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TITLE PAGE

HLA DRB1*15:01-DQB1*06:02-restricted human CD4+ T-cells are selectively activated with amoxicillin-peptide adducts

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Running title: T-cell responses to drugs

ABSTRACT

Amoxicillin-clavulanate is the most common cause of idiosyncratic drug-induced liver injury (DILI). Drug-specific CD4+ T-cells have been detected in patients with DILI, suggestive of an immune aetiology. Furthermore, genetic associations including the HLA DRB1*15:01-DQB1*06:02 haplotype influence susceptibility. Amoxicillin forms protein adducts that are postulated to activate T-cells, by conjugating with lysine residues. However, a role for such adducts has not been described. This study aimed to (1) investigate whether amoxicillin-modified HLA-DRB1*15:01-DQB1*06:02 binding peptides selectively activate DILI patient T-cells and (2) define the nature of the T-cell response with respective to antigen structure. Peptides carrying lysine residues for amoxicillin binding in positions (KP) 2-6 and anchors for the HLA-DRB1*15:01-DQB1*06:02 haplotype were designed. The amoxicillin-modified peptides were characterized by mass spectrometry prior to culturing with patient PBMC. T-cell clones were then tested for specificity with amoxicillin, unmodified- and amoxicillin-modified peptides, and structural variants. Amoxicillin-modified KP-2 and KP-3 peptide-specific CD4+ clones proliferated and secreted IFN-γ, IL-10, perforin and/or IL-17/IL-22 in a dose-dependent manner and displayed no cross-reactivity with amoxicillin, unmodified peptide or with positional derivatives. The T-cells response was HLA class II-restricted and the amoxicillin-modified peptides bound selectively to HLA-DRB1*15:01 and/or DQB1*06:02. To conclude, we show that amoxicillin-modified peptides bind to both components of the risk haplotype to stimulate DILI patient T-cells and describe the importance of the position of nucleophilic lysine residue in the HLA binding peptide sequence.

Key words: Drug-induced liver injury, T-lymphocytes, human, antigen

INTRODUCTION

Idiosyncratic drug-induced liver injury (DILI) is a major health concern resulting in significant patient morbidity and mortality. The delayed onset of DILI and the discovery of several associations with HLA Class I and Class II alleles in patients provides evidence that liver injury may be the result of a delayed-type immunological reaction. Notable examples include the association between HLA-B*57:01 and flucloxacillin-induced liver injury where 1 in every 500-1000 individuals expressing the allele will develop DILI (Daly *et al.,* 2009), and the more recently identified associations between the HLA-A*33 serotype and terbinafine-induced liver injury (Nicoletti *et al.,* 2017) and HLA-B*14:01 and HLA-B*35:01 and trimethoprim-sulfamethoxazole-induced liver injury (Li et al., 2020). Amoxicillin-clavulanate, the most common cause of idiosyncratic DILI, is associated with the HLA class I allele HLA-A*02:01 and the class II haplotype HLA-DRB1*15:01-DQB1*06:02 (Lucena *et al.,* 2011).

As a result of the genetic association studies, we have investigated whether drug-specific T-cells are detectable in peripheral blood mononuclear cells (PBMC) from patients with liver injury. Flucloxacillin-specific CD8+ T-cells were isolated from PBMC of HLA-B*57:01 positive patients and the T-cell proliferative response and cytokine release was restricted to the risk allele (Monshi *et al.,* 2013). Furthermore, CD8+ T-cell infiltrates have been identified in HLA-B*57:01 positive patient liver biopsies (Wuillemin *et al.,* 2014). A mixture of amoxicillin- and clavulanic acid-specific CD4+ T-cells have been isolated from PBMC of patients with amoxicillin-clavulanate-induced liver injury (Kim *et al.,* 2015). However, using the parent drugs (amoxicillin and clavulanic acid) to activate cloned T-cells it was not possible to elucidate

whether the drugs interacted with the HLA risk alleles identified in the genetic studies described above.

Drugs interact with HLA molecules and activate T-cells via two pathways (Meng *et al.,* 2018). In the hapten model, a drug must bind covalently to a protein. The protein adduct then undergoes processing within antigen presenting cells prior to display of the derived peptides on the cell surface within HLA molecules. Alternatively, the pharmacological interactions model suggests a drug can interact reversibly with HLA molecules and stimulate T-cells directly (Pichler *et al.,* 2011; White *et al.,* 2015; Illing *et al.,* 2016). In amoxicillin-clavulanate DILI patients, T-cells are stimulated with antigen presenting cells covalently modified with amoxicillin and the stimulation is dampened by inhibiting exogenous and endogenous protein processing (Kim *et al.,* 2015; Yaseen *et al.,* 2015). Additionally, multiple amoxicillin-modified proteins have been characterised in patient serum (Meng *et al.,* 2016; Ariza *et al.,* 2014). These data suggest that amoxicillin stimulates patient T-cells via the hapten model; however, there are still many unknown aspects. The exact peptide, group of peptides, or their source protein, that activates T-cells have not been identified. Also, whether amoxicillin is covalently-bound to stimulatory peptides and has any chemical interaction with the T-cell receptor is unclear.

The aims of this study were to (1) utilize mass spectrometric and peptide purification methods to synthesize positional derivatives of amoxicillin-modified designer HLA binding peptides, by placing lysine on different locations on the peptide backbone and (2) investigate their capacity to activate DILI patient T-cells. Peptides with anchor residues for HLA-DR15 and HLA-DQ6

were used to determine whether one or both of the HLA Class II alleles DRB1*15:01 and DQB1*06:02 associated with amoxicillin-clavulanate-induced liver injury are involved in the presentation of the drug antigen to patient T-cells.

MATERIALS AND METHODS

Design of amoxicillin-modified peptides

Amoxicillin-peptides were constructed using anchor residues for DR15 derived from the SYFPEITHI database based on HLA peptide elution data (Rammensee *et al.,* 1999). However, based on the known anchor residues for DQ6, the peptides would also be tolerated by DQ6 (Ettinger *et al.,* 1998). Peptides were designed with a poly-alanine backbone 12 amino acids in length with the addition of glutamic acid to improve peptide solubility. As β-lactams can bind to the peptide *n*-terminal amino group, peptides were amino acetylated to avoid non-specific binding. Lysine could be inserted in four locations within the binding motif, therefore, four positional derivatives of the peptide were designed (Fig. 1). Peptides were named according to the location of the lysine relative to the anchor residue leucine on the peptide backbone. For example, KP2 refers to the peptide where lysine is in position 2. Alanine was chosen as an 'inert' amino acid to incorporate into the sequence. This enabled clear chemical discrimination with locations with a drug-modified lysine to explore the role of drug-modification and modification position at TCR contact sites. If other amino acids were present, then it would have been more difficult to distinguish whether observations were due to the drug-modification or some additional binding effect caused by the other amino acids present.

Generation of amoxicillin-modified peptides

Peptides were purchased from SYN-Peptide Ltd as per the design specification (Fig. 1). Amoxicillin sodium (GlaxoSmithKline, Ware, UK) was incubated with the peptides at a 10:1

molar ratio for 24 hours at 37°C in 0.1mM Na_2CO_3 buffer, adjusted to pH10. Peptide modification was confirmed by mass spectrometric analysis following sample clean-up using C_{18} ZipTips according to the manufacturer's instructions.

HPLC purification of amoxicillin-modified peptides

Peptides were acidified with 0.1% formic acid and purified using reverse phase HPLC (Agilent 1200) and a Kinetex C18 (Phenomenex, 2.6µM; 100Å) column with the following gradient (min, %B; 0, 2%; 15, 50%; 15.10; 95%; 17, 95%; 17.10, 2%; 28, 2%). The amoxicillin-peptide peak was found at a retention time of 14.7 minutes with the amoxicillin-amoxicillin-peptide peak at 15.1 minutes (Sup. Fig. 1B). Multiple fractions were collected and dried using an Eppendorf concentrator before LC/MS/MS analysis or use in T-cell assays (Sup. Fig. 1C&E).

Mass spectrometric analysis

The free amoxicillin remaining in the amoxicillin-modified peptide fractions were quantified by mass spectrometry. Calibration standards were prepared at the following concentrations (100, 250, 500, 1000, 2500, 5000nM). All samples were diluted in 0.1% formic acid prior to analysis and spiked with the internal standard ampicillin (5µM). Samples and standards were analysed immediately by a QTRAP 4000 mass spectrometer (AB Sciex,) coupled with an Ultimate 3000 LC system (Dionex Corporation, Sunnyvale, CA). The multiple reaction monitoring transitions for each analyte were as following: amoxicillin 366.2/159.9 and 366.2/349.0, and ampicillin 350.2/160.2. Other MS parameters, such as voltage potential and

collision energy were optimised to achieve great sensitivity. Data acquisition and quantification were performed using Analyst 1.5 software.

