Definition of the nature and hapten threshold of the β-lactam antigen required for T cell activation *in vitro* and in patients[1]

Running title: T-cell responses to low molecular weight compounds

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Abstract

Covalent modification of protein by drugs may disrupt self-tolerance leading to lymphocyte activation. Determination of the threshold required for this process has not hitherto been possible. We have therefore performed quantitative mass spectrometric analyses to define the epitopes formed in tolerant and hypersensitive patients taking the β-lactam antibiotic piperacillin and the threshold required for T-cell activation. A hydrolysed piperacillin hapten was detected on 4 Lys residues of HSA isolated from tolerant patients. The level of modified Lys541 ranged from 2.6-4.8%. Analysis of plasma from hypersensitive patients revealed the same pattern and levels of modification 1-10 days after commencement of therapy. Piperacillin-responsive skin-homing CD4+ clones expressing an array of Vβ receptors were activated in a dose, time and processing-dependent manner; analysis of incubation medium revealed that 2.6% of Lys541 in HSA was modified when T-cells were activated. Piperacillin-HSA conjugates that had levels and epitopes identical to those detected in patients were shown to selectively stimulate additional CD4+ clones, which expressed a more restricted Vβ repertoire. To conclude, the levels of piperacillin-HSA modification that activated T-cells are equivalent to the ones formed in hypersensitive and tolerant patients, which indicates that threshold levels of drug antigen are formed in all patients. Thus, the propensity to develop hypersensitivity is dependent on other factors such as on the presence of T-cells within an individual's repertoire that can be activated with the β-lactam hapten and/or an imbalance in immune regulation.

Introduction

Human exposure to drug haptens results in a high prevalence of T-cell-mediated reactions often referred to as drug hypersensitivity (1). It has been postulated that covalent binding of the hapten to protein and the formation of neoantigens represents a crucial initiating event in the iatrogenic disease (2); however, there is a need to develop sensitive methods to determine the quantity of drug protein adducts formed in patients and to explore whether such adducts act as antigens to activate patient T-cells.

β-lactam antibiotics are the most common cause of drug hypersensitivity. They form adducts with protein through an irreversible covalent bond and as such represent an ideal drug class to investigate the relationship between hapten protein binding and induction of an immune response. For adduct formation, the β-lactam ring is targeted by reactive lysine residues in protein (3). Through evolution of bioanalytical technologies, mainly protein mass spectrometry, it has been possible to probe the nature of the drug protein interaction in greater detail. β-lactam antibiotics bind to extracellular protein, in particular human serum albumin (HSA),[2] with a degree of selectivity (4-9). Adduct formation on HSA is time- and concentration-dependent and modifications are detected at fewer than 10% of available nucleophilic lysine residues. The selective binding interaction does not only relate to pKa and therefore reactivity of the side-chain amino group as most adducts form at or around Sudlow sites, which are hydrophobic pockets involved in the non-covalent binding of drugs and endogenous molecules (10,11).

Preliminary studies indicated that T-cells from patients with hypersensitivity can be activated with β-lactam-HSA adducts and synthetic designer peptides modified with β-lactam haptens (12,13). It is well-known that antigen dose plays a crucial role in the severity of the

hypersensitive phenotype (14,15) and in determining the characteristics of the responding T-cell repertoire (16,17). Quantitative assessment of β-lactam hapten density on protein has been attempted previously (18,19); however, established approaches lack sensitivity and are unable to accurately monitor the level of β-lactam adduct formed. The use of liquid chromatography coupled with mass spectrometry (LC-MS) together with suitable internal standards is now the most widely accepted technique for quantification purposes. Therefore, the purpose of this study was to develop and utilize mass spectrometric methods to quantify the level of β-lactam protein binding in tolerant and hypersensitive patients and to define the association between adduct exposure and the drug-specific T cell response. To address this objective, T-cell clones were generated using PBMC cultured with parent drug, which forms conjugates with multiple proteins in culture (4,20) and a synthetic conjugate using a single protein carrier (HSA). This allowed us to analyse T-cell receptor usage, chemokine receptor expression and cross-reactivity. The study focused on piperacillin, an intravenous β-lactam antibiotic often administered to patients with cystic fibrosis for the treatment of recurrent respiratory infections. Hypersensitivity reactions have been reported to develop in approximately 30% of patients exposed to multiple courses of the drug (21). Moreover, we have recently reported on (1) the profile of drug protein conjugation at specific lysine residues with respect to dose and incubation time, (2) the formation of two forms of drug hapten (with intact or hydrolysed 4-ethyl-2,3-dioxopiperazine ring; Figure 1A), and (3) the presence of piperacillin hapten-specific CD4+ T-cells in approximately 75% of hypersensitive patients (7,8). Thus, piperacillin represents the ideal candidate to investigate the quantitative relationship between adducts formed in the circulation of patients and that required to activate T-cells *in vitro*.

Materials and methods

Patient details

Plasma samples were isolated from blood of ten tolerant patients with cystic fibrosis prior to piperacillin exposure and immediately after a standard 14 day treatment course (4.5g qds) and three piperacillin hypersensitive patients at the time the reaction was diagnosed. Clinical characteristics of the patients are summarized in Table 1. Plasma was aliquoted stored at -80^{o}C immediately after isolation for characterization and quantification if piperacillin hapten HSA adducts.

PBMC were also isolated from blood of 3 patients with historical delayed-onset piperacillin-mediated hypersensitivity. The patients' demographics, clinical features of reactions, skin testing and lymphocyte transformation test results are summarized in Table 2. Approval for the study was obtained from the Leeds local research ethics committee and informed written consent was received from participants prior to inclusion in the study.

Detection of piperacillin-specific PBMC responses

Proliferation of hypersensitive patients' PBMC (0.15 X10^{6} per well) against piperacillin (62.5–2000 μM) and tetanus toxoid (5 μg/ml; positive control) was measured using the lymphocyte transformation test (8). Proliferative responses were measured by the addition of [^{3}H]thymidine for the final 16h of the assay. Cells were cultured in RPMI 1640 medium, containing 10% AB serum, 100mM L-glutamine, 25mM HEPES, and 25μg/ml transferrin. 100μg/ml.

Synthesis of piperacillin-modified peptides

Our previous studies showed that selective modification of Lys541 was observed at low piperacillin concentrations, whereas at higher concentrations up to 13 lysine residues were modified, four of which (Lys190, 195, 432 and 541) were detected in patients' plasma. Thus, we developed a method to synthesize ATK(piperacillin)EQLK; an amino acid sequence incorporating Lys541 in HSA. Synthesis of the piperacillin-modified peptide was achieved by Fmoc chemistry in solution phase. Amino acid side chain protection was effected by the following: triphenilmethyl for glutamine; tert-butyl for aspartic acid, glutamic acid, and threonine; and benzyloxycarbonyl (Z) for C-terminus lysine. The synthesis was initiated with lys(Z)-OBn salt as shown in Figure 1. The coupling reactions were activated by means of addition of N,N'-dicyclohexylcarbodiimide, in the presence of 1-hydroxybenzotriazole and a base such as N-methylmorpholine. The Fmoc deprotection step was accomplished twice with 20% piperidine in chloroform for 10 min. The efficiency of these reactions was evaluated by the ninhydrin colorimetric reaction. Once the synthesis was complete, the deprotection processes were carried out following a series of sequential steps: firstly, benzyl or Z groups were removed by catalytic transfer hydrogenation; secondly, Fmoc was removed with 50% diethylamine in acetonitrile; and finally, cold diethyl ether was added to precipitate the peptide. The crude peptide was purified by semi-preparative HPLC on a Jupiter C18 column (10μm C18, 250 mm × 10mm, Phenomenex, Macclesfield, Cheshire, U.K.) with a linear gradient of 95-50% solvent A (0.05% trifluoroacetic acid in water) in solvent B (0.05% trifluoroacetic acid in acetonitrile) over 30 min at a flow rate of 5mL/min. The purity of peptide was determined by UV spectroscopy and the structure was characterised by MS/MS analysis. The 1H NMR spectra were recorded in $CDCl_3$ or MeOD at 400 MHz on a Bruker Advance NMR spectrometer.

