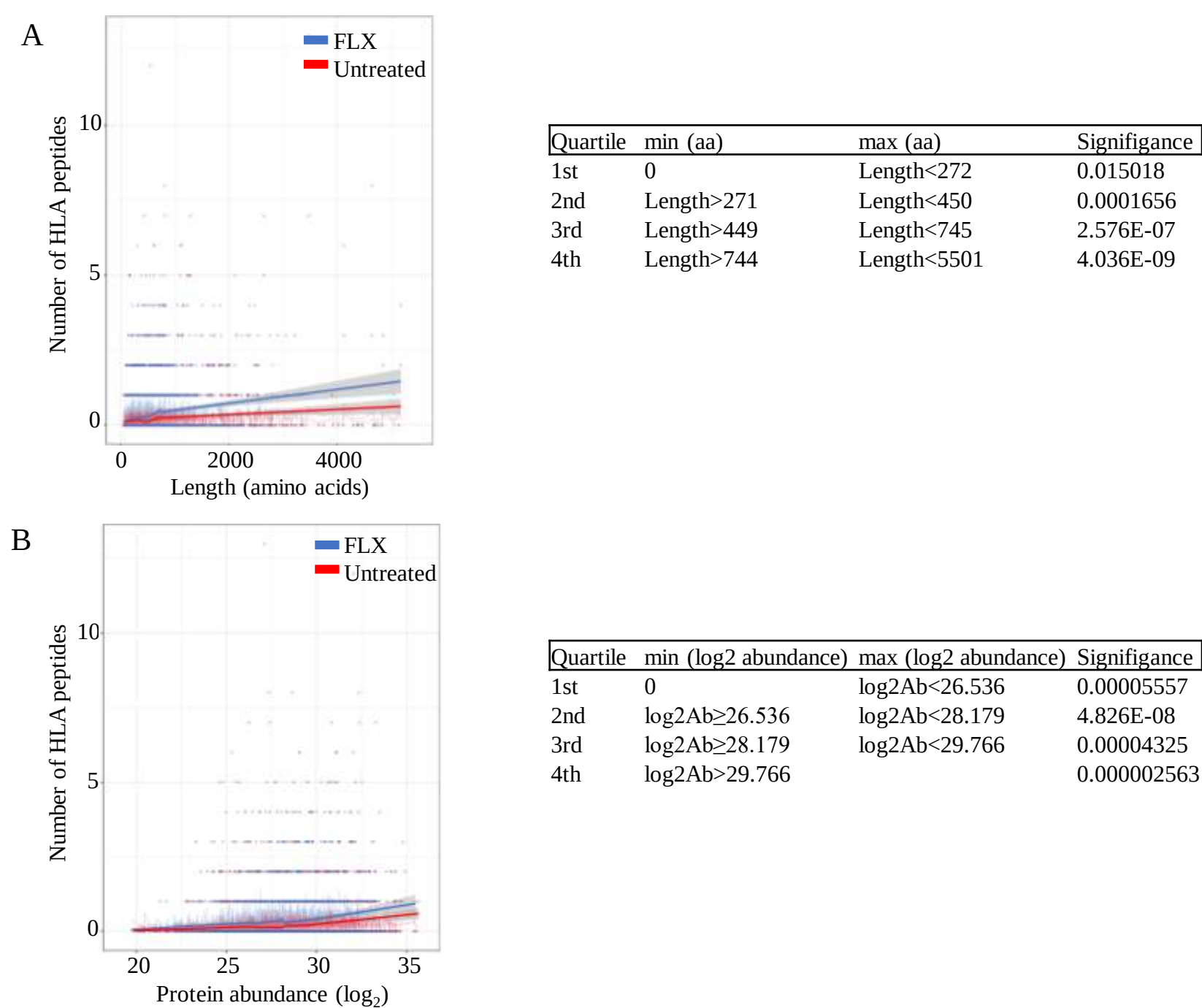


Figure S4. Linear trendlines from Figure 3 fitted in quartiles by protein length (A) or abundance (B) in relation to the number of HLA peptides in Untreated and FLX-treated samples. Quartiles and ANCOVA significance values are indicated in the corresponding data tables.

