

Millions of dots: violet makes your plot more interesting
New eBioscience™ Super Bright antibody conjugates

[Learn more](#)

invitrogen
by Thermo Fisher Scientific



This information is current as of June 14, 2017.

Definition of the Nature and Hapten Threshold of the β -Lactam Antigen Required for T Cell Activation In Vitro and in Patients

Xiaoli Meng, Zaid Al-Attar, Fiazia S. Yaseen, Rosalind Jenkins, Caroline Earnshaw, Paul Whitaker, Daniel Peckham, Neil S. French, Dean J. Naisbitt and B. Kevin Park

J Immunol 2017; 198:4217-4227; Prepublished online 24 April 2017;
doi: 10.4049/jimmunol.1700209
<http://www.jimmunol.org/content/198/11/4217>

References This article **cites 37 articles**, 13 of which you can access for free at:
<http://www.jimmunol.org/content/198/11/4217.full#ref-list-1>

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



Definition of the Nature and Hapten Threshold of the β -Lactam Antigen Required for T Cell Activation In Vitro and in Patients

Xiaoli Meng,* Zaid Al-Attar,* Fiazia S. Yaseen,* Rosalind Jenkins,* Caroline Earnshaw,* Paul Whitaker,[†] Daniel Peckham,[†] Neil S. French,* Dean J. Naisbitt,* and B. Kevin Park*

Covalent modification of protein by drugs may disrupt self-tolerance, leading to lymphocyte activation. Until now, determination of the threshold required for this process has not been possible. Therefore, we 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 hydrolyzed piperacillin hapten was detected on four lysine residues of human serum albumin (HSA) isolated from tolerant patients. The level of modified Lys⁵⁴¹ ranged from 2.6 to 4.8%. Analysis of plasma from hypersensitive patients revealed the same pattern and levels of modification 1–10 d after the commencement of therapy. Piperacillin-responsive skin-homing CD4⁺ clones expressing an array of V β 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 V β 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 Ag are formed in all patients. Thus, the propensity to develop hypersensitivity is dependent on other factors, such as 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. *The Journal of Immunology*, 2017, 198: 4217–4227.

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 represent 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 Ags 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, primarily protein mass spectrometry (MS), 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), with a degree of selectivity (4–9). Adduct formation on HSA is time- and concentration-dependent, and modifications are detected at <10% of available nucleophilic lysine residues. The selective binding interaction does not only relate to pKa and reactivity of the side-chain amino group as most adducts are formed at or around “Sudlow sites,” which are hydrophobic pockets involved in the noncovalent 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 Ag 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 MS (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 use MS 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

*Medical Research Council Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, The University of Liverpool, Liverpool L69 3GE, United Kingdom; and [†]Regional Adult Cystic Fibrosis Unit, St James's Hospital, Leeds LS9 7TF, United Kingdom

ORCID: 0000-0002-0111-5382 (Z.A.-A.); 0000-0002-3730-1136 (R.J.); 0000-0001-7723-1868 (D.P.); 0000-0002-9376-3482 (N.S.F.).

Received for publication February 9, 2017. Accepted for publication March 29, 2017.

This work was supported by Grant PJ533 from the Cystic Fibrosis Trust. Central funds were obtained from the Centre for Drug Safety Science, which is supported by the Medical Research Council (Grant G0700654).

Address correspondence and reprint requests to Prof. B. Kevin Park, Medical Research Council Centre for Drug Safety Science, Department of Molecular and Clinical Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, Liverpool L69 3GE, U.K. E-mail address: bkpark@liv.ac.uk

Abbreviations used in this article: ACN, acetonitrile; CF, cystic fibrosis; HSA, human serum albumin; LC-MS, liquid chromatography coupled with MS; LTT, lymphocyte transformation test; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem MS; Z, benzyloxycarbonyl.

Copyright © 2017 by The American Association of Immunologists, Inc. 0022-1767/17/\$30.00

generated using PBMCs 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 analyze TCR usage, chemokine receptor expression, and cross-reactivity. The study focused on piperacillin, an i.v. β -lactam antibiotic that is often administered to patients with cystic fibrosis (CF) for the treatment of recurrent respiratory infections. Hypersensitivity reactions have been reported to develop in ~30% of patients exposed to multiple courses of the drug (21). Moreover, we have recently reported on the profile of drug–protein conjugation at specific lysine residues with respect to dose and incubation time, the formation of two forms of drug hapten (with an intact or hydrolyzed 4-ethyl-2,3-dioxopiperazine ring; Fig. 1A), and the presence of piperacillin hapten-specific CD4⁺ T cells in ~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 adducts required to activate T cells in vitro.