Isolation of HSA from plasma

HSA was isolated by affinity chromatography. In brief, a POROS anti-HSA affinity cartridge (Applied Biosystems, Foster City, CA, USA) attached to a Vision Workstation (Applied Biosystems) was used to affinity capture HSA which was then eluted with 12 mM hydrochloric acid. Protein was methanol precipitated, and analysed by reversed phase LC-MS.

Mass spectrometric analysis of piperacillin-modified HSA

Analyses were performed on a 5500 QTRAP® hybrid triple-quadrupole/linear ion trap instrument with Nanospray® II source (AB SCIEX, Foster City, CA, USA) and automated in-line liquid chromatography (U3000 HPLC system, 5 mm C18 nano-precolumn and 75 µm x 15 cm C18 PepMap column, Dionex) via a 10 µm inner diameter PicoTip emitter (New Objective). A gradient from 2% ACN/0.1% formic acid (v/v) to 50% ACN/0.1% formic acid (v/v) in 70 min was applied at a flow rate of 300 nL/min. The ionspray potential was set to 2200-3500 V, the nebulizer gas to 18 and the interface heater to 150 ^{o}C. MRM transitions specific for drug modified peptides were selected as follows: the m/z values for all possible modified peptides with a missed cleavage at the modified lysine residue were used together with a fragment mass of 160 corresponding to the cleaved thiazolidine ring of the drug. MRM transitions were acquired at unit resolution in both the Q1 and Q3 quadrupoles to maximize specificity. They were optimized for collision energy and collision cell exit potential, and the dwell time was 50 ms. MRM survey scans were used to trigger enhanced product ion scans of piperacillin-modified peptides, with Q1 set to unit resolution, dynamic fill selected, and dynamic exclusion for 20 s. Notwithstanding the disparity in the ionisation efficiency of the peptides, relative MRM peak areas for each of the modified peptides were determined by MultiQuant software version 2.0 (AB SCIEX). The total ion count for the whole digest for

each sample was normalised to an internal synthetic peptide ATKEQLK that contains unmodified K541: in this way, the MRM signals were adjusted for differences in sample loading on-column. Relative quantification of modified peptides was performed by comparing the relative normalized MRM peak areas for each of the modified residues across samples. To achieve the absolute quantity of piperacillin modification formed *in vitro*, in cell culture medium, and in patients, synthetic piperacillin-modified peptide ATK(Pip)EQLK was spiked into tryptic HSA digests to construct calibration curves. Six calibration standards containing peptide ATK(pip)EQLK (30, 75, 150, 225, 375, 750 fmol) were prepared. The quantities of piperacillin modification in samples were calculated against calibration curve.

Synthesis of drug-modified albumin conjugates

Synthetic drug HSA conjugates were generated for functional studies by incubating drugs (piperacillin, penicillin G, and amoxicillin) with HSA at molar ratio of 2:1-250:1 for 24 h in phosphate buffer. The conjugates were purified by ultracentrifuge with a 3,000 MW cut off centrifugal filter (Amicon Ultra-15, Millipore, UK) according to the manufactory's protocol. Briefly, 600 µL conjugates were added to the filter device followed by addition of 14 mL phosphate buffer (10mM, pH7.4). The device was centrifuged at 5000Xg for 20 min and the filtrate was discarded. The process of washing was repeated 6 times until the concentration of the free drug has been sufficiently reduced. The concentration of free drug in the filtrate from the last wash was determined by LC-MS and was found to be below 2.5nM.

Generation of T-cell clones

Antigen-specific T-cells were enriched by culturing PBMC with piperacillin or the piperacillin albumin adduct (generated using a molar ratio of 250:1 [drug:protein] for 24 h) for 14 days. IL-2 (60 U/ml) was added to maintain antigen specific proliferation. T-cells were

cloned by serial dilution using established methodology without purification of CD4+ or CD8+ T-cells (8). To test the specificity of the clones, T-cells (5 X 10^4) were incubated with irradiated antigen-presenting cells (Epstein–Barr virus- transformed B-cell lines; 1 X 10^4) and piperacillin (2mM) or a piperacillin albumin adduct (2mg/ml) for 48 h. [^{3}H]thymidine (0.5μCi) was added, and 16 h later proliferation was measured by scintillation counting. TCR Vβ protein expression was measured by flow cytometry using the IOTest Beta Mark TCR Vβ repertoire kit (Immunotech, Beckman Coulter, UK). Data was analysed using Cyflogic software (CyFlo Ltd., Finland). Cell phenotyping was performed using antibodies against CD4, CD8, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CCR10, CXCR1, CXCR3, CXCR6 and CLA.

Characterization of piperacillin-specific T-cell responses and quantification of piperacillin albumin binding

The addition of piperacillin to cell culture medium results in modification of albumin, and a multitude of other proteins, that could act as antigenic determinants. To quantify the relationship between piperacillin protein binding *in vitro* and the activation of T-cells, drug-responsive CD4+ clones were cultured with (1) antigen presenting cells and titrated concentrations of piperacillin (0.01-2mM) and (2) antigen presenting cells pulsed with titrated concentrations of piperacillin (0.1-2mM) for 1-48h. In the latter experiments, antigen presenting cells were washed with medium (3x5ml) and cultured with clones in the absence of free drug. Finally, antigen presenting cells were fixed with glutaraldehyde to block protein processing. Supernatant was collected from each experiment to quantify piperacillin HSA adducts. The proliferative response of the clones was measured by the addition of [^{3}H]thymidine.

Characterization of piperacillin albumin adduct-responsive T-cell clones and quantification of piperacillin albumin binding

Experiments were also conducted using piperacillin albumin adducts to quantify the level of protein binding required to activate CD4+ T-cells. Importantly, these experiments used clones generated by culturing PBMC with a synthetic piperacillin-HSA conjugate. First, clones were incubated with antigen presenting cells and titrated concentrations of either soluble piperacillin or the piperacillin albumin adduct generated using a molar ratio of 250:1 (drug:protein) for 24 h (0.25-2mg/ml). Unmodified albumin subjected to the same extraction protocol was used as a control. Second, clones were incubated with antigen presenting cells and piperacillin albumin adducts generated using molar ratios of 2:1-250:1 (drug:protein) for 24 h (2mg/ml). Third, antigen presenting cells were fixed with glutaraldehyde to block protein processing and the generation of new MHC associated peptide antigens. Finally, crossreactivity was assessed by incubating clones with antigen presenting cells and piperacillin, penicillin G or amoxicillin albumin adducts generated using molar ratios of 100:1-250:1 (drug:protein) for 24 h (2mg/ml). The proliferative response of the clones was measured by the addition of [^{3}H]thymidine.

Statistical analysis

Multiple clones (up to 13 per experiment) were used to analyse T-cell responses. Experiments were conducted in duplicate or triplicate. Mean±SD was calculated, and statistical analysis was performed using paired T test (Sigmaplot 12 software).

Results

Synthesis of piperacillin-modified albumin peptides

To quantify drug hapten albumin binding a piperacillin-modified HSA peptide incorporating the amino acid sequence around Lys541 (ATK[Pip]EQLK) was synthesized using Fmoc chemistry (Figure 1A). The presence of a doubly charged ion at *m/z* 676.8 on the MS/MS spectrum (Figure 1B), corresponding to the peptide ATKEQLK with an additional mass of 535 amu, demonstrated that a hydrolysed piperacillin-modified peptide was formed. The peptide sequence was confirmed by a product ion spectrum that generated partial singly charged y and b series ions. The modification was defined by b3* (peak at m/z 517) and b4* (peak at m/z 646), with addition of 216 amu, providing evidence that piperacillin is attached to K541 (Figure 1B). Purification of crude peptide products by HPLC afforded peptide ATK(piperacillin)EQLK in greater than 90% purity.