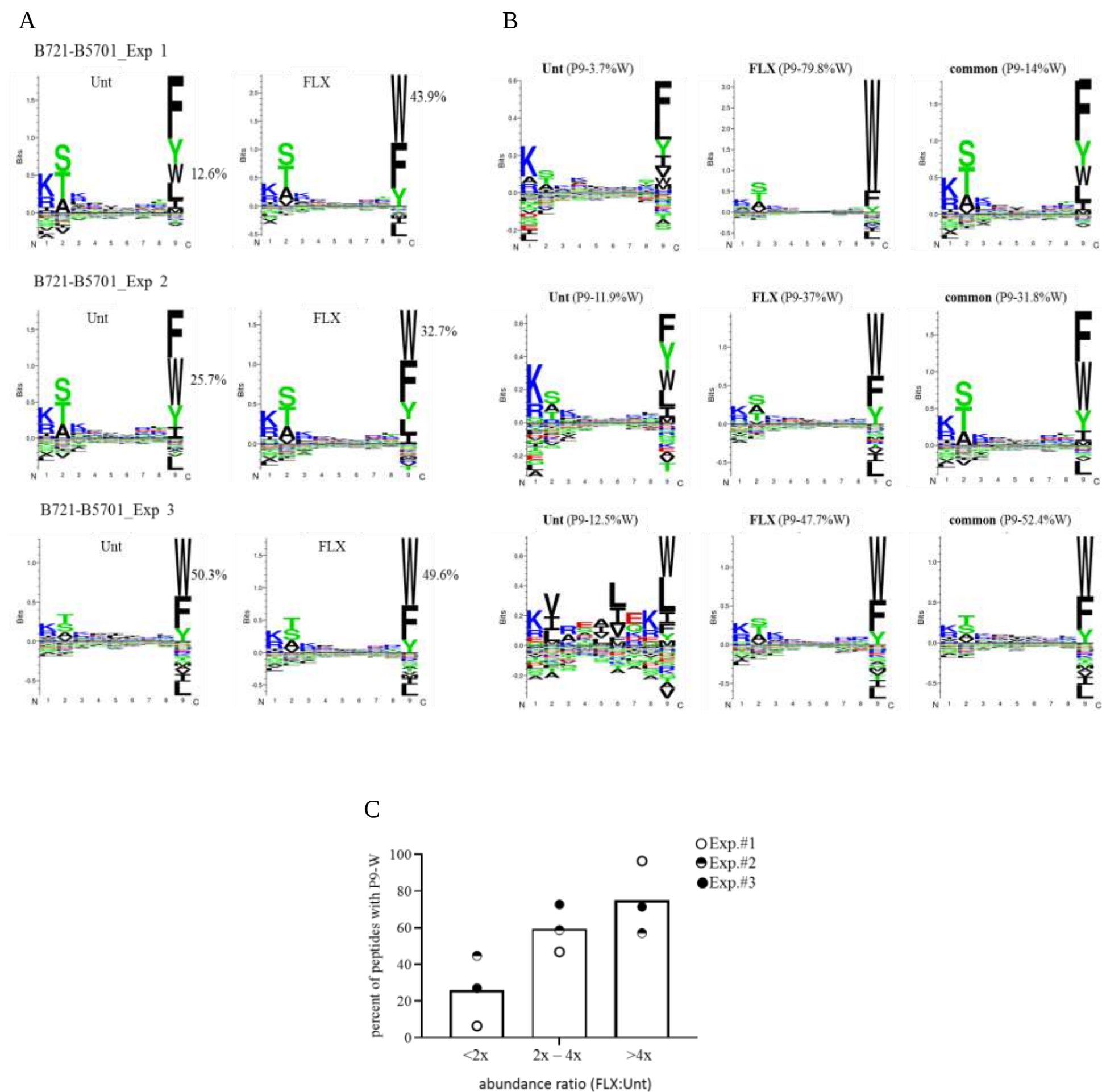
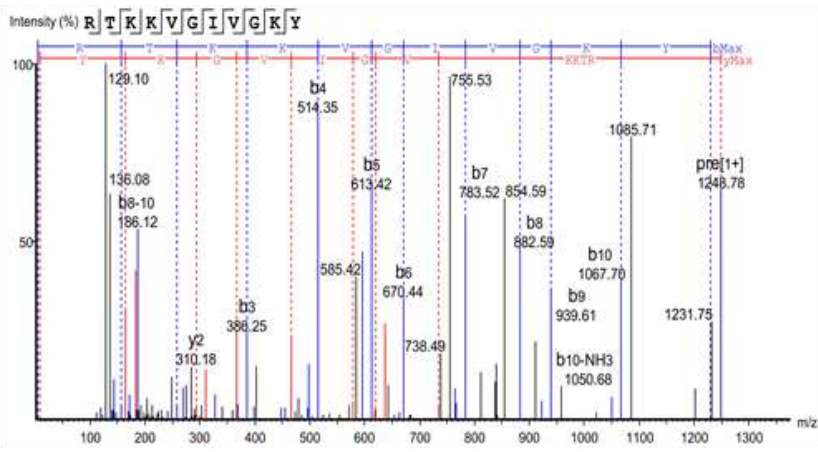