Mass spectrometric characterisation of the un-purified peptides identified three major peaks including unmodified peptide, amoxicillin-modified peptide and the amoxicillin-amoxicillin (dimer)-peptide (Sup.Fig1G). Unmodified peptides and the modified peptides were characterised using previously described methods on a TripleTOF 5600 (AB Sciex) mass spectrometer (Jenkins *et al.,* 2013). Briefly, samples were delivered into the mass spectrometer by automated in-line reversed phase liquid chromatography, using an Eksigent NanoUltra cHiPLC System (AB Sciex) mounted with a trap and analytical column (15 cm × 75 µm) packed with ChromXP C18−CL 3 µm. A NanoSpray III source was fitted with a 10 µm inner diameter PicoTip emitter (New Objective). Samples were loaded in 0.1% formic acid onto the trap, which was then washed with 2% acetonitrile/0.1% formic acid for 10 min at 2 µL/min before switching in-line with the analytical column. A gradient of 2−50% (v/v) acetonitrile/0.1% (v/v) formic acid over 90 min was applied to the column at a flow rate of 300 nL/min. Spectra were acquired automatically in positive ion mode using information-dependent acquisition, using mass ranges of 400−1600 Da in MS and 100−1400 Da in MS/MS. Up to 25 MS/MS spectra were acquired per cycle (approximately 10 Hz) using a threshold of 100 counts per s, with dynamic exclusion for 12 s and rolling collision energy. Modified peptides were identified by filtering for specific fragment ions in PeakView 1.2.0.3 (AB Sciex) and manual inspection of the spectra. Positional derivatives had an equal mass, therefore key fragment ions are highlighted to distinguish their sequences (Sup. Figs. 3-6).

Human subjects

Patients with amoxicillin-clavulanate-induced liver injury were enrolled from the DILIGEN study (Lucena *et al.,* 2011) with the clinical characteristics of hepatocellular / mixed type injury. Chemagic magnetic separation (Chemagen, Baesweiler, Germany) was used to extract genomic DNA followed by high-resolution HLA-typing carried out by the Histogenetics Laboratory (Histogenetics, Ossining, NY). The HLA type and demographics of the patients have been described previously (6). One patient expressed the HLA-DRB1*15:01, HLA-DQB1*06:02 haplotype (HLA-A*02:01/03:01, HLA B*07:02/57:01, HLA-DRB1*15:01/07:01, HLA-DQB1*03:03/06:02, HLA-DQA1*01:02/02:01) and PBMC from this patient were used to generate clones to explore the immunogenicity of the amoxicillin-modified peptides.

Healthy donors positive or negative for the risk haplotype or expressing either HLA-DRB1*15:01 or HLA-DQB1*06:02 were also recruited to the study to generate Epstein-Barr virus (EBV)-transformed B-cells as antigen presenting cells for HLA allele restriction studies. The HLA genotype of the donors is shown in Sup. Table 1. Approval for the study was acquired from the Liverpool local research ethics committee and informed written consent was obtained from each donor.

Generation of T-cell clones and EBV-transformed B-cell lines

PBMC (10^6 cells/well; 0.5ml) were cultured with amoxicillin (1.5mM; 0.5ml) or amoxicillin-modified peptides (100μM; 0.5ml) in RPMI 1640 supplemented with 10% human AB serum (Class A; Innovative Research Inc., Novi, MI), 10mM of L-glutamine, 25mM of HEPES and 25

µg/mL of transferrin (Sigma-Aldrich, Gillingham, UK). PBMCs were fed with media containing human interleukin (IL)-2 (PeproTech, London, UK) added on days 6 and 9. On day 13, cells were seeded into 96 well plates as described in previous studies (Wu *et al.,* 2007). EBV-transformed B-cell lines were generated by transforming PBMC with supernatant from the EBV-producing cell line B95.8. EBV-transformed B-cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100mM L-glutamine, penicillin and streptomycin (Sigma-Aldrich, Gillingham, UK).

Antigen specificity of the clones was assayed by culturing T-cells ($5x10^4$ cells/well, 50µL) with autologous EBV-transformed B-cells lines ($1x10^4$ cells/well, 50µL) and amoxicillin (1.5mM; 100µL) or amoxicillin-modified peptides (5µM; 100µL). Proliferation was assessed by the addition of [^{3}H]-thymidine (0.5 µCi/well, 5 Ci/mmol; Morovek Biochemicals, Brea, CA) for the last 16 hours of the culture period. Incorporated radioactivity was measured by scintillation counting. Clones with a stimulation index greater than 2 were expanded using a stimulation cocktail made with T-cell culture media containing IL-2 (200I IU/ml) PHA (5µg/ml) and irradiated allogeneic PBMCs ($5x10^5$). T-cell clones were maintained through the addition of IL-2 (200I IU/ml) every two days until further experiments were carried out.

T-cell specificity assays

T-cell clones were subjected to a further test to confirm specificity to amoxicillin-modified peptides in comparison to unmodified peptides. This and all subsequent T-cell assays underwent the same co-culture procedure; clones ($5x10^4$ cells/well) were cultured with or

without autologous irradiated EBV-transformed B-cells as the antigen presenting cell ($1x10^4$ cells/well) in the presence of amoxicillin-modified peptides (25-50μM) and unmodified peptides (up to 500μM). Proliferation and cytokine secretion were measured using [^{3}H]-thymidine incorporation and the Enzyme Linked ImmunoSpot Assay (ELISpot – IFN-γ, IL-13, IL-5, perforin, IL-22, IL-17, IL-10, granzyme B), performed according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). CD4+/CD8+ and chemokine receptor (CCR 1-6 & 8-10, CXCR 3, CD69, E-CAD and CLA) phenotyping was performed using flow cytometry (BD FACs Canto II) by staining with fluorescent antibodies (R&D Biosystems). Due to limited quantities of the modified peptides, the concentration used in subsequent mechanistic studies was 10μM, the lowest concentration required to gain a significant response in all clones. This was determined by titrating the concentration of the amoxicillin-modified peptides (0.01 – 10μM) and analysis of proliferation. Only CD4+ T-cells were taken for further assessment.

Cross-Reactivity studies

In initial experiments clones responsive towards a specific amoxicillin-modified peptide and irradiated EBV-transformed B-cells were cultured with the other positional derivative peptides (with and without amoxicillin modification) and proliferation was measured by [^{3}H]thymidine incorporation. Subsequently, a number of peptide reagents were generated using the same synthesis, purification and characterization processes as the amoxicillin-modified peptides and used to characterize T-cell cross-reactivity in proliferation or cytokine release assays. These included ampicillin-modified-KP2 peptide, amoxicillin-modified HSA peptide K^{204} and several amoxicillin dimer-modified peptides. Furthermore, HLA binding

inhibition was performed by culturing increasing concentrations (10-300μM) of unmodified peptides with the amoxicillin-modified peptides (10μM) prior to analysis of proliferation and cytokine release.

HLA-restriction studies

HLA class I and HLA class II (including HLA class II DR and DQ subtypes) blocking antibodies (and isotype controls; BD Biosciences, Oxford, UK) were incubated with EBV-transformed B-cells and T-cell clones 30min prior to addition of amoxicillin-modified peptides and analysis of proliferation and/or IFN-γ release by ELIspot. To test which specific HLA molecules the amoxicillin-modified peptides interact with prior to activating T-cells, the HLA-typed Liverpool Healthy Volunteer Cohort was accessed and donors were selected based on the presence or absence of specific co-amoxiclav HLA risk alleles (HLA-DRB1*15:01 and/or HLA-DQB1*06:02). EBV-transformed B-cell lines were generated from frozen PBMCs, irradiated, and used in proliferation assays with the amoxicillin-modified peptides and T-cell clones.

Toxicity Assay

The toxicity of the drug-modified peptides was tested by incubating freshly isolated PBMC ($1.5x10^6$; 200μl) from healthy volunteers for 4 days after which PHA (5μg/ml) was added and proliferation measured using [^{3}H]-thymidine. Toxicity was measured as an inhibition of proliferative activity observed with cultures in the absence of peptide upon stimulation with PHA.

RESULTS

Characterization of amoxicillin-modified peptides

Amoxicillin-modified peptides (KP2-6, with the number referring to the position of the drug-modified lysine with respect to the leucine anchor residue [Fig. 1]) were successfully generated, purified and characterized using mass spectrometry. Although the mass of each peptide was the same, key fragment ions derived from the cleavage of the peptide backbone were identified for each modified peptide to distinguish positional derivatives and the amoxicillin peak. Ions which represent amino acids with the mass addition of amoxicillin are highlighted for each amoxicillin-modified peptide (Sup. Figs. 3-6).

Each batch of peptides generated was analysed to quantify unbound amoxicillin. The amoxicillin-modified peptides were found to contain approximately 2-3% unbound amoxicillin correlating to 1.5μM amoxicillin with the highest concentration of amoxicillin-modified peptide used in T-cell assays (Sup. Fig. 2). To put this into perspective for the range of amoxicillin-modified peptide concentrations used in this study: 100μM amoxicillin-modified peptide contained 3.21 ± 0.4 μM free amoxicillin; 25μM amoxicillin-modified peptide contained 0.8 ± 0.1 μM amoxicillin; and 10μM, the concentration most frequently used in T-cell assays, contained 0.32μM ± 0.04 μM amoxicillin remaining. This was well below the concentration of amoxicillin which we have previously shown could activate T-cells (8μM). The amoxicillin-modified and unmodified peptides subjected to the same extraction and purification protocols were assayed for direct toxicity towards PBMC. No direct PBMC toxicity was observed with the amoxicillin-modified-peptide concentrations used to stimulate T-cells (0.3-300 μM) (Sup. Fig. 2).

Generation of amoxicillin-modified peptide-specific CD4+ T-cell clones

T-cell cloning was performed following antigen-driven T-cell enrichment by treating PBMC with the amoxicillin-modified peptides (KP2-6) for 13-14 days. Individual T-cells were serially diluted and expanded with repeated mitogen stimulation. T-cell colonies reaching approximately 0.5-1.0x10^6 cells were assessed for antigen-specific proliferation. In total, over 900 colonies were tested for reactivity against the amoxicillin-modified peptides; colonies with a stimulation index (SI) greater than 2 were deemed potentially responsive and were expanded for further testing.