Quantitative analysis of piperacillin-modified albumin in tolerant and hypersensitive patients

In contrast to the two hapten structures detected on piperacillin albumin adducts generated *in vitro* (cyclised and hydrolysed; Figure 2A), the hydrolysed form was the only hapten detected on albumin Lys residues in tolerant patient plasma, with triptic peptides incorporating Lys541 and Lys190 being the dominant sites of modification (results not shown). A total of four lysine residues, namely Lys190, Lys432, Lys 525, and Lys541, were detected in all patients (Figure 2B). A calibration curve was constructed using the synthetic piperacillin-Lys541 peptide to quantify piperacillin albumin binding in tolerant patient plasma after a 14 day treatment course (Figure 2C); the analyses were conducted on three separate occasions. The level of modification of Lys541 in total HSA ranged from 2.7-4.7% (mean 3.86%); with 6 patients displaying modification levels of 4% or more (Figure 2D).

The analysis was repeated with plasma from 3 patients that developed hypersensitivity 1, 6, and 10 days after commencement of piperacillin therapy. Piperacillin albumin adducts formed rapidly in patients, with 2% modification detected within 24 h (patient 032; Figure 2E & F). The level of modification in patients that developed hypersensitivity 6 and 10 days after treatment commenced matched those circulating in tolerant patients (Figure 2E & F).

Generation of piperacillin-responsive T-cell clones and characterization of the minimal quantity of piperacillin hapten albumin binding required for T cell activation

Seventy seven piperacillin-responsive clones were isolated from 3 LTT positive patients with a history of piperacillin-mediated maculopapular exanthema (Table 2). The majority of clones were CD4+; 4 clones expressed CD8, while 2 clones expressed CD4 and CD8 receptors. Fifteen distinct Vβ T-cell receptors were detected on 53 out of 64 clones analysed. T-cell receptor Vβ 5.1, 13.1 and 17 were detected with the greatest frequency (Figure 3A). As described recently (22) clones expressed several chemokine receptors, including CCR4, 9 and 10, and CXCR3 (Figure 3B). Soluble piperacillin, which forms conjugates with multiple proteins in culture medium, activated the clones in a concentration-dependent manner (0.5-4mM; Figure 3C).

Twenty CD4+ clones were used to study the relationship between piperacillin hapten protein binding and T-cell activation. Since these clones were not activated with synthetic piperacillin-HSA adducts, albumin was used as a surrogate protein to relate the level of piperacillin adduct formed to the activation of T-cells. First, the piperacillin concentration in the proliferation assay was reduced to establish a threshold for T-cell stimulation. A weak piperacillin-specific T-cell proliferative response was detected with 5/9 clones at a

concentration of 0.01mM (Figure 3 D & E). However, statistical significance was only reached with concentrations of 0.1mM and above (Figure 3D). Coinciding with the T-cell response at 0.1mM piperacillin, approximately 1% of piperacillin-modified K541 was detectable after 48h. Increasing the concentration of piperacillin resulted in higher levels of piperacillin hapten-modified Lys 541, with 4.7% modification detected at 2mM piperacillin. Moreover, piperacillin hapten albumin binding was detected at earlier time-points (4-24h) with piperacillin concentrations of ranging from 0.5-4mM (Figure 3F). As described previously, fixation of antigen presenting cells, which blocks protein processing (24-27), prevented the piperacillin-specific activation of all clones (results not shown).

In contrast to our previous study using a piperacillin-albumin conjugate purified through solvent precipitation (free piperacillin concentration, 150nM) (7), clones were not activated with the albumin conjugate used herein (Figure 3G). As described above, free piperacillin was removed from the conjugate by repeated ultrafiltration and the non-covalently bound drug concentration was less than 2.5nM.

The level of piperacillin hapten adduct required for triggering T-cells was determined by pulsing antigen presenting cells with piperacillin (2mM) for 1, 4, 24, and 48h prior to washing and exposure of the antigen presenting cells to clones. Antigen-presenting cells pulsed with piperacillin for 1 and 4 h did not stimulate a proliferative response and only low levels of piperacillin-modified Lys541 was detected (Figure 4). In contrast, 24 and 48h pulsed antigen presenting cells stimulated all clones to proliferate and the strength of the induced response was equivalent to or stronger than that seen with the soluble drug (Figure 4). From this data it can be seen that 2.8% of Lys541 in HSA is modified by piperacillin when protein adducts are generated to activate T-cell proliferative responses.

Generation of piperacillin HSA adduct-responsive T-cell clones

Since clones generated through the culture PBMC of with the parent compound are activated via a hapten mechanism with piperacillin protein adducts other than piperacillin-modified albumin, additional experiments were conducted to generate piperacillin-albumin conjugate-responsive clones. A piperacillin HSA adduct generated by culturing piperacillin and HSA at a ratio of 250:1 for 24h, prior to purification by ultracentrifugation, was used to activate patient PBMC. After 2 weeks in culture, T-cells were cloned by serial dilution. 22 CD4+ and 1 CD8+ clone were stimulated to proliferate with the piperacillin HSA adduct. As one would expect with the limited number of drug-modified peptide epitopes that can be generated from the piperacillin-albumin conjugate, the clones displayed a restricted pattern of Vβ receptor expression; Vβ 9 was expressed on 15 clones, while other clones expressed Vβs 2, 17 and 20 (Figure 5A). Thirteen piperacillin HSA adduct-responsive clones were analysed for chemokine receptor expression. High expression of CCR2, 4, 8, 9, 10 and CXCR3 was detected (Figure 5B).

Clones were stimulated to proliferate with titrated concentrations of the piperacillin HSA adduct (0.25-2mg/ml [Figure 5C]; concentrations of 0.1mg/ml and below did not activate clones) via a pathway dependent on protein processing (i.e., glutaraldehyde fixation of antigen presenting cells inhibited the antigen-specific proliferative response [results not shown]). Importantly, unmodified HSA processed in the same way as the piperacillin HSA adduct did not activate the clones (Figure 5D). Ten well-growing clones were expanded and used to determine the minimum level of piperacillin hapten binding required to activate T-cells. Piperacillin HSA adducts generated at drug:HSA ratios of 10:1 – 250:1 were found to stimulate the clones to proliferate (Figure 5E), with the strength of the response increasing

with increasing levels of piperacillin hapten modification. Although piperacillin-HSA adducts are generated spontaneously in cell culture medium containing free drug, only 50% of the clones were activated with soluble drug, and the strength of the proliferative response was significantly weaker (Figures 5F & G). Despite this, the concentration of piperacillin required to activate the clones and the kinetics for T-cell activation (i.e., the antigen presenting cell pulse duration) were the same as described with clones depicted in figures 3 and 4.

The synthetic piperacillin-HSA conjugates were characterised by mass spectrometry and western blot. Epitope profiling showed that both cyclised and hydrolysed hapten were formed; piperacillin-modified K541 was the major site of binding (Figure 5H & I). The levels of hydrolysed piperacillin-modified K541 increased with higher concentrations of piperacillin, ranging from 3.6% at the lowest T-cell stimulatory concentration to 23.5% at a drug:protein ratio of 250:1 (Figure 5J). The concentration-dependent binding of piperacillin was also mirrored using western blotting (Figure 5K).

Piperacillin HSA adduct-responsive T-cell clones are not activated with other β-lactam HSA adducts

Penicillin G, amoxicillin and piperacillin HSA adducts were generated by culturing the drugs with HSA at 100:1 and 250:1 ratios for 24h. Epitope profiles showed that the same subset of Lys residues was targeted by all three drugs; however, the relative level of binding differed at the individual sites of modification (Figure 6A & B). Piperacillin HSA adduct-responsive clones were not stimulated to proliferate with either penicillin G or amoxicillin HSA adducts (Figure 6C & D).

Discussion

It is currently impossible design drugs with no immunological liability; furthermore, it is very difficult to predict which individuals will develop hypersensitivity when exposed to a therapeutic treatment regimen. One of the predominant problems is the complexity of processes that deliver drug-derived antigens to the T-cell receptor. *In vitro* analyses have revealed that certain drugs bind directly to the MHC peptide binding cleft and/or pre-bound peptides to activate T-cells (26-31). However, the dominant pathway for drugs such as the β-lactam antibiotics (23,26,32-34) and sulphonamides (35-37) involves the formation of a protein adduct with the drug hapten bound irreversibly to specific amino acid residues on non-MHC associated protein. The protein adduct is processed by antigen presenting cells liberating peptides that associate with MHC molecules to activate T-cells.