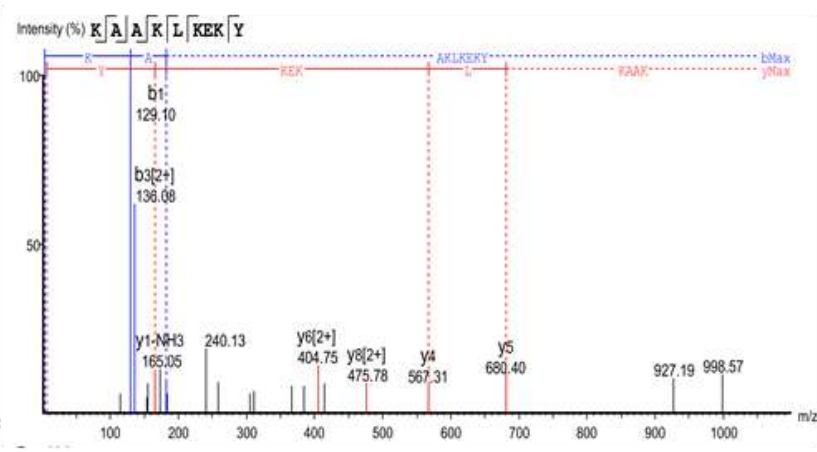
Figure S5. Qualitative and quantitative analysis of 9-mer peptides containing tryptophan (W) in P9 for 3 independent experiments– (A-B) Frequency of amino acids in 9-mer sequences of the immunopeptidome of B721-5701 cells untreated (Unt) or treated with FLX (FLX) for 5 days, including the total number of 9-mers for each treatment (A), or 9-mers unique for Unt, unique for FLX or common to both treatments (B). Percent values correspond to the fraction of W in P9. (C) Percent of common peptides with P9-W (9-mer) increased in abundance by more than 4x, 2-4x or less than 2x in FLX immunopeptidome from 3 independent experiments. Abundance values correspond to ion counts of common peptides and presented as a ratio between FLX and Unt samples.