Clones were initially tested using a relatively low concentration of 5μM amoxicillin-modified peptide. Cloning with amoxicillin-modified peptides-KP2 and KP3 identified 5/240 colonies from each serial dilution with an SI>2 (Fig 2A & B). Of these, four CD4+ (Fig. 2C) T-cell clones displayed strong proliferative responses and cytokine release (IFN-γ and IL-13) in response to the amoxicillin-modified peptides tested at 25-50 μM (50 μM; SI Range = 12-25, P<0.005) (Fig. 2D,E). Amoxicillin-modified peptide-KP3 clones 35 and 90 showed low levels of reactivity with the unmodified KP3 peptide, though, at much higher concentrations (500μM). In contrast amoxicillin-modified-KP2 peptide-responsive clones were not activated with the unmodified peptide. Dose titration of amoxicillin-modified peptides demonstrated proliferative responses at concentrations as low as 1-10 μM (Fig. 3A) and IFN-γ secretion at 0.1 μM (Fig. 3B). In addition to IFN-γ, all clones secreted the cytolytic molecule perforin, IL-10 and IL-22 following antigen treatment and expressed the chemokine receptors CCR4 and CXCR3 (Sup. Fig. 7). The amoxicillin-modified-KP3 clones also secreted IL-17.

Amoxicillin-modified-KP2 and KP3 responsive clones were assessed for reactivity with unmodified or modified positional derivatives, HSA and amoxicillin-modified HSA. Clones KP2-63, KP2-91 and KP3-35 were stimulated to proliferate with amoxicillin-modified-KP2 or –KP3, but they did not cross-react with the other peptides, proteins or free amoxicillin. Amoxicillin-modified KP3-90 clone proliferated readily in the presence of amoxicillin-modified KP3 and weakly with unmodified KP3, unmodified KP6 and amoxicillin-modified-KP6 (Fig. 4).

Cloning to amoxicillin-modified-KP5 and –KP6 resulted in 0/208 and 2/216 colonies (Fig. 5A and B). On repeated testing, a single amoxicillin-modified-KP6 clone, was found to proliferate strongly (50μM; SI=7.4; P=0.0005) in a dose-dependent manner and secrete IFN-γ and IL-13 (Fig. 5C, D). Proliferative responses and cytokine release were not observed with unmodified KP6, amoxicillin-modified or unmodified positional derivatives, amoxicillin-modified-HSA or the free drug (Fig. 5D). Interestingly, the clone expressed CD8+ and was therefore not interacting with the DR15-DQ6 haplotype. Therefore, no further investigation was performed on this amoxicillin-modified peptide-specific clone.

Structural features of the amoxicillin-modified-KP2 and KP3 peptide-specific T-cell response

The structural features of the amoxicillin-modified-KP2 and KP3 peptide-specific T-cell response was further investigated in a series of mechanistic studies. First, amoxicillin dimer-KP2 and KP3 peptides were characterised, purified and incorporated into the T-cell assay. In these peptides, the lysine residue was modified with a dimerized amoxicillin moiety (Sup. Fig.

8). This was deemed important as the dimerized adduct has been identified in patient serum (Meng *et al.,* 2016). Amoxicillin-modified-KP2 and –KP3 clones were stimulated to proliferate in the presence of peptides modified with amoxicillin and the amoxicillin dimer (Fig. 6A). Secondly, amoxicillin-modified-KP2 and -KP3 peptide-responsive clones were incubated with free drug and 100μM of the unmodified peptide to determine whether the peptide conjugate is formed in sufficient quantities under cell culture conditions to activate T-cells. T-cell proliferative responses were not detected (results not shown). Thirdly, T-cell stimulatory amoxicillin-modified-KP2 and –KP3 peptides (10μM) were co-incubated with increasing concentrations of unmodified peptide in an HLA binding competition assay. With an increase in unmodified peptide, the response to modified peptide was significantly dampened with a 4.25-fold (P=0.0046) decrease in amoxicillin-modified-KP2 clones at high concentrations of unmodified KP2. Amoxicillin-modified-KP3 clone 90 showed cross-reactivity with the unmodified KP3 and therefore, the proliferative response was only dampened by 2.1-fold (P=0.0067) in the presence of 100μM unmodified peptide. Furthermore, the proliferative response began to increase with higher concentrations (200μM) of the unmodified peptide (Fig. 6B).

Previous studies have shown strong reactivity of amoxicillin-specific T-cell clones with the structurally similar the β-lactam antibiotic ampicillin at the same drug concentrations (Kim *et al.*, 2015). Therefore, fourthly, ampicillin-modified-KP2 was generated, purified and characterised (Sup. Fig. 9), no cross reactivity was observed even at 10-fold higher concentrations of ampicillin-modified KP2 peptide (Fig. 6C). Finally, an experiment was designed to explore whether a peptide derived from HSA containing a similar HLA-

DRB1*15:01-DQB1*06:02 binding motif, and amoxicillin in the same position, could cross-react with the amoxicillin-modified-KP3 clones. Amoxicillin-modified HSA^{K204} chosen for its similarity with the KP3 peptide, was synthesized, characterised and purified (Sup. Fig. 10). The amoxicillin-modified-KP3 clones were not stimulated to proliferate in the presence of titrated concentrations of the amoxicillin-modified-HSA^{K204} peptide (Fig. 6D).

Assessment of HLA Restriction

Anti-MHC class I and II blocking antibody experiments revealed that activation of amoxicillin-modified-KP2 and -KP3-responsive clones was class II restricted (Fig. 7). While the peptides were designed to bind DRB1*15:01, the anchor amino acids could also be accommodated by the other component of the amoxicillin-clavulanate haplotype DQB1*06:02. Thus, to characterize the HLA molecules that the peptides were interacting with to activate the clones, EBV-transformed B-cells were generated from 6 healthy donors: 3 expressing the HLA risk haplotype and 3 expressing other HLA -DR and -DQ alleles. Strong proliferative responses were observed when clones (amoxicillin-modified-KP2 and -KP3 responsive) were incubated with the amoxicillin-modified peptides and autologous antigen presenting cells or antigen presenting cells from the donors expressing HLA-DRB1*15:01-DQB1*06:02. In contrast, the strength of the proliferative response with antigen presenting cells from donors negative for the HLA risk haplotype was similar to that observed in the absence of antigen presenting cells (Fig. 8A).

To determine whether the peptides were interacting with HLA-DRB1*15:01 and/or HLA-DQB1*06:02 antigen presenting cells were generated from donors positive for HLA-DRB1*15:01 but negative for HLA-DQB1*06:02 and vice versa. Amoxicillin-modified-KP2 peptide-responsive clones showed strong reactivity with peptide and antigen presenting cells expressing either risk allele. In contrast, amoxicillin-modified-KP3 peptide-responsive clones showed strong proliferative responses to the peptide only in the presence of antigen presenting cells from donors expressing DQB1*06:02 (Fig. 8B). Blocking experiments with anti-HLA-DR and –DQ antibodies confirmed activation of clones with amoxicillin-modified KP3 peptide was restricted to HLA-DQ (Sup. Fig. 11).

DISCUSSION

Amoxicillin-clavulanate is the most common cause of idiosyncratic DILI in the USA and Europe and hence the most important form of DILI to study (Andrade *et al*., 2005; Chalasani *et al*., 2008). Pharmacogenomic investigations have reported a significant association between the HLA class II haplotype DRB1*15:01-DQB1*06:02 and susceptibility to amoxicillin-clavulante-induced DILI (Hautekeete *et al.,* 1999; O'Donohue *et al.,* 2000; Donaldson *et al.,* 2010; Andrade *et al.,* 2004). Furthermore, we have isolated and characterized amoxicillin- and clavulanic acid-responsive T-cells, from patients with DILI, in terms of phenotype and function (Kim *et al.,* 2015; Yaseen *et al.,* 2015). However, unlike our previous study of flucloxacillin-induced DILI in patients expressing HLA-B*57:01 (Monshi *et al.,* 2013), we were not able to show that either amoxicillin or clavulanic acid interact with HLA-DRB1*15:01 or HLA-DQB1*06:02 to activate CD4+ T-cells. In fact, to date, it has not been possible to link HLA class II genetic associations to the activation of T-cells and the iatrogenic disease for any form of DILI. Thus, the objective of this study was to utilize fully-characterized drug-modified peptides, instead of the parent drug, as a source of antigen to probe T-cell responses and to investigate whether the peptides bind with a degree of selectivity to components of the amoxicillin-clavulante HLA class II haplotype to stimulate T-cells. We present evidence that amoxicillin-modified peptides bind to both HLA-DRB1*15:01 and HLA-DQB1*06:02 to stimulate DILI patient T-cells and describe in detail the importance of the position of nucleophilic lysine residue that amoxicillin modifies in the HLA binding peptide sequence.

The approach that we used derives from an early study by Weltzein and colleagues who synthesized benzylpenicillin-modified peptides containing anchor residues that interact with

the HLA class II binding cleft with the addition of a lysine residue to bind the drug. Multiple positional derivatives of these peptides were designed by placing lysine on different locations on the peptide backbone (Padovan *et al.,* 1997). Importantly, our peptide sequences were designed with the help of the SYFPEITHI database to contain anchor residues for both HLA-BRB1*15:01 and HLA-DQB1*06:02. In total, 4 peptides were used to synthesize amoxicillin and amoxicillin dimer adducts. Lysine residues for amoxicillin modification were located in positions 2,3, 5 and 6 with respect to the leucine anchor (Fig. 1).