Our previous studies have demonstrated that the β-lactam antibiotic piperacillin covalently modifies Lys residues at drug binding "Sudlow sites" on HSA in plasma (8). Moreover, piperacillin stimulates hypersensitive patient circulating and skin resident T-cells to proliferate and secrete effector molecules, including the tissue-specific cytokine IL-22 (7,22). El-Ghaiesh et al (7) found that all piperacillin-responsive clones were activated with a piperacillin HSA adduct containing 150nM piperacillin bound non-covalently to the protein. We repeated these experiments and found comparable results (data not shown). However, in the present study using a fully characterised, and highly purified HSA adduct, for which we have used low molecular weight mass spectrometry to exclude the presence of non-covalently-associated piperacillin above an analytical limit of 2.5nM, we can now rigorously demonstrate two classes of clones from the same patient: firstly, clones stimulated by a single protein (albumin) adduct *per se*; and secondly, clones stimulated as a result of modification of proteins present in the incubation by addition of the parent drug. CD4+ T-cells expressing

386 skin homing receptors such as CCR4 and CCR10 were selectively activated with either the
387 piperacillin albumin adduct or the parent drug, via a hapten mechanism. All clones were
388 activated with piperacillin-pulsed antigen presenting cells. Furthermore, fixation of antigen
389 presenting cells, which blocks protein processing, inhibited the activation of T-cells with
390 piperacillin. Thus, we did not identify clones that were activated with the parent drug bound
391 directly to MHC via a P-I mechanism. The piperacillin albumin adduct-responsive clones
392 displayed a more restricted profile of Vβ receptors, which may relate to the limited number of
393 epitopes available for MHC T-cell receptor binding (approximately 10-12), when compared
394 with the parent drug, which in theory could modify 1000s of serum and/or cellular proteins
395 within the culture medium. Analysis of piperacillin hapten binding in patient's plasma and *in*
396 *vitro* revealed that piperacillin binds to HSA within 24 h at comparable levels. Thus,
397 antigenic determinants with the potential to activate T-cells and cause tissue injury are
398 formed in all patients exposed to the drug.
399
400 The availability of piperacillin hapten-specific clones with specificity for albumin adducts
401 lead us to investigate whether the level of piperacillin HSA modification differs in tolerant
402 and hypersensitive individuals exposed to the same treatment regimen. To do this, a synthetic
403 drug hapten peptide standard incorporating amino acid residues found in the native protein
404 (38,39) was generated. 3.9% of Lys 541 in HSA isolated from plasma of tolerant patients
405 exposed to piperacillin for 14 days was modified with the piperacillin hapten. The level of
406 Lys541 binding ranged from 2.7-4.7%, indicating that there is a 2 fold difference in exposure
407 to piperacillin HSA adducts in patients exposed to the same treatment regimen. Somewhat
408 surprisingly, the piperacillin hapten with a hydrolysed 4-ethyl-2,3-dioxopiperazine ring was
409 the only moiety bound to HSA in patient plasma, which indicates that this form of the hapten
410 almost certainly interacts with T-cell receptors expressed on antigen-specific T-cells.

411

412 The level of Lys541 modification on albumin from patients sampled on the day of
413 hypersensitivity diagnosis (days 1, 6 and 10) matched those circulating in tolerant patients,
414 which indicates that the level of covalent binding in all patients exposed to piperacillin is
415 sufficient to activate T-cells; however, only a portion of patients develop hypersensitivity.
416 Thus, the propensity to develop hypersensitivity may be dependent on the presence of T-cells
417 within an individual's repertoire that can be activated with the β-lactam hapten and/or an
418 imbalance in immune regulation. Interestingly, piperacillin albumin adducts were formed
419 rapidly after commencement of piperacillin therapy. After day 1 of treatment, the level of
420 Lys541 modification exceeded 2%.

421

422 These data quantifying piperacillin protein adducts in plasma led us to measure the threshold
423 level of HSA modification using piperacillin concentrations required to activate T-cells.
424 CD4+ T-cell clones were cultured with antigen presenting cells and titrated concentrations of
425 piperacillin to ascertain the lowest drug concentration associated with a significant
426 proliferative response. Piperacillin-modified K541 was detectable at 0.1mM, the lowest
427 concentration associated with T-cell proliferative responses. A dose-dependent increase in the
428 level of modification was observed with piperacillin concentrations associated with a 20-fold
429 increase in the proliferation of T-cells (i.e., 0.5-4mM). Importantly, equivalent levels of
430 Lys541 modification were detected in patient plasma and *in vitro* with piperacillin
431 concentrations that stimulated a T-cell response. To estimate the absolute levels of HSA
432 binding at the earliest possible time that T-cells are activated, antigen-presenting cells were
433 pulsed with piperacillin (2mM) for 1-48h prior to washing and exposure of clones to the
434 pulsed cells. Antigen presenting cells pulsed with piperacillin for 24h stimulated all clones to

proliferate and the strength of the response was stronger to that seen with the soluble drug. At this time-point 2.8% of Lys541 was modified with the piperacillin hapten.

Since T-cell clones cultured with soluble piperacillin are activated by the drug hapten bound covalently to protein carriers other than HSA, a synthetic β-lactam HSA adduct was generated to (1) assess the relationship between the level of hapten binding and the T-cell response and (2) study T-cell reactivity with other β-lactam protein adducts. Piperacillin HSA adducts were generated with 3.6-23.5% Lys541 modification by culturing piperacillin with HSA at ratios of 10:1-250:1. The response of all piperacillin HSA adduct-responsive clones was blocked by gluturaldehyde fixation indicating that the clones were activated via a hapten mechanism involving the generation of antigenic HSA-derived peptides. Furthermore, the response was restricted to the piperacillin hapten structure as other β-lactam HSA adducts did not activate the T-cells. In line with our previous findings, the preference of β-lactam antibiotics for different lysine residues in albumin is driven at least in part by the initial non-covalent interaction, which positions the drug in a favourable position to facilitate covalent binding. The three-dimensional shape of the drug as well as its inherent chemical reactivity therefore determines selectivity of covalent binding and as demonstrated in figure 6 the activation of T-cells. An increase in the level of piperacillin modification at Lys541 correlated with the strength of the T-cell proliferative response (r^2=0.96). Clones were initially activated with an adduct generated at a drug:protein ratio of 10:1; quantitative mass spectrometry revealed that 3.6% of Lys541 was modified with the piperacillin hapten.

Collectively, our data reveal that the level of drug hapten protein binding in tolerant and hypersensitive patients exposed to a therapeutic treatment regimen is sufficient to activate T-cells. Thus, it is important that future research focuses on why most patients do not develop a

460 drug antigen-specific T-cell response and clinical manifestations of hypersensitivity. To do
461 this, we have recently initiated a prospective investigation of piperacillin hypersensitivity.
462 Bloods samples are being collected during repeated drug courses and when patients develop
463 an adverse event to define the quantitative relationship between antigen formation and the
464 factors that control the balance between immune tolerance and activation.
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612

Footnotes

[1]This work was funded by a grant from the Cystic Fibrosis Trust (PJ533). Central funds were obtained from the Centre for Drug Safety Science supported by the MRC (G0700654).

[2]Abbreviations: human serum albumin, HSA; peripheral blood mononuclear cells, PBMC.

Figure legends

Figure 1. Synthesis of a piperacillin-modified peptide incorporating Lys541. (A) Scheme showing the synthetic pathway for piperacillin modified K541 peptide ATK(Pip)EQLK. (B) MS/MS spectra of synthetic peptide ATK(Pip)EQLK with characteristic fragment ions from piperacillin circled.