Materials and Methods

Patient details

Plasma samples were isolated from blood of 10 tolerant patients with CF, prior to piperacillin exposure and immediately after a standard 14-d treatment course (4.5 g, four times a day), and 3 piperacillin-hypersensitive patients at the time that the reaction was diagnosed. Clinical characteristics of the patients are summarized in Table I. Plasma was aliquoted and stored at -80°C immediately after isolation for characterization and quantification of piperacillin hapten HSA adducts.

PBMCs were also isolated from the blood of three patients with a history of delayed-onset piperacillin-mediated hypersensitivity. The patients' demographics, clinical features of reactions, skin testing, and lymphocyte transformation test (LTT) results are summarized in Table II. 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' PBMCs (0.15×10^6 per well) against piperacillin (62.5–2000 μM) and tetanus toxoid (5 $\mu\text{g}/\text{ml}$; positive control) was measured using the LTT (8). Proliferative responses were measured by the addition of [³H]thymidine for the final 16 h of the assay. Cells were cultured in RPMI 1640 medium containing 10% AB serum, 100 mM L-glutamine, 25 mM HEPES, and 25 $\mu\text{g}/\text{ml}$ transferrin.

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, 4 of which (Lys¹⁹⁰, Lys¹⁹⁵, Lys⁴³², and Lys⁵⁴¹) were detected in patient 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: triphenylmethyl 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 Fig. 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 performed twice with 20% piperidine in chloroform for 10 min. The efficiency of these reactions was evaluated by a ninhydrin colorimetric reaction. Once the synthesis was complete, the deprotection processes were carried out following a series of sequential steps: benzyl or Z groups were removed by catalytic transfer hydrogenation, Fmoc was removed with 50% diethylamine in acetonitrile (ACN), and cold diethyl ether was added to precipitate the peptide. The crude peptide was purified by semipreparative HPLC on a Jupiter C18 column (10 μm C18, 250 mm $\times$ 10 mm; Phenomenex, Macclesfield, 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 ACN) over 30 min at a flow rate of 5 ml/min. The purity of peptide was determined by UV

spectroscopy and the structure was characterized by tandem MS (MS/MS) analysis. The ¹H nuclear magnetic resonance spectra were recorded in CDCl₃ or MeOD at 400 MHz on a Bruker AVANCE NMR spectrometer.

Isolation of HSA from plasma

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

MS analysis of piperacillin-modified HSA

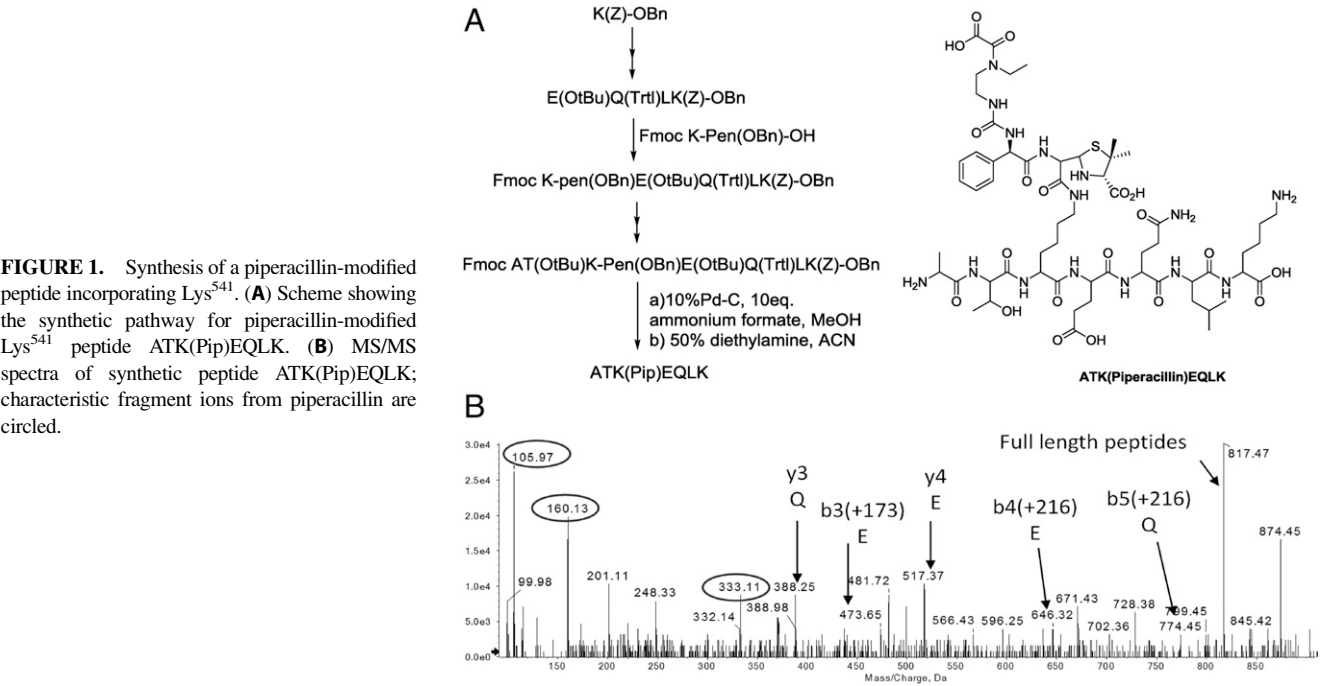
Analyses were performed on a 5500 QTRAP hybrid triple-quadrupole/linear ion trap instrument with a Nanospray II source (AB SCIEX, Foster City, CA) and automated in-line liquid chromatography (U3000 HPLC system, 5 mm C18 nano precolumn and 75 $\mu\text{m} \times 15$ cm C18 PepMap column; Dionex) via a 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 was set to 18, and the interface heater was set to 150°C . Multiple reaction monitoring (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 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 ionization efficiency of the peptides, relative MRM peak areas for each of the modified peptides were determined using MultiQuant software version 2.0 (AB SCIEX). The total ion count for the whole digest for each sample was normalized to an internal synthetic peptide ATK(EQLK) that contains unmodified Lys⁵⁴¹; 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, and 750 fmol) were prepared. The quantities of piperacillin modification in samples were calculated against a 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 ultracentrifuging with a 3000 m.w. cut-off centrifugal filter (Amicon Ultra-15; Millipore, Watford, U.K.), according to the manufacturer's protocol. Briefly, 600 μl of conjugates was added to the filter device, followed by the addition of 14 ml of phosphate buffer (10 mM [pH 7.4]). The device was centrifuged at $5000 \times g$ for 20 min, and the filtrate was discarded. The washing process was repeated six times until the concentration of the free drug had been sufficiently reduced. The concentration of free drug in the filtrate from the last wash was determined by LC-MS to be <2.5 nM.