A

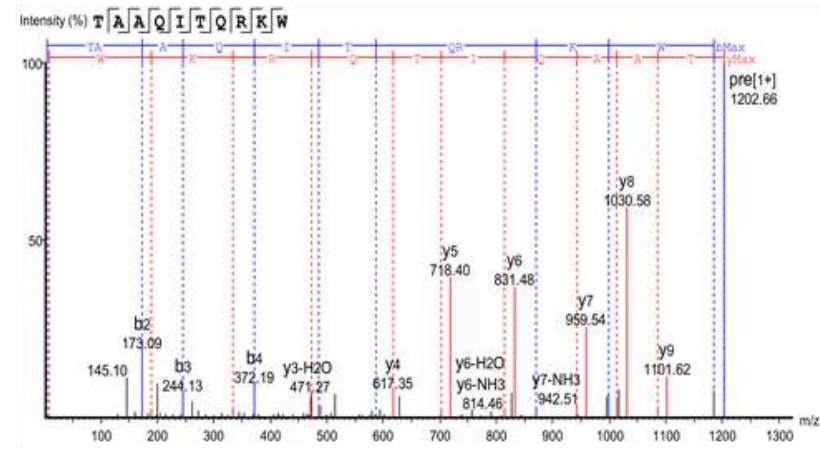
RTKK



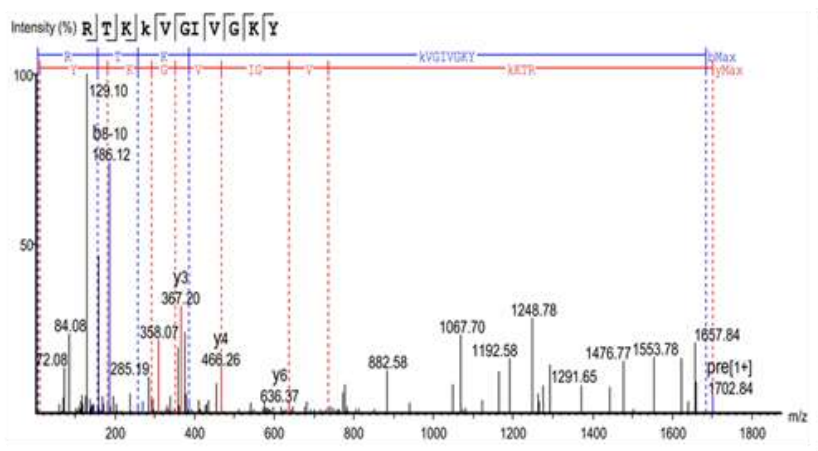
KAA



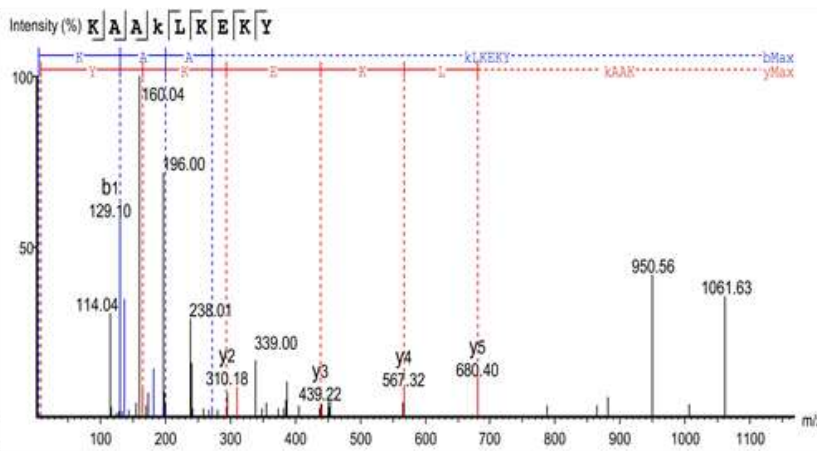
TAA



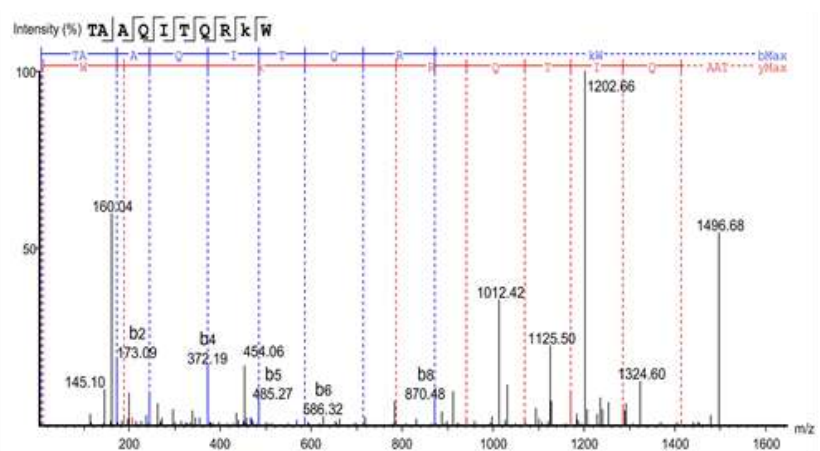
FLX-RTKK_K4



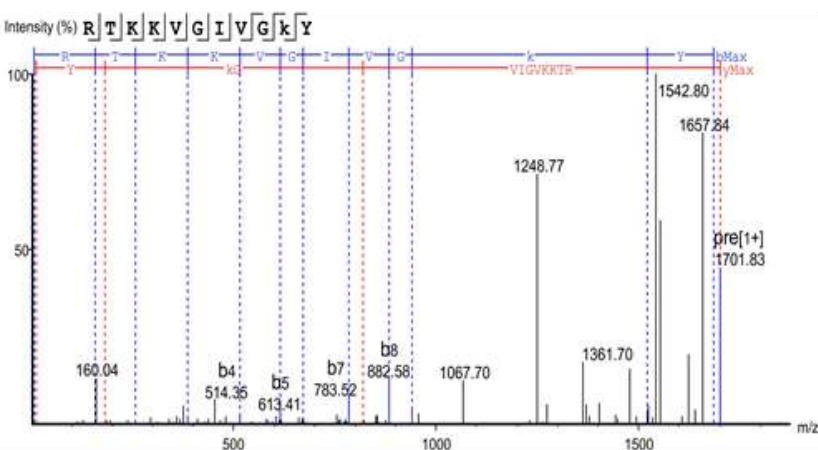
FLX-KAA_K4



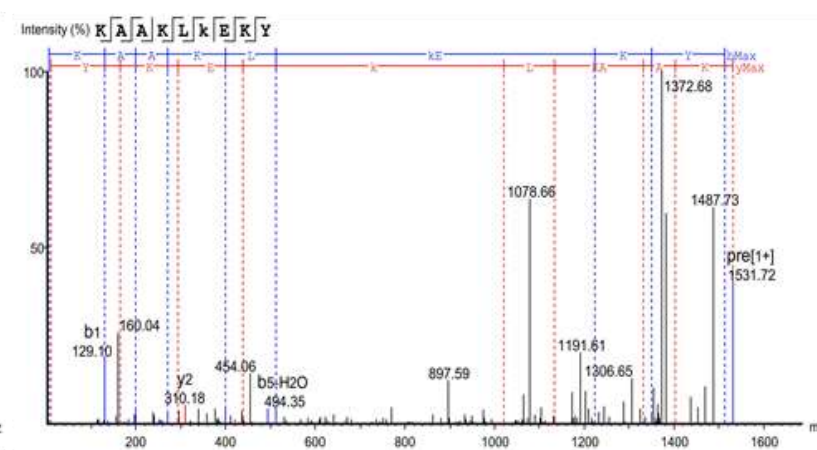
FLX-TAA_K9



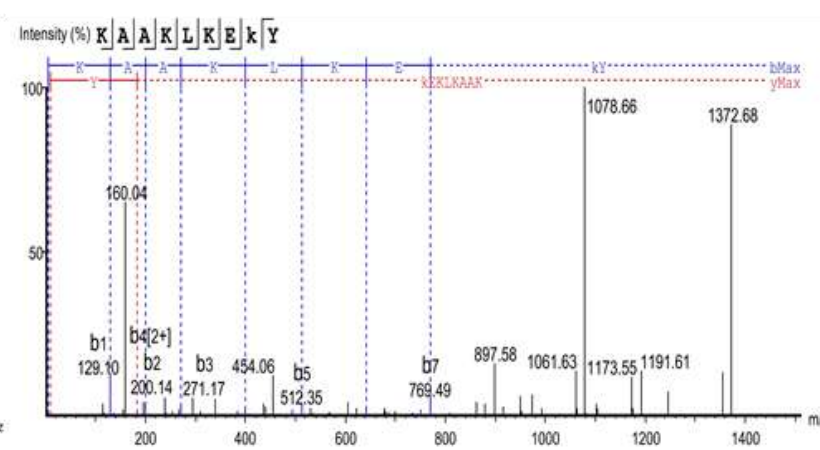
FLX-RTKK_K10



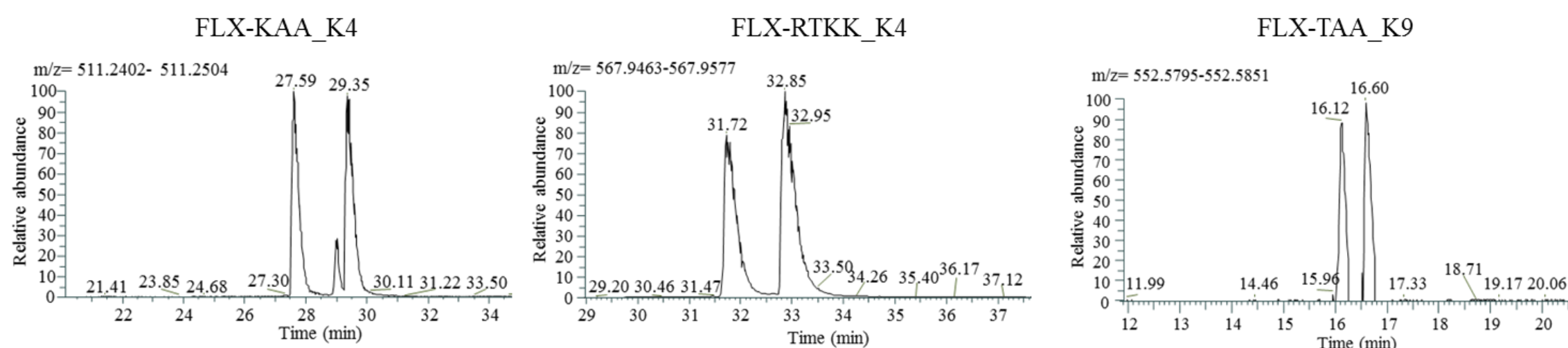
FLX-KAA_K6



FLX-KAA_K8



B



C

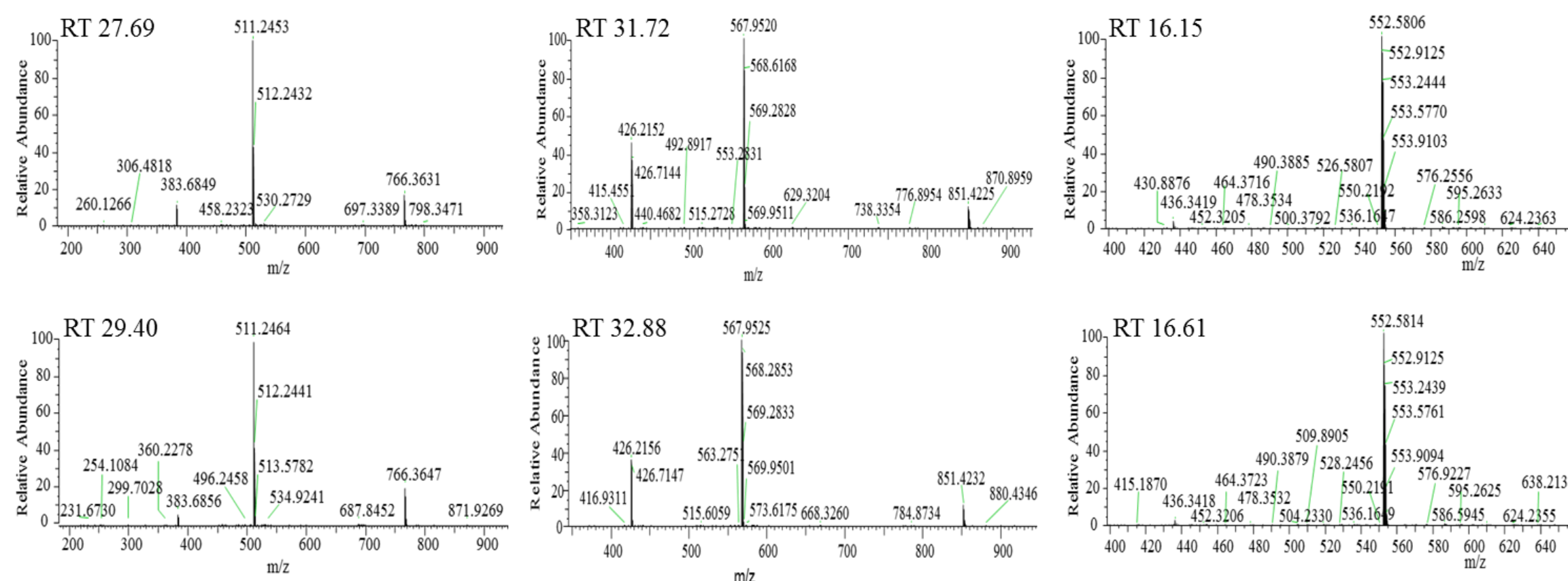


Figure S6. Quality control and isoform characterization of unmodified and chemically synthesized FLX-modified peptides. The FLX-modified RTKK, KAA and TAA peptide sequences were synthesized using FLX Fmoc-conjugated lysine residues. **(A)** Quality control of the synthetic peptides was conducted by LC-MS and compared to the unmodified peptide sequences obtained from the cell immunopeptidome (top panels, unmodified sequences; middle and bottom panels, chemically synthesized modified sequences). “k” corresponds to K+453 (FLX) . **(B)** LC-MS (ms1) chromatograms for three of the synthetic peptides, showing two main isoform peaks with different retention times (RT) for each peptide preparation. **(C)** LC-MS spectra of the isoform peaks with different RT showing identical mass for the corresponding peptides in **(B)** (top and bottom, respectively). m/z of FLX-KAA_K4: 511.24/3+, 766.36/2+; FLX-RTKK_K4: 567.95/3+, 851.42/2+; FLX-TAA_K9: 552.58/3+, 828.36/2+ (not shown).