Figure 2. Piperacillin hapten structures and absolute quantification of piperacillin hapten albumin binding in tolerant and hypersensitive patient plasma. (A) Scheme showing the 2 potential piperacillin haptens bound covalently to protein. (B) Model of albumin showing piperacillin lysine binding sites in patients. (C) Standard curve constructed using synthetic piperacillin-modified K541 peptide with concentrations ranging from 0.05μM to 1.5μM. (D, E) Absolute level of piperacillin-modified K541 peptide detected in plasma from (D) tolerant and (E) hypersensitive patients. Mass spectrometric analysis was repeated on 3 separate occasions. (F) Comparison of piperacillin K541 binding in hypersensitive (●) and tolerant (◆) patients showing day of analysis.

Figure 3. Processing-dependent activation of piperacillin-responsive CD4+ clones from hypersensitive patients and quantification of piperacillin albumin binding. (A, B) Flow cytometric analysis of (A) T-cell receptor Vβ and (B) chemokine receptor expression on pipercillin-responsive clones. (C, D) T-cell clones were cultured with irradiated antigen presenting cells and piperacillin ([C] 0.5-4mM; [D] 0.01-100μM) and proliferative responses were measured by [^{3}H]thymidine uptake. (E) Representative proliferative response of 2 clones shown in D. (F) The absolute level of time-dependent piperacillin-modified K541 peptide detected in culture supernatant. (G) T-cell clones were cultured with irradiated

643 antigen presenting cells and piperacillin or HSA adducts and proliferative responses were
644 measured by [^{3}H]thymidine uptake.
645
646 **Figure 4. Time-dependent activation of CD4+ clones with piperacillin-pulsed antigen**
647 **presenting cells and quantification of piperacillin albumin binding.** (A) T-cell clones
648 (n=8) were cultured with antigen presenting cells pulsed with piperacillin (2mM; 1-48h) and
649 proliferative responses were measured by [^{3}H]thymidine uptake. Antigen presenting cells
650 were subjected to repeated washing with drug-free medium prior to exposure to clones. (B)
651 The absolute level of time-dependent piperacillin-modified K541 peptide detected in antigen
652 presenting cell culture supernatant.
653
654 **Figure 5. Processing-dependent activation of piperacillin-modified albumin responsive**
655 **CD4+ clones from hypersensitive patients and quantification of piperacillin albumin**
656 **binding.** (A, B) Flow cytometric analysis of (A) T-cell receptor Vβ and (B) chemokine
657 receptor expression on pipercillin-responsive clones. (C) T-cell clones were cultured with
658 irradiated antigen presenting cells and piperacillin-modified HSA (generated using a molar
659 ratio of 250:1 drug:protein for 24h) and proliferative responses were measured by
660 [^{3}H]thymidine uptake. (D) Proliferative response of T-cell clones cultured with unmodified
661 and piperacillin-modified HSA and antigen presenting cells. (E) Proliferative response of T-
662 cell clones cultured with antigen presenting cells and piperacillin-modified HSA adducts
663 generated using different molar ratios of drug:protein. (F, G) Proliferative response of T-cell
664 clones cultured with antigen presenting cells and piperacillin-modified HSA adducts or
665 piperacillin ([F] combined data; [G] representative clones]. (H, I) Relative quantification of
666 (H) cyclysed and (I) hydrolysed forms of the piperacillin hapten generated at different lysine
667 residues on the piperacillin albumin adduct (drug:protein ratios of 10:1-250:1). (J) The

668 absolute level of piperacillin-modified K541 peptide detected on the piperacillin albumin
669 adductd generated at drug:protein ratios of 10:1-250:1. (K) Western blot analysis of the
670 piperacillin albumin adducts.
671
672 **Figure 6. Piperacillin-albumin adduct-responsive CD4+ clones are not activated with**
673 **alternative β-lactam albumin adducts.** (A,B) Relative mass spectrometric quantification of
674 Benzyl penicillin, amoxicillin and piperacillin haptens formed at different lysine residues *in*
675 *vitro* when the drug was incubated at drug:protein ratios of (A) 100:1 and (B) 250:1. (C,D) T-
676 cell clones (n=5) were cultured with irradiated antigen presenting cells and benzyl penicillin,
677 amoxicillin, or piperacillin-modified HSA (generated using a molar ratio of (C) 100:1 or (D)
678 250:1 drug:protein for 24h) and proliferative responses were measured by [^{3}H]thymidine
679 uptake.
680
681

Table 1. Clinical characteristics of the drug tolerant and hypersensitive patients used in the mass spectrometric analyses

F: female; M: male; MPE: maculopapular exanthema; NT: not tested; ID: intradermal; LTT: lymphocyte transformation test.
+: SI 2-5; ++: SI 5-10; +++: SI 10-20; ++++: >20
[a] Days after commencement of therapy that plasma HSA was analyzed by mass spectrometry for piperacillin covalent binding.

Patient ID	Drug	Reaction	Clinical features	Time to reaction (days)	Day of analysis[a] (days)	No. of courses prior to reaction
			Hypersensitive patients			
032	Tazocin	Maculopapular rash	Age 33, Male; df508/df508; Chronic Pseudomonas Infection; FEV1 2.27 litres (57% predicted); Pancreatic insufficient; CF related diabetes mellitus	1	1	8
040	Tazocin	Facial rash, pruritis	Age 20, Female; df508/df508; Chronic Pseudomonas infection; FEV1 0.43 litres (15% predicted); Pancreatic insufficient; CF related diabetes mellitus	6	6	6
059	Tazocin	Maculopapular rash	Age 26, Female; df508/unknown; Chronic Pseudomonas infection; FEV1 1.84 litres (54%); Pancreatic insufficient	10	10	8
			Tolerant patients			
006	Tazocin	-		-	14	n/a
016	Piperacillin	-		-	14	n/a
020	Piperacillin	-		-	14	n/a
030	Tazocin	-		-	14	n/a
026	Tazocin	-		-	14	n/a
022	Tazocin	-		-	14	n/a
009	Tazocin	-		-	14	n/a
017	Tazocin	-		-	14	n/a
029	Tazocin	-		-	14	n/a
025	Tazocin	-		-	14	n/a

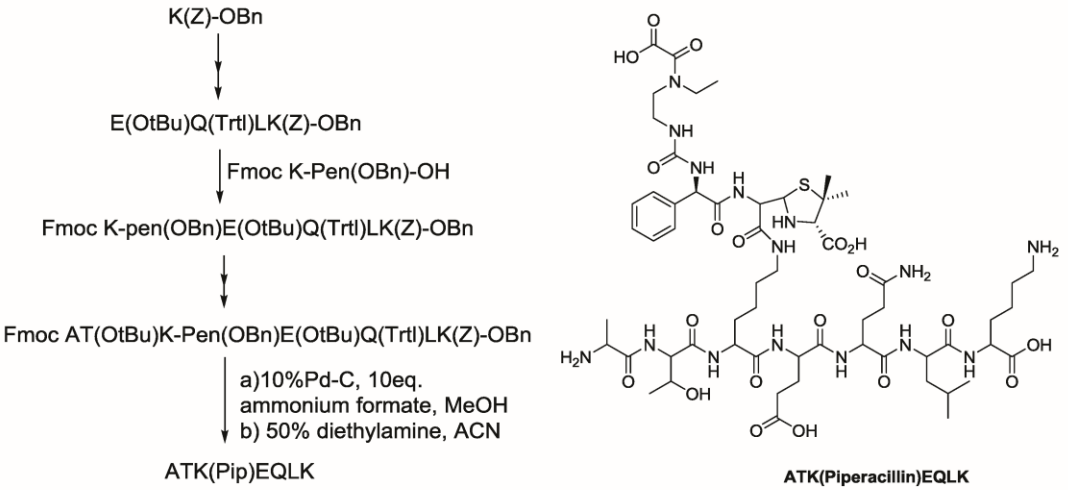
Table 2. Clinical characteristics of the hypersensitive patients used in the immunological studies

F: female; M: male; MPE: maculopapular exanthema; LTT: lymphocyte transformation test.
+: SI 2-5; ++: SI 5-10; +++: SI 10-20; ++++: >20