Amoxicillin-modified-KP2 and –KP3 CD4+ clones isolated from the patient with liver injury were stimulated to proliferate and secrete IFN-γ in a dose-dependent manner in the presence of the modified peptides. Interestingly, position 2 and 3 have been proposed as critical T-cell receptor contact sites in previous studies exploring the immunogenicity of myelin basic protein-derived peptides in HLA-DRB1*15:01+ patients with multiple sclerosis (Wucherpfennig *et al.,* 1994). Amoxicillin-modified-KP2 peptide-responsive clones were not activated with the unmodified peptide, whereas very low levels of proliferation and IFN-γ release were observed with the amoxicillin-modified-KP3 peptide-responsive clones. Therefore, the carrier peptide must form part of the antigenic determinant for certain clones. The hapten could sit above the HLA binding peptide when interacting with T-cell receptors or alternatively be accommodated in a pocket of the HLA binding groove where it would induce steric stress and the possible presentation of peptides in an unnatural conformation. The dimerized amoxicillin-modified peptide proved a useful tool to investigate the HLA binding interaction as it is unlikely that the large drug structure (approximately the size of 5 amino acids) could be accommodated in an HLA pocket. As the dimerized peptide adduct stimulated

all amoxicillin-modified peptide-responsive clones to proliferate it is probable that the drug component of the amoxicillin-modified peptide protrudes from the HLA molecule and faces the T-cell receptor. Collectively, these data confirm that drug hapten modification of peptides generates novel antigenic determinants for T-cells as described originally by Padovan *et al* (1997) and more recently by Azoury et al (Azoury *et al.,* 2018) with benzyl penicillin. However, they also generate the concept that hapten modification enhances the intrinsic immunogenicity of endogenous peptides.

CD4+ clones reactive towards amoxicillin-modfiied-KP5 and –KP6 peptides were not detected. However, one CD8+ clone was stimulated with the drug-modified-KP6 peptide. These data raise the intriguing possibility that drug-modified HLA class II binding peptides also activate CD8+ cells in a truncated or spliced form following antigen processing through modification of HLA class I molecules. Furthermore, the patient is also positive for HLA-A*0201, which is part of the wider risk haplotype. It is plausible that this peptide, could be presenting via this allele. It would be interesting to study the structure of the peptide that activates CD8+ T-cells; however, this was beyond the scope of the current investigations.

Cross-reactivity studies were performed to explore the nature of the antigenic epitope that interacts with the CD4+ T-cells. Interestingly, none of the amoxicillin-modified peptide-responsive clones were activated with soluble amoxicillin. This differed from the observations of Padovan et al using T-cells from benzylpenicillin allergic patients where drug-modified peptide-specific T-cells were also activated with the free drug (Padovan *et al.,* 1997). Peptide sequences chosen for this study were synthetic using a simple alanine backbone, an

amoxicillin-modifiable lysine residue and relevant anchor amino acids to mimic the HLA binding interaction of multiple natural peptides to allow us to detect as many amoxicillin-modified peptide-specific T-cells as possible. We did synthesize a natural amoxicillin-modified HSA-derived HLA binding peptide with similar anchor residues to our designer KP3 peptide. However, this peptide did not activate the clones. Thus, in on-going experiments we are beginning to elute the thousands of HLA class II bound peptides from amoxicillin-treated antigen presenting cells to characterize the drug-modified and unmodified immunopeptidome. These studies will allow us to identify natural amoxicillin-modified HLA class II binding peptides for a more detailed assessment of T-cell reactivity.

The lack of cross-reactivity between amoxicillin-modified-KP2 peptide-responsive and amoxicillin-modified-KP3 peptide-responsive clones indicate that T-cell activation is highly specific with respect to the drug position within the HLA peptide binding groove. Furthermore the replacement of amoxicillin with ampicillin in the KP2 peptide did not activate the clones. The only difference in the structures of ampicillin and amoxicillin is a single hydroxyl group.

HLA-restriction studies were conducted to investigate the role of the risk haplotype in the CD4+ T-cell response to the amoxicillin-modified peptides. All clones were activated with the amoxicillin-modified peptides in the presence of antigen presenting cells expressing the risk haplotype, whereas with donors expressing other HLA-DRB1 and -DQB1 alleles, the strength of the proliferative response was similar to that seen in the absence of antigen presenting cells. Moreover, using antigen presenting cells expressing only one component of the HLA risk haplotype and HLA class II sub-type blocking experiments, we demonstrated that clones

responsive towards amoxicillin-modfiied-KP2 peptide may be activated through an interaction with DQB1*06:02 as well as DRB1*1501, while clones responsive towards amoxicillin-modfiied-KP3 peptide require a selective interaction with DQB1*06:02. HLA allele restriction was not observed in previous experiments with CD4+ T-cell clones responsive toward soluble amoxicillin (Kim *et al.,* 2015; Yaseen *et al.,* 2015). This difference presumably relates to the high milli-molar concentrations of amoxicillin used to activate T-cells, which will either saturate the binding cleft of multiple HLA class II molecules and/or modify numerous proteins that contain peptide sequences for multiple HLA class II molecules. Unfortunately, patients and volunteers were not screened for the DRB5 allele, of which DRB5*01:01 is also a known component of the wider risk haplotype. While it is plausible these peptides may also be able to interact with this allele, reports have demonstrated a low degree of overlap in the peptide repertoires for DRB1*15:01 and DRB5*01:01 (Scholz *et al.,* 2017). However, this would be an area of interest for future studies to better understand the risk haplotype.

HLA is thought to be one of factors influencing susceptibility to DILI, and while certain patients are carriers of the HLA risk allele(s), many carriers of the allele(s) will not have liver injury and there will be some who have liver injury but are not carriers of the allele(s). T-cell activation and differentiation is highly influenced by regulatory pathways involving receptor ligand interactions (e.g., PD-1, CTLA-4), Tregs and cytokines with certain cytokines providing a shift towards certain cellular phenotypes. An analysis of the secretory molecules release by the amoxicillin-modified peptide-responsive CD4+ clones identified the cytolytic molecule perforin and alongside the regulatory cytokine IL-10. While it is atypical for CD4+ clones to have cytolytic properties, aberrant expression of MHC Class II on hepatocytes has been

observed in the context of autoimmune disease (Herkel *et al.,* 2003). As such, it is plausible that this could provide some clinical relevance for a CD4 mediated process for hepatotoxicity and a reason for the class II association. The analysis of MHC class II expression in liver biopsies from hypersensitive patients would be an interesting avenue of further study. Furthermore, amoxicillin-modified-KP2 responsive cells were IL-22 positive and IL-17 negative, which alludes to a Th22 phenotype, while the amoxicillin-modified-KP3 responsive cells were IL-22 positive and IL-17 positive indicative of a Th17 phenotype. IL-17 induces an inflammatory response while IL-22 may be involved in regeneration (Eyerich *et al.,* 2010). An analysis of cell surface chemokine receptor expression shows a similar profile between the clones with an upregulation of CCR4 which is involved in allergic inflammation and also CXCR3 which is expressed in response to an adaptive immune response. CXCR3 is also a key receptor upregulated in the liver (Oo *et al.,* 2010). Ultimately, it would be interesting to see the role that these cells play in the wider context of drug allergy adding to the growing list of aberrant T-cell responses observed.

To summarize, our data provide proof-of-concept evidence that drug haptenated peptides bind selectively to HLA class II risk alleles associated with a clinically relevant form of idiosyncratic DILI to activate CD4+ patient T-cells. Furthermore, we have defined the nature of the T-cell response with respect to drug structure and the position of the hapten modification on the HLA binding peptide. While our designer peptides are not derived from natural proteins they represent an important tool to identify antigenic determinants and ultimately define drug-peptide 'toxicophores' involved in the patient T-cell response. Also, the availability of powerful bioinformatics tools such as PROSITE, can help match these

'toxicophores' to natural proteins which contain these epitopes. Now that it has been established that amoxicillin-modified HLA-DRB1*15:01-DQB1*06:02 binding peptides activate T-cells, further structural analyses that are ongoing will help elucidate the nature of the receptor binding interaction. Furthermore, HLA class II tetramers incorporating the amoxicillin-modified peptide sequences are being developed for flow cytometry-based assays to quantify antigen-specific T-cell numbers in naïve, tolerant and hypersensitive patients and for the rapid diagnosis of this drug-specific iatrogenic disease.

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List of abbreviations: drug-induced liver injury, DILI; peripheral blood mononuclear cells, PBMC; Epstein-Barr virus, EBV, IL, interleukin.

FIGURE LEGENDS

Figure 1. Sequences of amoxicillin-modified HLA-binding peptides. (A) Criteria for the design and construct of drug-modified peptides. (B) Sequences of four drug-modified peptides with lysine in different locations called positional derivatives. Amoxicillin-modified peptides were named according to the location of lysine. Anchor residues were derived from the SYFPEITHI database.