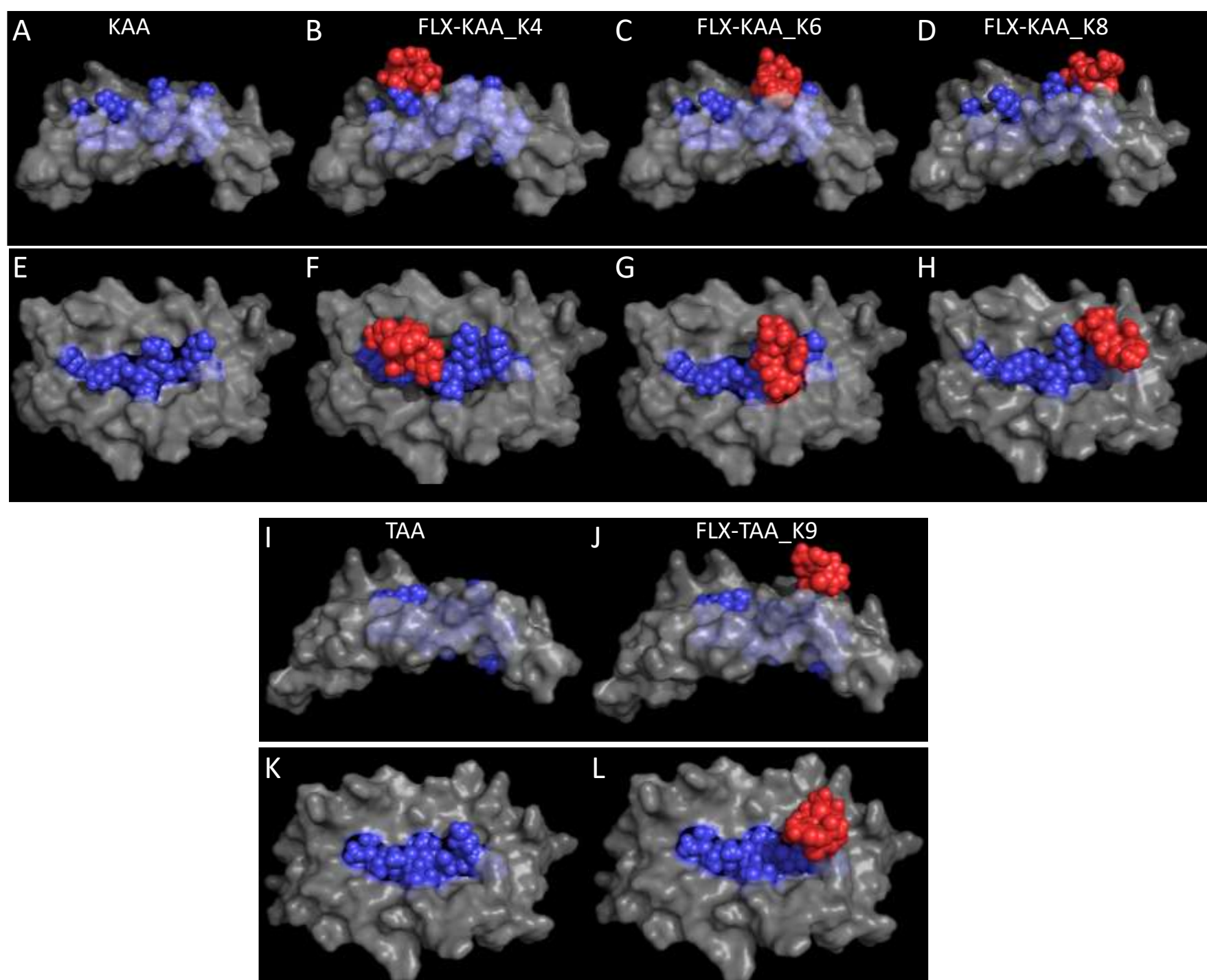
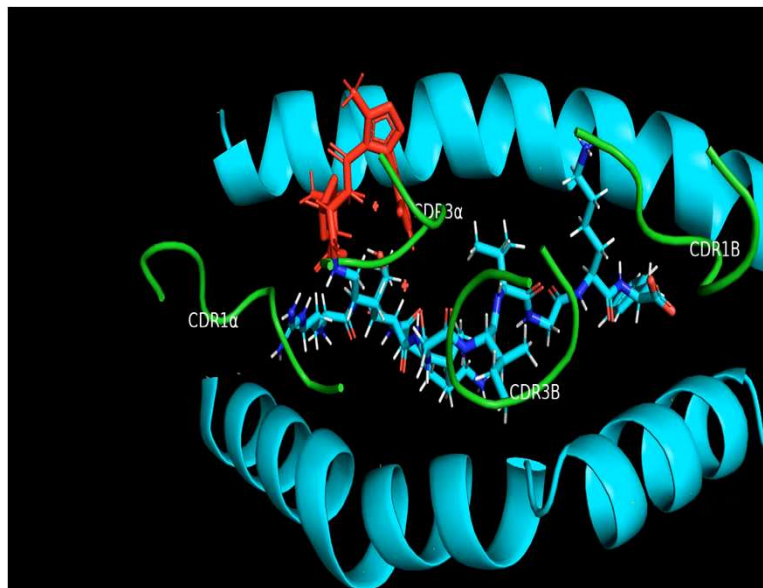


Figure S7. *In silico* prediction of the interactions of parent and FLX-KAA and FLX-TAA peptides with HLA-B*57:01 cleft using PyMOL (v2.3.4) and OpenBable (v3.0.0) software. The LF9 peptide (PDB:2RFX) was modified with the KAA residues, and the TW10 peptide sequence (PDB:5V5M) was modified with the TAA peptide residues. Lateral (A-D & I-J) and apical (E-H & K-L) views of the HLA complex with the unmodified KAA (A&E), FLX-KAA_K4 (B&F), FLX-KAA_K6 (C&G) and FLX-KAA_K8_K10 (D&H). Also, TAA (I&K) and FLX-TAA_K9 (J&L). Peptide sequences are represented in blue spheres, drug is represented by red spheres and $\alpha 1\alpha 2$ domains of the HLA are depicted in gray color.