Patient ID	Age (years) / Gender	Clinical features	Drug	Reaction	Delay between course initiation and reaction (days)	Time since reaction (years)	Delayed intradermal readings	Piperacillin LTT
A	26/M	Chronic Pseudomonas infection; FEV1 2.87 litres (68% predicted); BMI 23; CF related diabetes; 5 days of intravenous antibiotics over last 12 months	Piperacillin Aztreonam Ceftazidime Meropenem	MPE/Fevers MPE Delayed Angioedema MPE	9 4 3 5	12 10 10 10	- - - -	++
B	28/M	Chronic Pseudomonas infection; FEV1 0.61lites (15% predicted); BMI 16; Osteoporosis; 197 days of intravenous antibiotics over past 12 months	Piperacillin	MPE	11	9	+ve at 24 hours	+++
C	27/M	Chronic Pseudomonas infection; FEV1 1.1lites (28% predicted); BMI 17; Osteoporosis; 96 days intravenous antibiotics over past 12 months	Ceftazidime Piperacillin	MPE MPE/fever	5 2	9 5	- +ve at 48 hours	++++

Figure 1

A


K(Z)-OBn
E(OtBu)Q(Trtl)LK(Z)-OBn
Fmoc K-Pen(OBn)-OH
Fmoc K-pen(OBn)E(OtBu)Q(Trtl)LK(Z)-OBn
Fmoc AT(OtBu)K-Pen(OBn)E(OtBu)Q(Trtl)LK(Z)-OBn
a)10%Pd-C, 10eq.
ammonium formate, MeOH
b) 50% diethylamine, ACN
ATK(Pip)EQLK
ATK(Piperacillin)EQLK

B

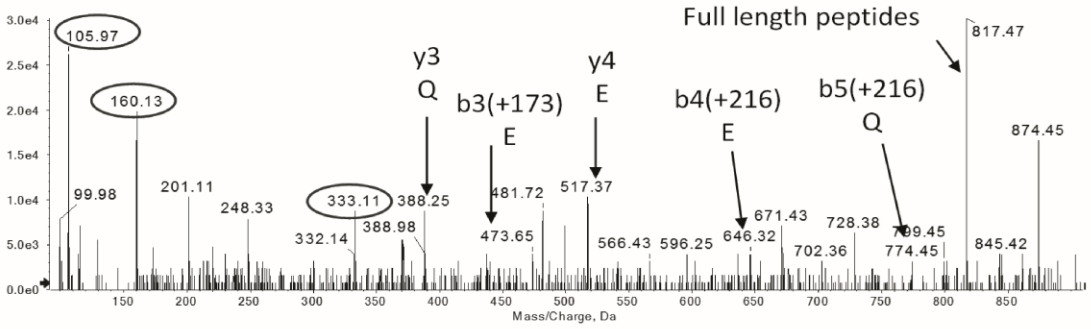

Full length peptides
y3
Q
b3(+173)
E
y4
E
b4(+216)
E
b5(+216)
Q
105.97
160.13
99.98
201.11
248.33
333.11
388.25
388.98
332.14
473.65
481.72
517.37
566.43
596.25
646.32
671.43
702.36
728.38
774.45
817.47
845.42
874.45
Mass/Charge, Da

Figure 2

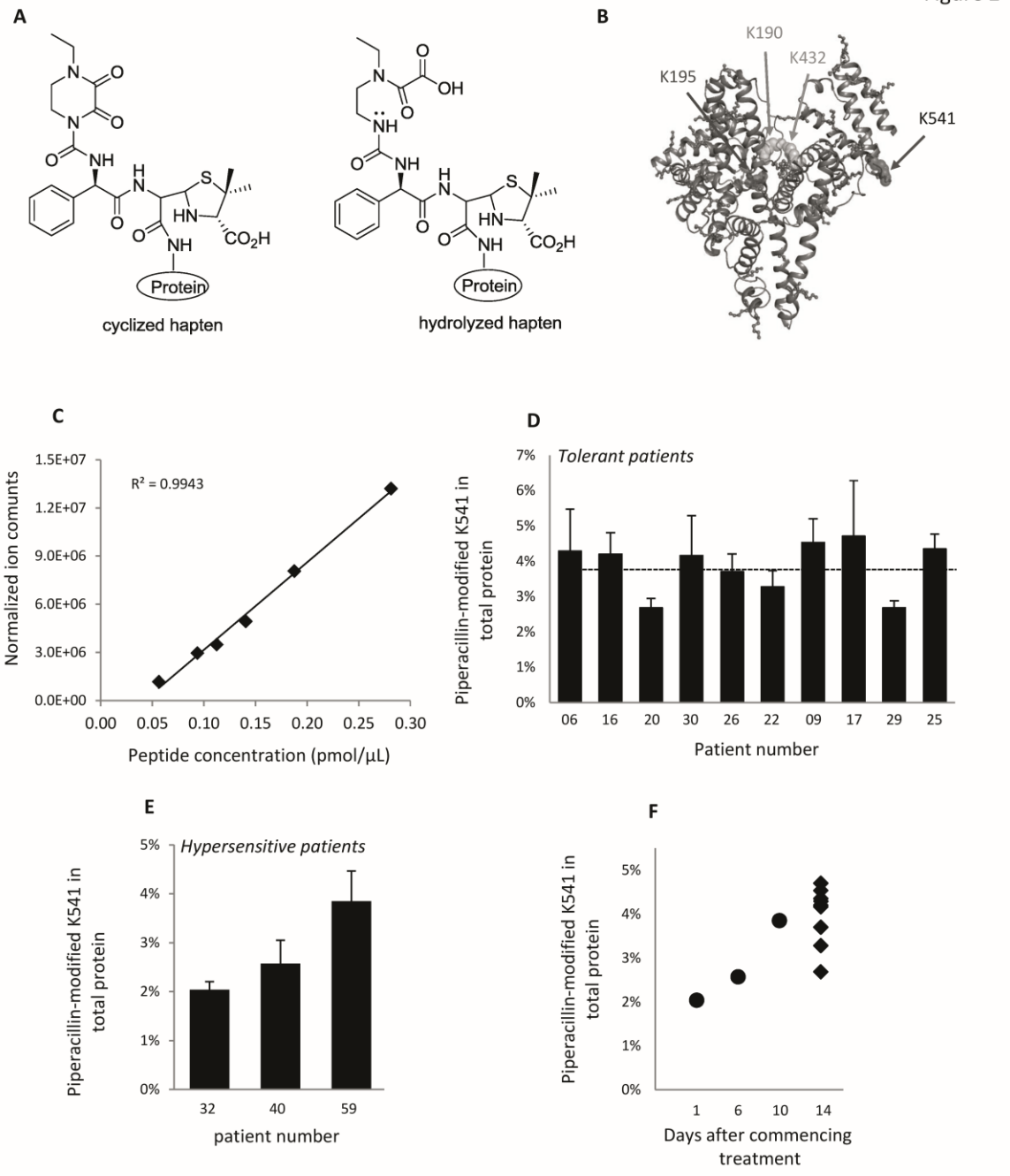

A
cyclized hapten
hydrolyzed hapten
Protein
B
K190
K432
K195
K541
C
Normalized ion comunts
R² = 0.9943
Peptide concentration (pmol/µL)
D
Tolerant patients
Piperacillin-modified K541 in total protein
Patient number
06 16 20 30 26 22 09 17 29 25
E
Hypersensitive patients
Piperacillin-modified K541 in total protein
patient number
32 40 59
F
Piperacillin-modified K541 in total protein
Days after commencing treatment
1 6 10 14