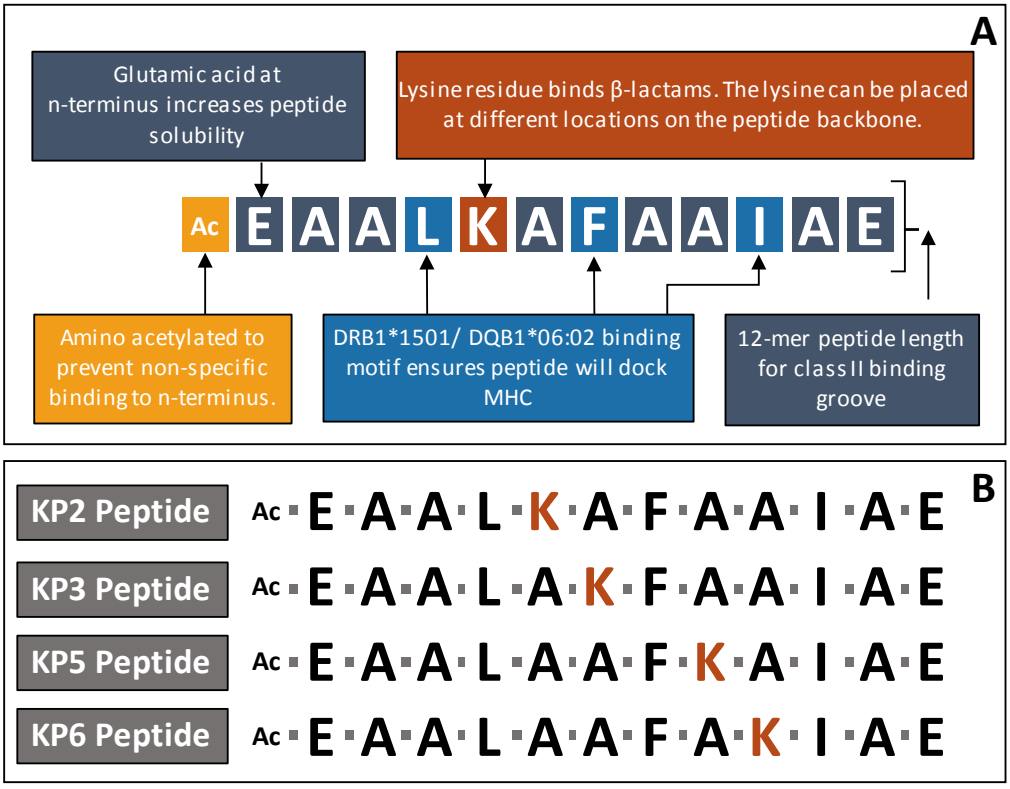
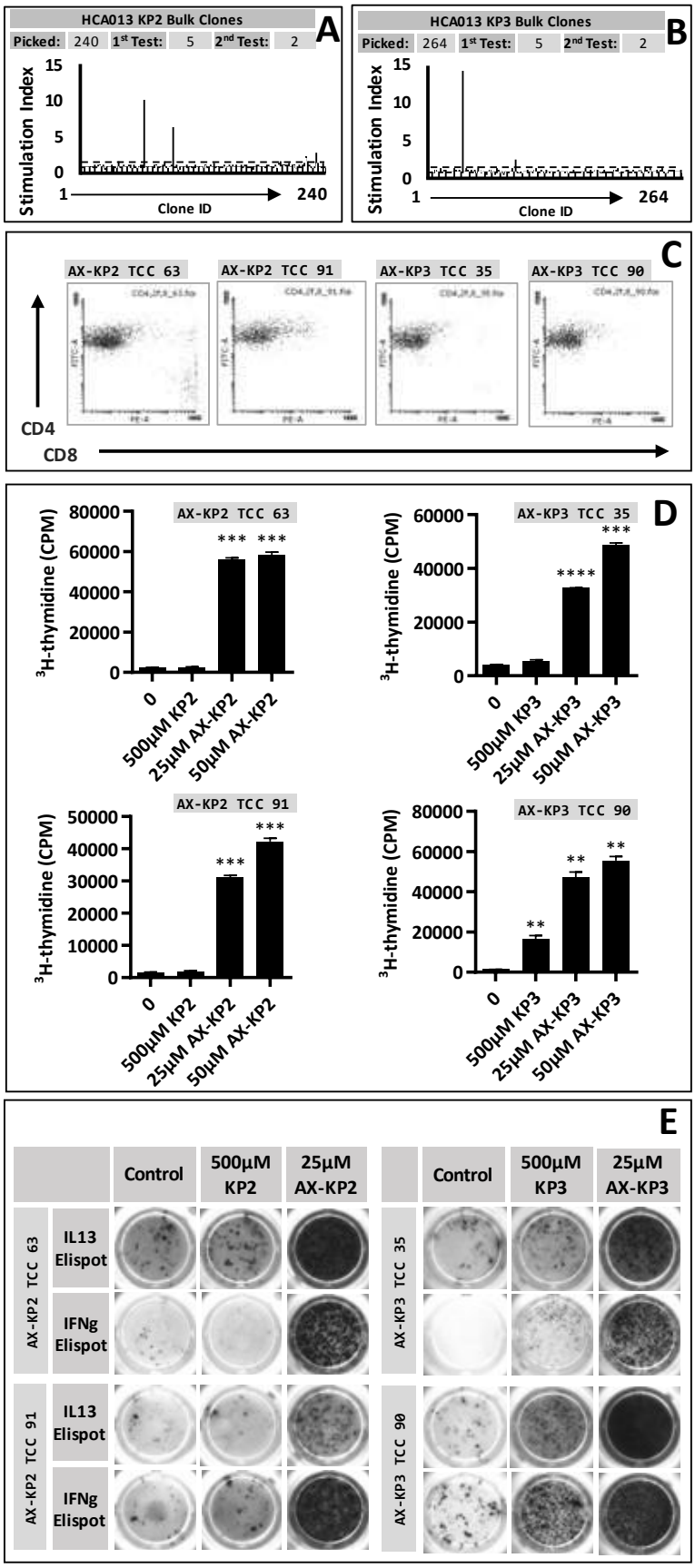


Figure 2. CD4+ T-cell clones responsive towards amoxicillin-modified KP2 & KP3 peptides. PBMC were cultured individually with the peptides for 14 days prior to cloning by serial dilution and repetitive mitogen stimulation. Proliferation of T-cell clones with (A) amoxicillin-modified KP2 or (B) amoxicillin KP3 peptides and irradiated autologous EBV-transformed B-cells as antigen presenting cells. This initial experiment was conducted in duplicate. Proliferative responses were measured using [^{3}H]thymidine and recorded as a stimulation index (proliferation in test incubations with drug/ proliferation in control incubations with medium alone) and SI > 1.5 deemed a positive response. (C) Phenotype characterisation of amoxicillin-modified peptide-specific clones using flow cytometry. (D) Proliferation of amoxicillin-modified KP2 and KP3 peptide specific T-cell clones with amoxicillin-modified and unmodified KP2 and KP3 peptides. Clones were incubated with modified or unmodified peptides and irradiated autologous antigen presenting cells for 48h. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures. (E) IFN-γ and IL-13 cytokine secretion from amoxicillin-modified peptide-specific T-cell clones. Clones were incubated with amoxicillin-modified or unmodified peptides and cytokine secretion was visualized using ELIspot.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240
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264
C
AX-KP2 TCC 63
AX-KP2 TCC 91
AX-KP3 TCC 35
AX-KP3 TCC 90
CD4
CD8
D
AX-KP2 TCC 63
AX-KP3 TCC 35
AX-KP2 TCC 91
AX-KP3 TCC 90
3H-thymidine (CPM)
0
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25μM AX-KP2
50μM AX-KP2
500μM KP3
25μM AX-KP3
50μM AX-KP3
E
Control
500μM KP2
25μM AX-KP2
500μM KP3
25μM AX-KP3
IL13 Elispot
IFNg Elispot
AX-KP2 TCC 63
AX-KP2 TCC 91
AX-KP3 TCC 35
AX-KP3 TCC 90

Figure 3. Dose-dependent stimulation of CD4+ T-cell clones with amoxicillin-modified KP2 and KP3 peptides. Stimulation of the clones was measured using [^{3}H]thymidine and ELIspot for the detection of (A) proliferation and (B) IFN-γ secretion, respectively.