A

FLX-RTKK_K4



B

FLX-RTKK_K10

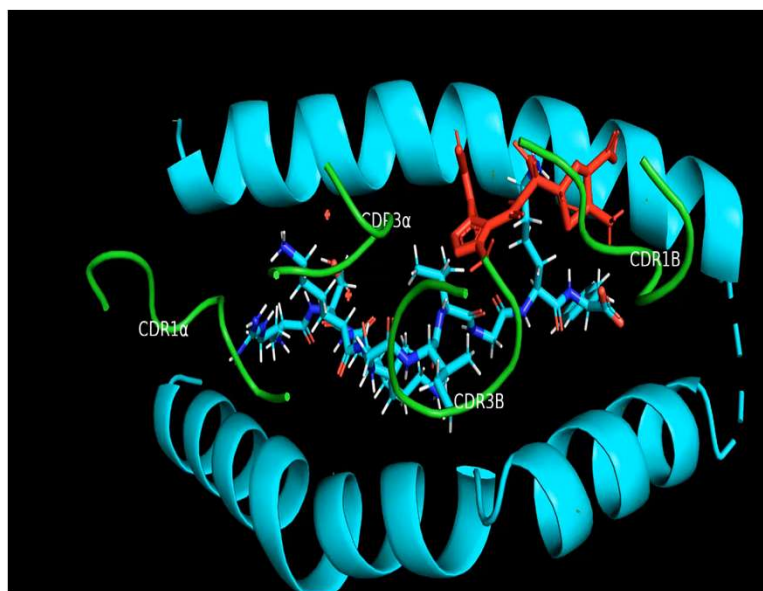


Figure S8. Spatial representation videos of the HLA/peptide complex relative to the TCR - FLX (in red) conjugated to the RTKK peptide (in blue backbone) lysine in position 4 clashes with the CDR3V- α subunit of the model KAF-TCR (PDB ID: 2YPL) (in green) **(A)** as opposed to the drug conjugated in the lysine in position 10 **(B)**. HLA α 1 and α 2 helixes are shown in light blue. 3-D structures were visualized in PyMOL software.

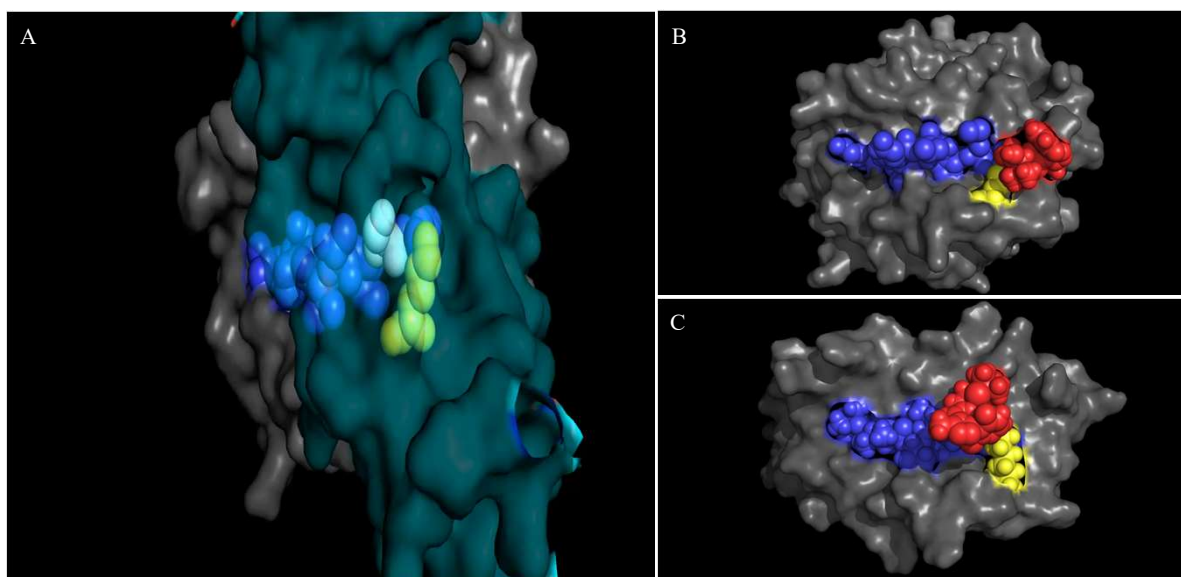


Figure S9. *In silico* prediction of the potential interference of FLX with HLA/killer cell immunoglobulin-like receptor (KIR)-3DL on NK cells. (A) HLA (PDB ID: 2YPK) /KIR-3DL (PDB ID: 3VH8) interaction showing the spatial proximity of HLA-K146 and the PΩ-1 of the peptide and their location on the contact area among HLA and KIR (click on video play). **(B)** FLX-conjugated in K146 residue in HLA molecule. HLA pocket is occupied by KAF11 peptide (PDB ID:2YPK). **(C)** FLX-TAA_K9 peptide in the HLA pocket. In all the figures, HLA is represented in gray, KIR-3DL in teal, peptides are in blue, the HLA-K146 residue is in yellow, and the PΩ-1 residue of the peptide is shown in white. FLX is represented in red. 3-D structures were visualized in PyMOL software.