Figure 3

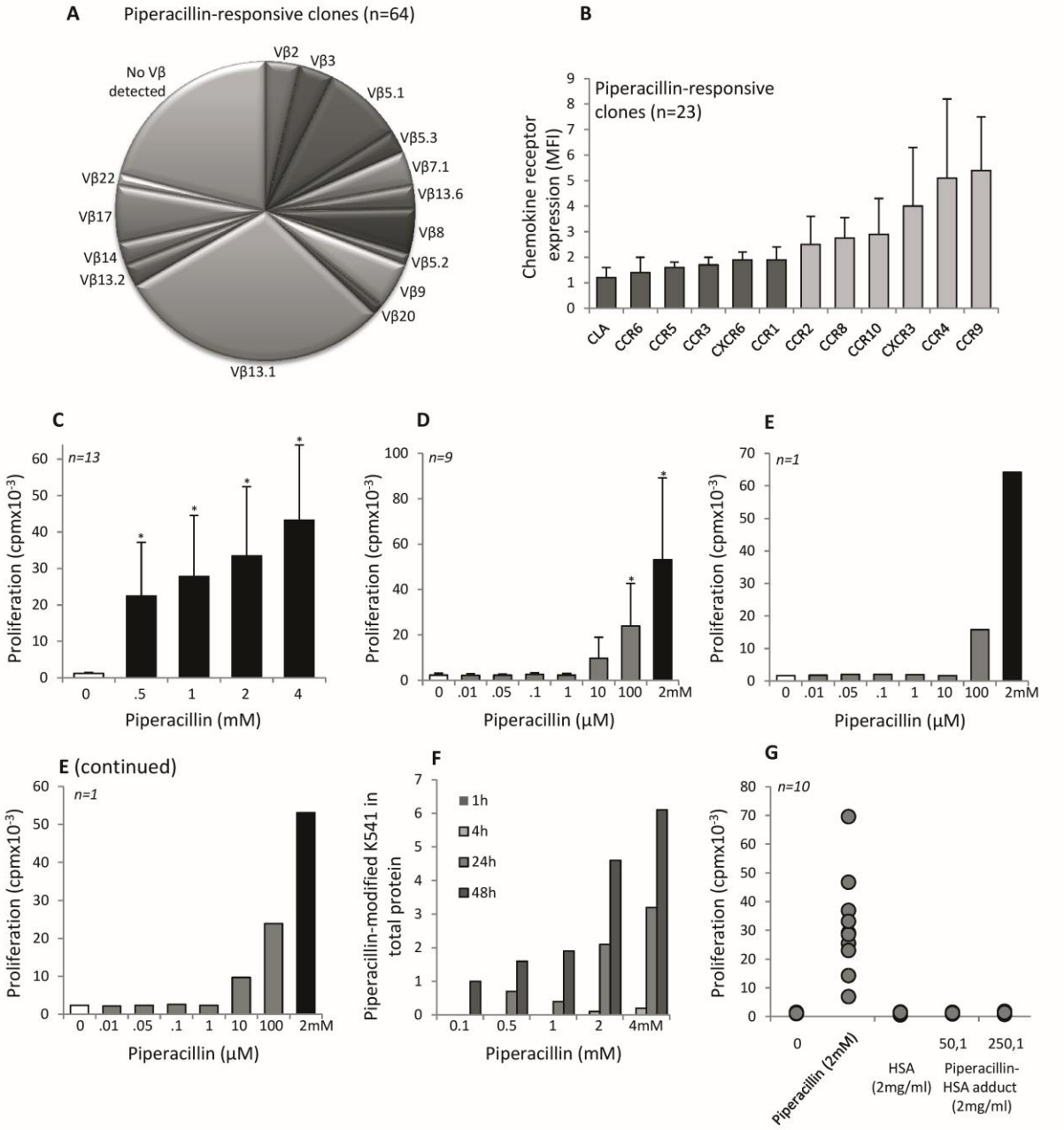

A
Piperacillin-responsive clones (n=64)
No Vβ detected
Vβ2
Vβ3
Vβ5.1
Vβ5.3
Vβ7.1
Vβ13.6
Vβ8
Vβ5.2
Vβ9
Vβ20
Vβ13.1
Vβ13.2
Vβ14
Vβ17
Vβ22
B
Piperacillin-responsive clones (n=23)
Chemokine receptor expression (MFI)
CLA
CCR6
CCR5
CCR3
CXCR6
CCR1
CCR2
CCR8
CCR10
CXCR3
CCR4
CCR9
C
n=13
Proliferation (cpmx10-3)
Piperacillin (mM)
D
n=9
Proliferation (cpmx10-3)
Piperacillin (μM)
E
n=1
Proliferation (cpmx10-3)
Piperacillin (μM)
E (continued)
n=1
Proliferation (cpmx10-3)
Piperacillin (μM)
F
1h
4h
24h
48h
Piperacillin-modified K541 in total protein
Piperacillin (mM)
G
n=10
Proliferation (cpmx10-3)
Piperacillin (2mM)
HSA (2mg/ml)
Piperacillin-HSA adduct (2mg/ml)

Figure 4

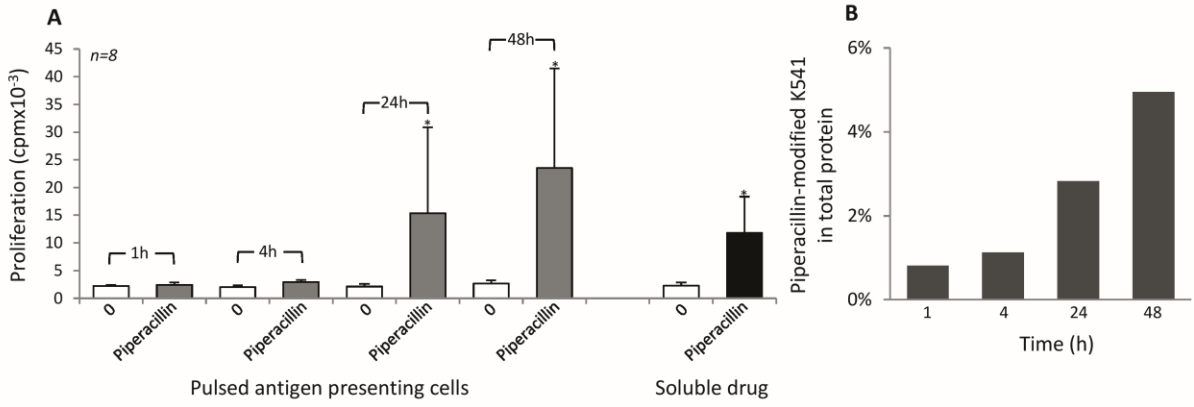

A
n=8
Proliferation (cpmx10-3)
45
40
35
30
25
20
15
10
5
0
1h
4h
24h
48h
0
Piperacillin
Pulsed antigen presenting cells
Soluble drug
B
Piperacillin-modified K541 in total protein
6%
4%
2%
0%
1
4
24
48
Time (h)