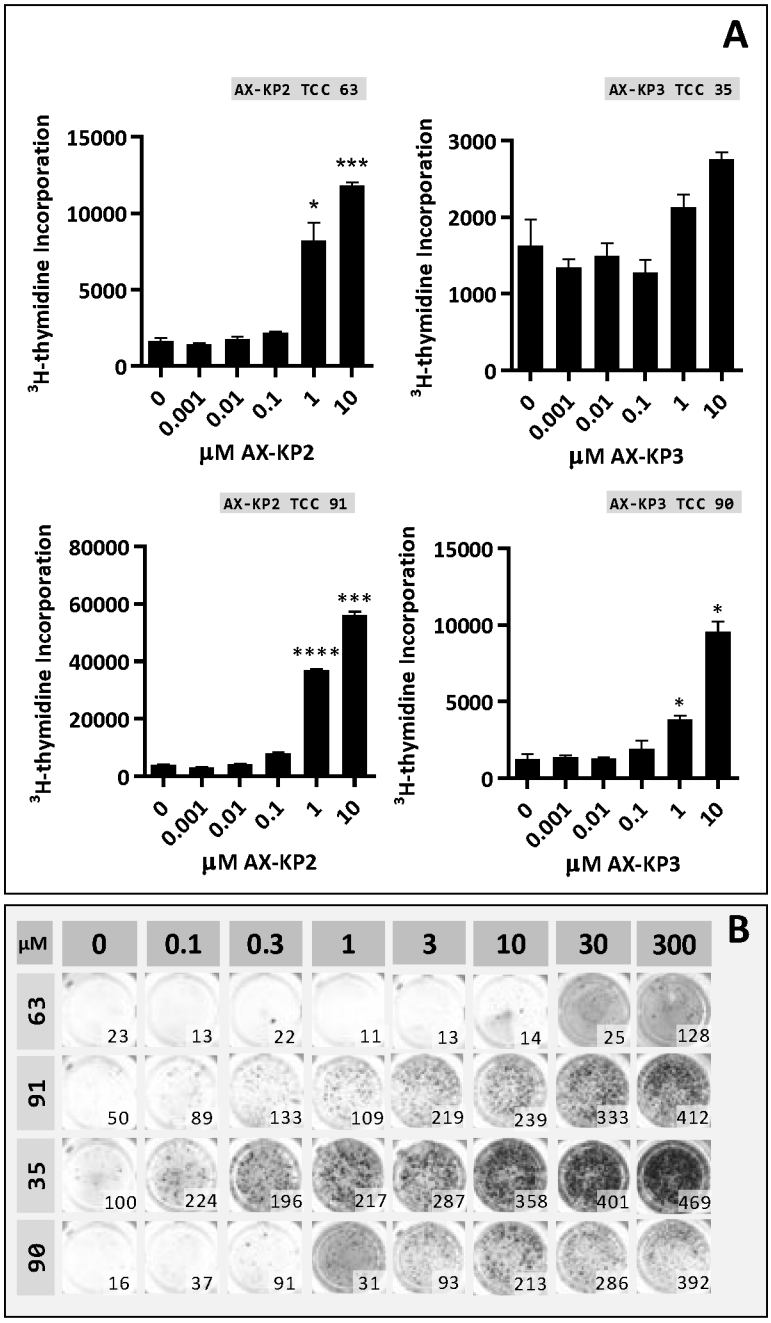


Figure 4. Cross reactivity of amoxicillin-modified KP2 and KP3 responsive CD4+ T-cell clones. Clones were incubated with modified or unmodified KP2-6 peptides, amoxicillin, HSA or amoxicillin-modified HSA and irradiated autologous antigen presenting cells for 48h. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures.

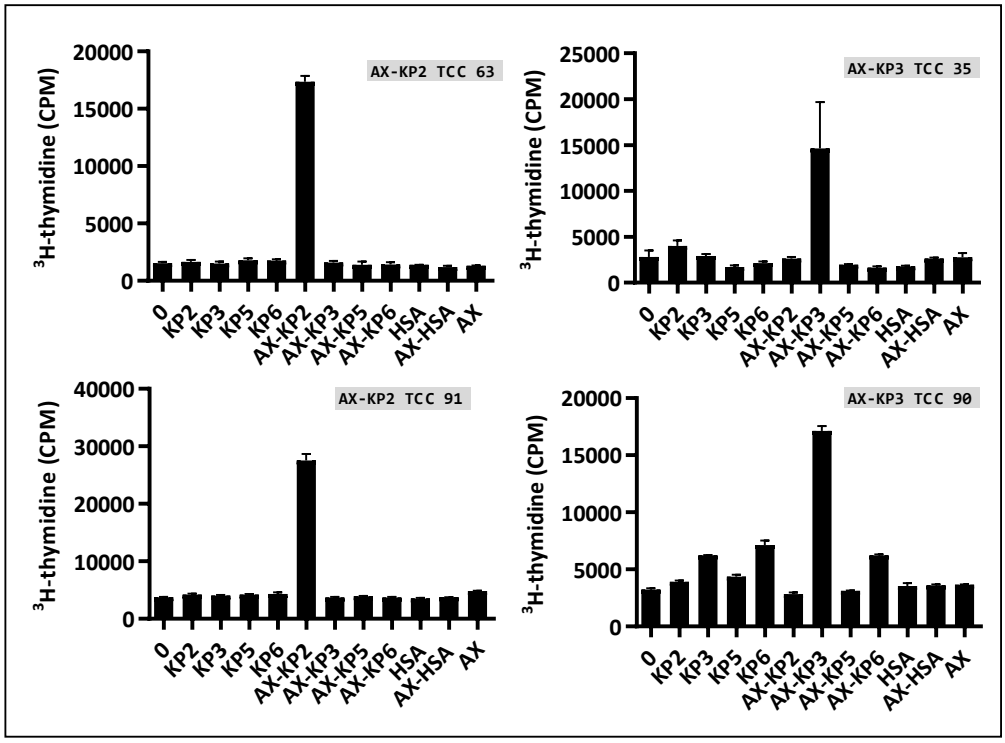
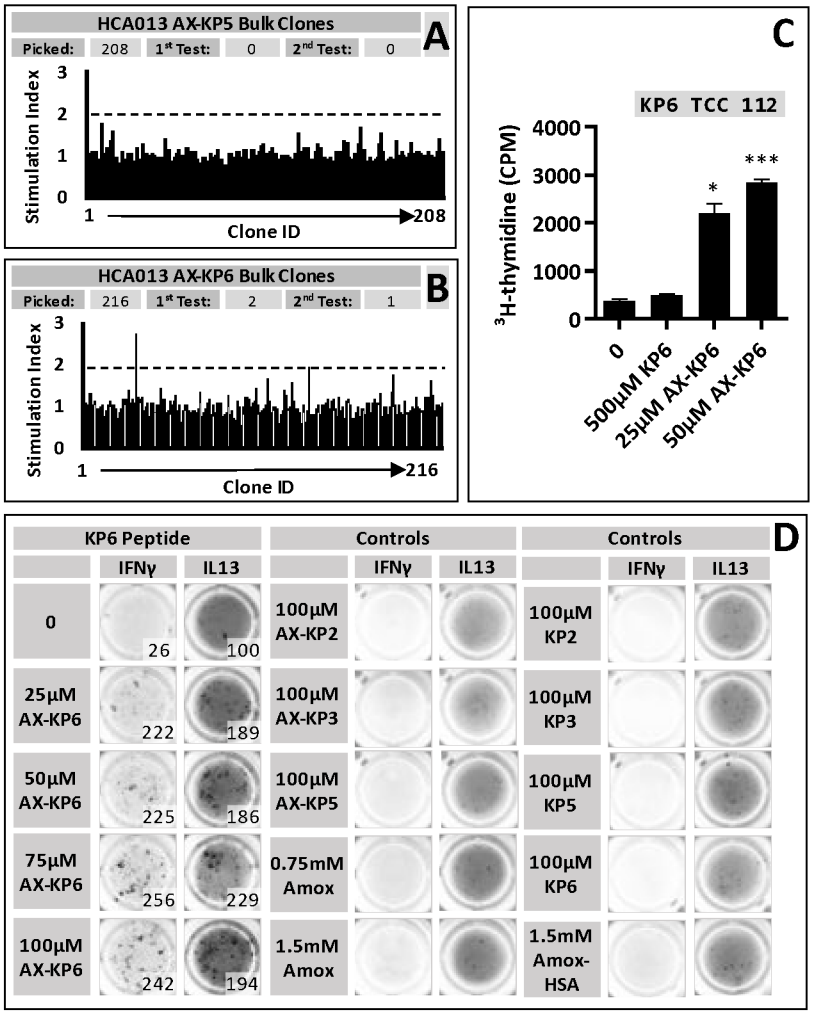