Figure 5

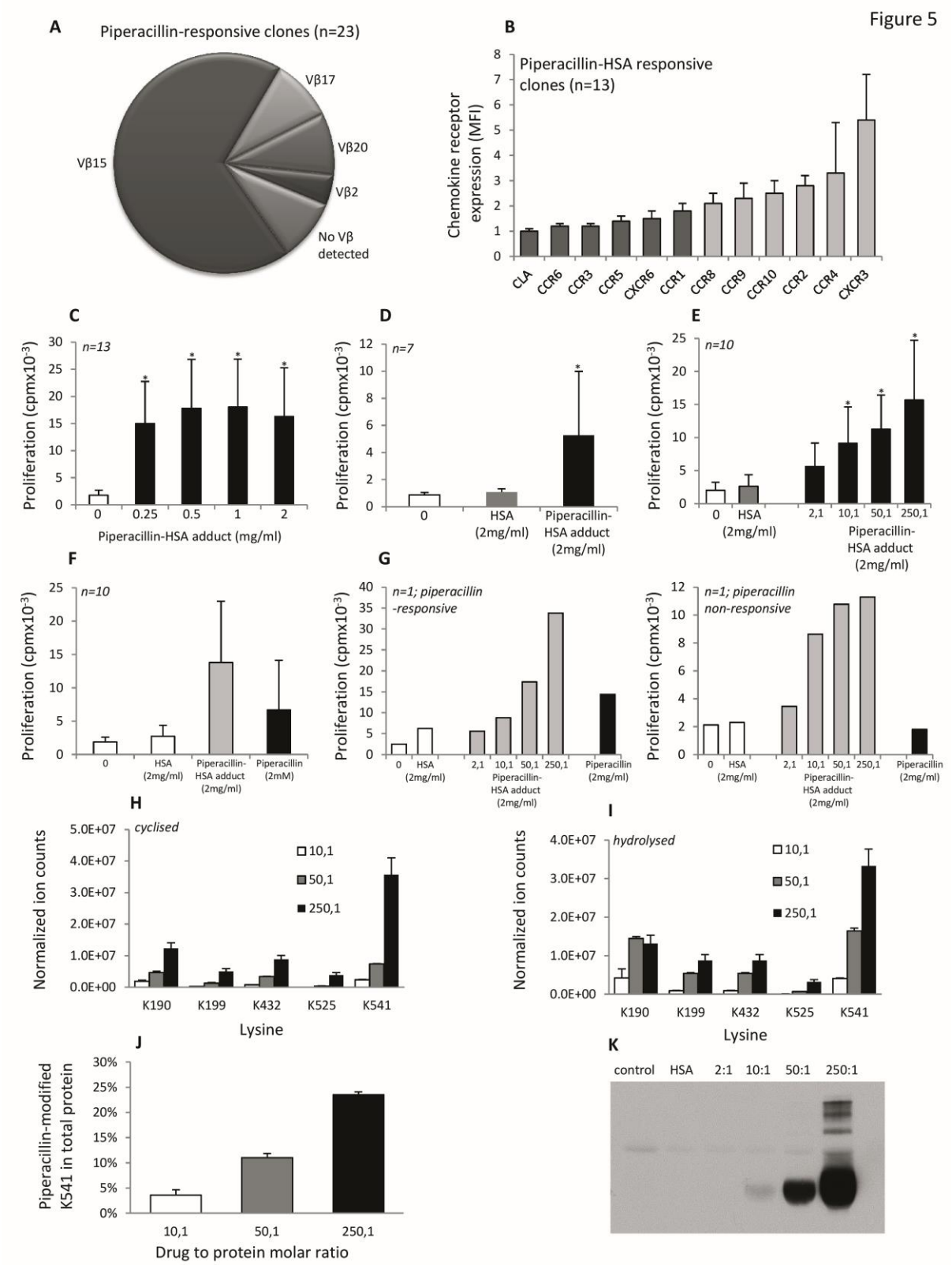
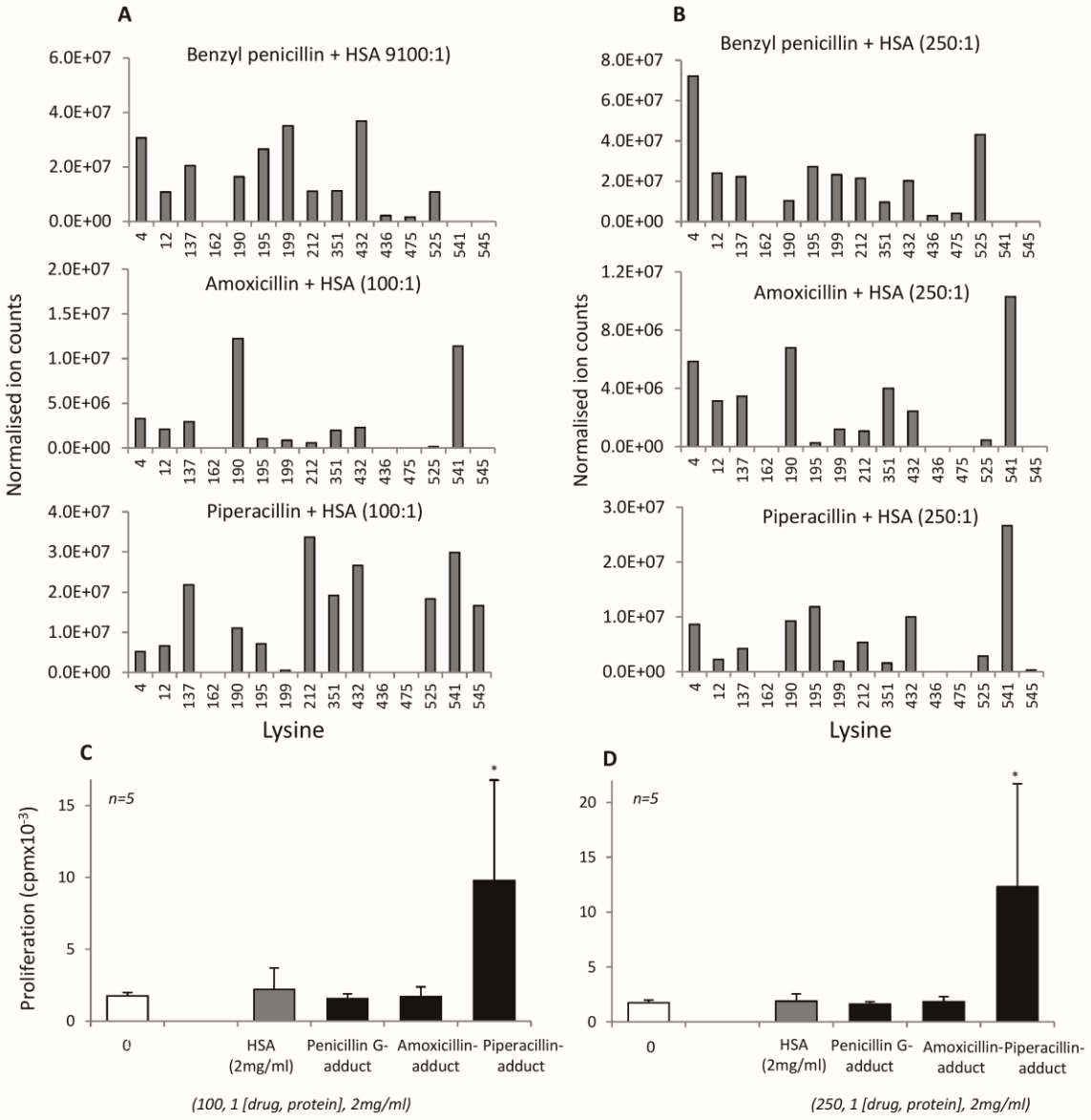

A
Piperacillin-responsive clones (n=23)
Vβ17
Vβ20
Vβ15
Vβ2
No Vβ detected
B
Piperacillin-HSA responsive clones (n=13)
Chemokine receptor expression (MFI)
CLA
CCR6
CCR3
CCR5
CXCR6
CCR1
CCR8
CCR9
CCR10
CCR2
CCR4
CXCR3
C
n=13
Proliferation (cpmx10-3)
Piperacillin-HSA adduct (mg/ml)
D
n=7
HSA (2mg/ml)
Piperacillin-HSA adduct (2mg/ml)
E
n=10
F
n=10
Piperacillin (2mM)
G
n=1; piperacillin -responsive
n=1; piperacillin non-responsive
H
cyclised
Normalized ion counts
10,1
50,1
250,1
K190
K199
K432
K525
K541
Lysine
I
hydrolysed
J
Piperacillin-modified K541 in total protein
Drug to protein molar ratio
K
control
HSA
2:1
10:1
50:1
250:1

Figure 6


A
Benzyl penicillin + HSA 9100:1)
Amoxicillin + HSA (100:1)
Piperacillin + HSA (100:1)
Normalised ion counts
Lysine
B
Benzyl penicillin + HSA (250:1)
Amoxicillin + HSA (250:1)
Piperacillin + HSA (250:1)
Normalised ion counts
Lysine
C
n=5
Proliferation (cpmx10-3)
0
HSA (2mg/ml)
Penicillin G-adduct
Amoxicillin-adduct
Piperacillin-adduct
(100, 1 [drug, protein], 2mg/ml)
D
n=5
0
HSA (2mg/ml)
Penicillin G-adduct
Amoxicillin-adduct
Piperacillin-adduct
(250, 1 [drug, protein], 2mg/ml)