Figure 5. CD8+ T-cell clone responsive towards amoxicillin-modified KP6 peptide. PBMC were cultured individually with the amoxicillin-modified KP5 and KP6 peptides for 14 days prior to cloning by serial dilution and repetitive mitogen stimulation. Proliferation of T-cell clones with (A) amoxicillin-modified KP5 or (B) amoxicillin KP6 peptides and irradiated autologous EBV-transformed B-cells as antigen presenting cells. This initial experiment was conducted in duplicate. Proliferative responses were measured using [^{3}H]thymidine and recorded as a stimulation index (proliferation in test incubations with drug/ proliferation in control incubations with medium alone) and SI > 1.5 deemed a positive response. (C) Proliferation of amoxicillin-modified KP6 peptide-specific T-cell clone with amoxicillin-modified and unmodified KP6 peptide. Clones were incubated with modified or unmodified peptides and irradiated autologous antigen presenting cells for 48h. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures. (D) IFN-γ and IL-13 cytokine secretion from the amoxicillin-modified peptide-specific T-cell clone. Clones were incubated with amoxicillin-modified or unmodified peptides and cytokine secretion was visualized using ELIspot.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Stimulation Index
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Picked: 216
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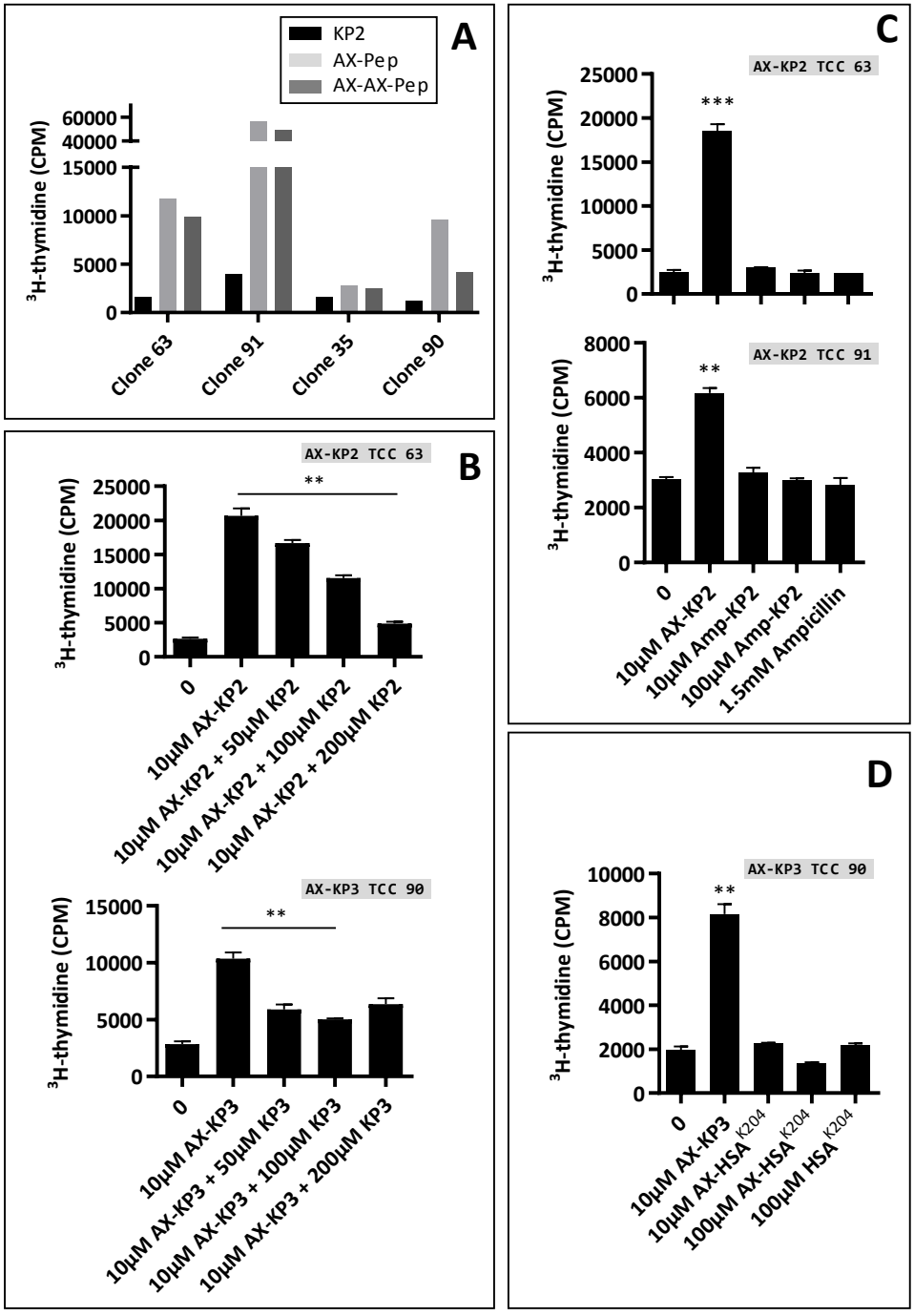
B
Stimulation Index
Clone ID
1
216
C
KP6 TCC 112
3H-thymidine (CPM)
0
500µM KP6
25µM AX-KP6
50µM AX-KP6
D
KP6 Peptide
Controls
Controls
IFNγ
IL13
0
25µM AX-KP6
50µM AX-KP6
75µM AX-KP6
100µM AX-KP6
26
100
222
189
225
186
256
229
242
194
100µM AX-KP2
100µM AX-KP3
100µM AX-KP5
0.75mM Amox
1.5mM Amox
100µM KP2
100µM KP3
100µM KP5
100µM KP6
1.5mM Amox-HSA

Figure 6. Structural features of the amoxicillin-modified-KP2 and KP3 peptide-specific CD4+ T-cell response. Activation of amoxicillin-modified-KP2 or KP3 peptide-specific T-cell clones with (A) the amoxicillin dimer-modified KP2 peptide, (C) the ampicillin-modified KP2 peptide and (D) the amoxicillin-modified peptide derived from HSA-K^{204} with similar anchor positions to KP3. Clones were incubated with or without the peptides and antigen presenting cells for 48h. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures. (B) Peptide binding competition assay. Amoxicillin-modified peptide specific T-cell clones were cultured with the amoxicillin-modified peptides (10μM) in the presence of increasing concentration of unmodified peptide for 48h. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures.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AX-AX-Pep
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40000
15000
10000
5000
0
Clone 63
Clone 91
Clone 35
Clone 90
B
AX-KP2 TCC 63
**
^{3}H-thymidine (CPM)
25000
20000
15000
10000
5000
0
0
10μM AX-KP2
10μM AX-KP2 + 50μM KP2
10μM AX-KP2 + 100μM KP2
10μM AX-KP2 + 200μM KP2
AX-KP3 TCC 90
**
^{3}H-thymidine (CPM)
15000
10000
5000
0
0
10μM AX-KP3
10μM AX-KP3 + 50μM KP3
10μM AX-KP3 + 100μM KP3
10μM AX-KP3 + 200μM KP3
C
AX-KP2 TCC 63
***
^{3}H-thymidine (CPM)
25000
20000
15000
10000
5000
0
AX-KP2 TCC 91
**
^{3}H-thymidine (CPM)
8000
6000
4000
2000
0
0
10μM AX-KP2
10μM Amp-KP2
100μM Amp-KP2
1.5mM Ampicillin
D
AX-KP3 TCC 90
**
^{3}H-thymidine (CPM)
10000
8000
6000
4000
2000
0
0
10μM AX-KP3
10μM AX-HSA K204
100μM AX-HSA K204
100μM HSA K204

Figure 7. HLA-restricted activation the amoxicillin-modified-KP2 and KP3 peptide-specific CD4+ T-cell clones. Amoxicillin-modified peptide-specific clones were incubated with the drug-modified peptides and antigen presenting cells in the presence of HLA class I and class II blocking antibodies or relevant isotype control for 48h. IFN-γ secretion was visualized by ELIspot.

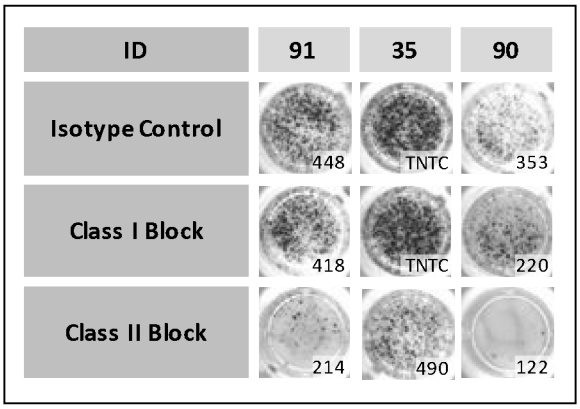

Figure 8. HLA-DR15-DQ6 restricted activation the amoxicillin-modified-KP2 and KP3 peptide-specific CD4+ T-cell clones. (A) Amoxicillin-modified peptide-specific clones were incubated with the drug-modified peptides and antigen presenting cells containing the DR15-DQ6 haplotype or haplotype negative controls. (B) Amoxicillin-modified peptide-specific clones were incubated with the drug-modified peptides and antigen presenting cells expressing either HLA-DR15 or -DQ6. Proliferative responses were measured using [^{3}H]thymidine and recorded as mean ± SD cpm of triplicate cultures.

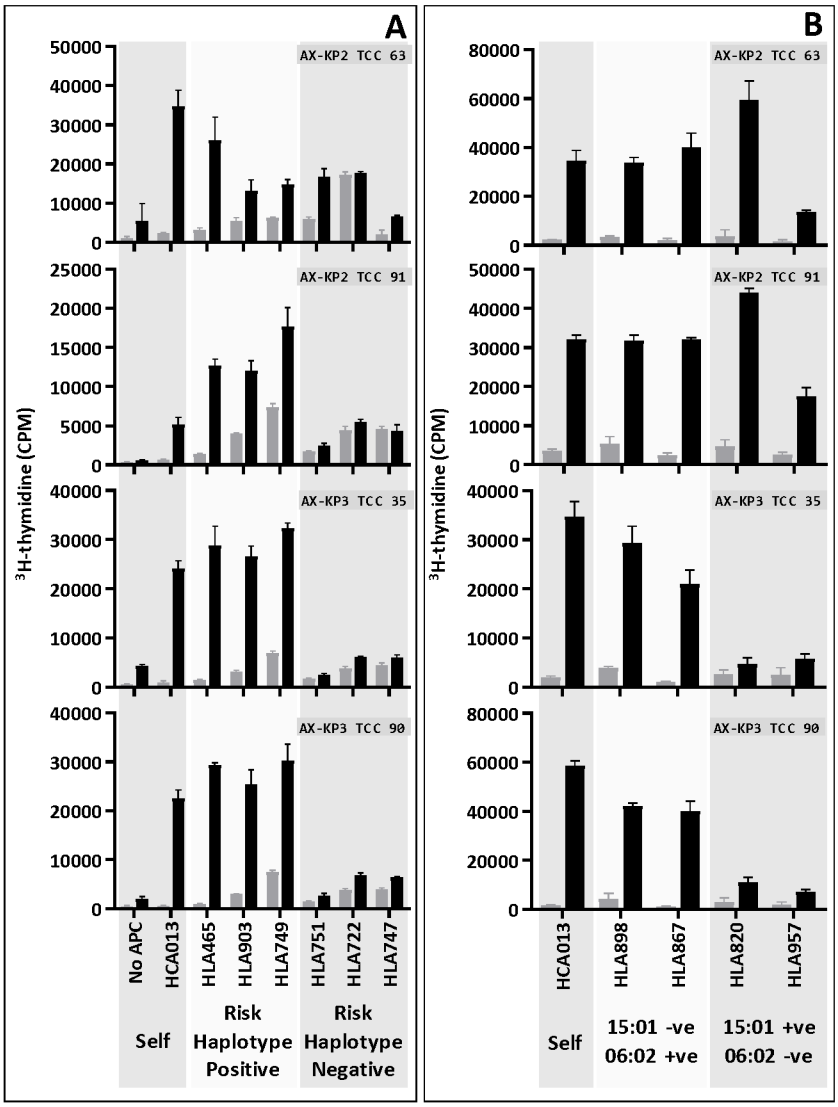

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Figure 1

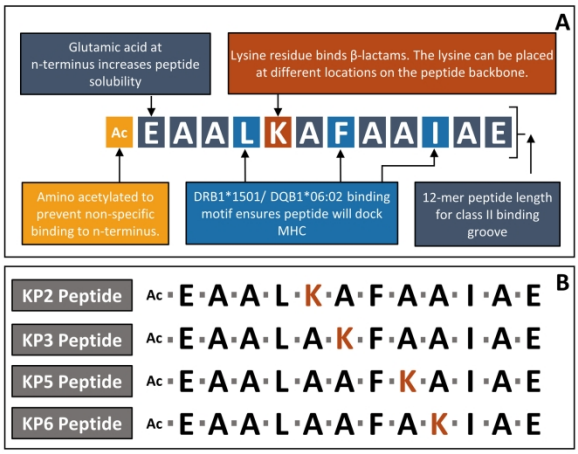

Figure 1

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Figure 2

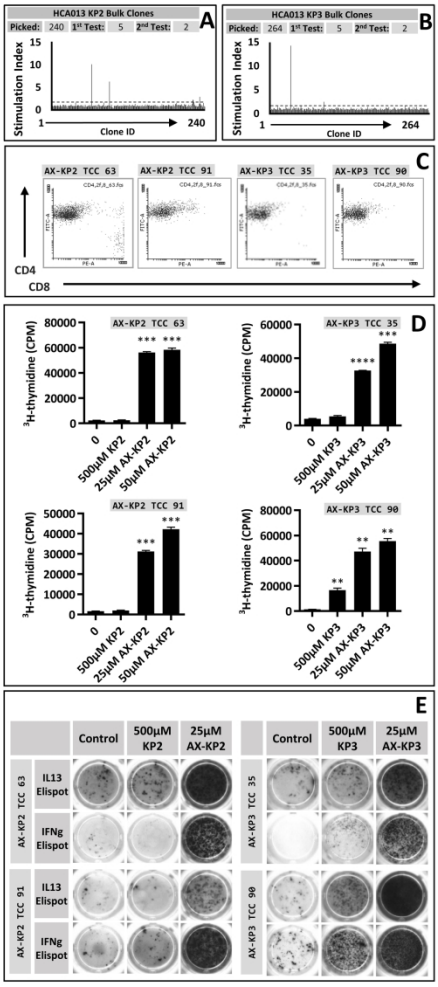

Figure 2

209x297mm (300 x 300 DPI)

Figure 3

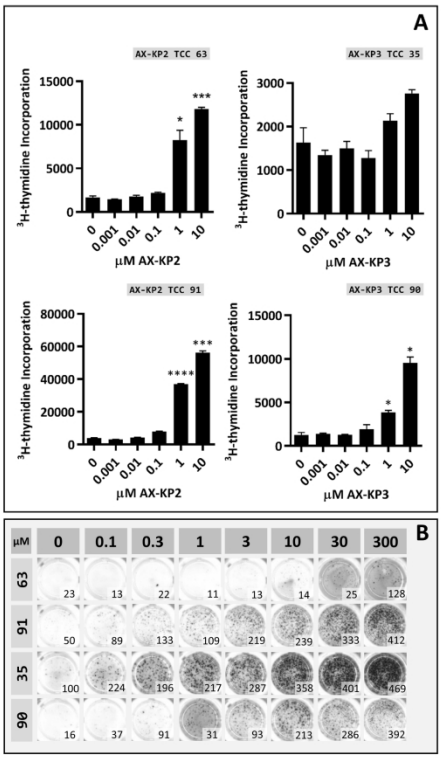

Figure 3

209x297mm (300 x 300 DPI)

Figure 4

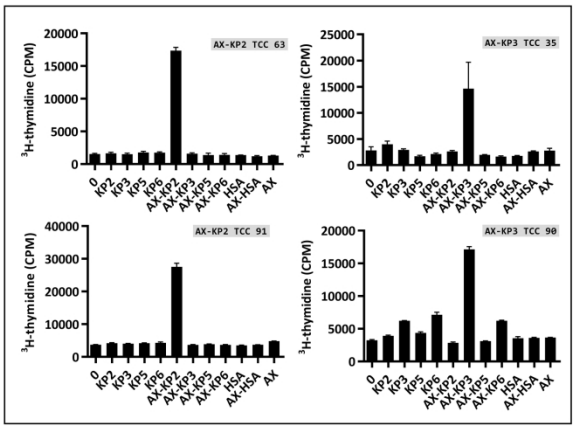

Figure 4

209x297mm (300 x 300 DPI)

Figure 5

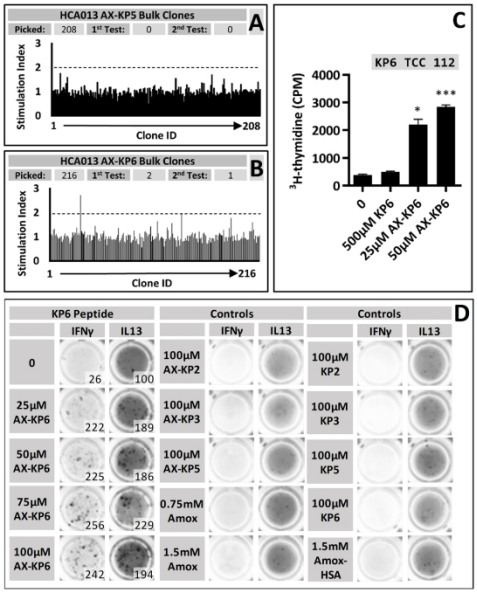

Figure 5

209x297mm (300 x 300 DPI)

Figure 6

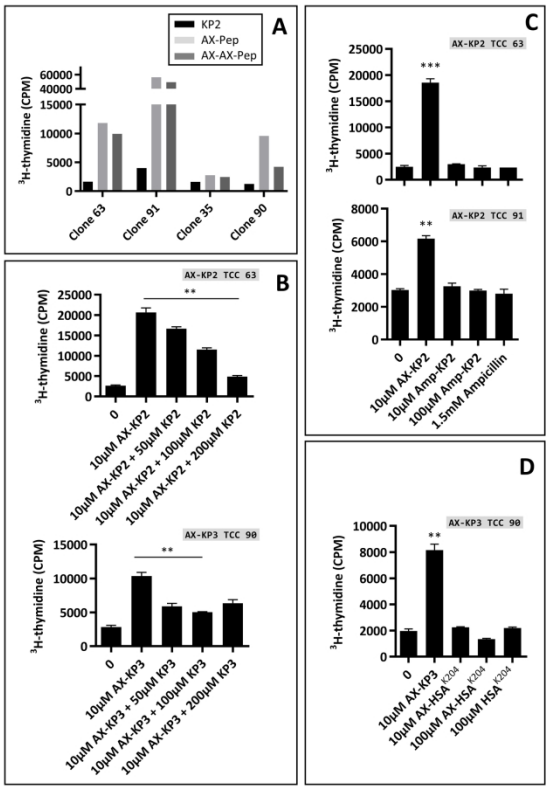

Figure 6

209x297mm (300 x 300 DPI)

Figure 7

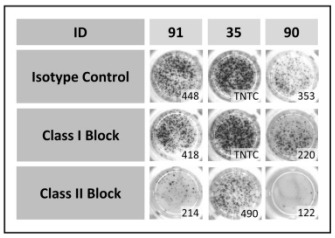

Figure 7

209x297mm (300 x 300 DPI)

Figure 8

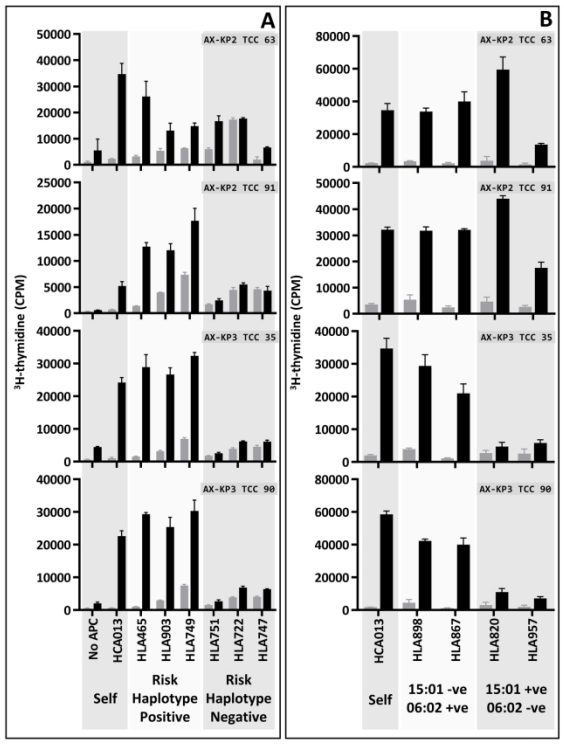

Figure 8

209x297mm (300 x 300 DPI)