Generation of T cell clones

Ag-specific T cells were enriched by culturing PBMCs with piperacillin or the piperacillin albumin adduct (generated using a molar ratio of 250:1 [drug/protein] for 24 h) for 14 d. IL-2 (60 U/ml) was added to maintain Ag-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 (5×10^4) were incubated with irradiated APCs (EBV-transformed B cell lines; 1×10^4) and piperacillin (2 mM) or a piperacillin albumin adduct (2 mg/ml) for 48 h. [³H]Thymidine (0.5 μCi) was added, and proliferation was measured 16 h later by scintillation counting. TCR V β protein expression was measured by flow cytometry using the IOTest β Mark TCR V β repertoire kit (Immunotech, Beckman Coulter, High Wycombe, U.K.). Data were analyzed using Cyflog software (CyFlo, Kirkkonummi, Finland). Cell phenotyping was performed using Abs 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, as well as 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 APCs and titrated concentrations of piperacillin (0.01–2 mM) or with APCs pulsed with titrated concentrations of piperacillin (0.1–2 mM) for 1–48 h. In the latter experiments, APCs were washed with medium (3 × 5 ml) and cultured with clones in the absence of free drug.

Finally, APCs 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 [³H]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

Table I. Clinical characteristics of the hypersensitive and drug-tolerant patients used in MS analyses

Patient Identification	Drug	Reaction	Clinical Features	Time to Reaction (d)	Day of Analysis ^a	No. of Courses Prior to Reaction
Hypersensitive patients						
032	Tazocin	Maculopapular rash	Age 33 y, male; df508/df508; chronic <i>Pseudomonas</i> infection; FEV1 = 2.27 l (57% predicted); pancreatic insufficient; CF-related diabetes mellitus	1	1	8
040	Tazocin	Facial rash, pruritis	Age 20 y, female; df508/df508; chronic <i>Pseudomonas</i> infection; FEV1 = 0.43 l (15% predicted); pancreatic insufficient; CF-related diabetes mellitus	6	6	6
059	Tazocin	Maculopapular rash	Age 26 y, female; df508/unknown; chronic <i>Pseudomonas</i> infection; FEV1 = 1.84 l (54%); pancreatic insufficient	10	10	8
Tolerant patients						
006	Tazocin	n/a		n/a	14	n/a
016	Piperacillin	n/a		n/a	14	n/a
020	Piperacillin	n/a		n/a	14	n/a
030	Tazocin	n/a		n/a	14	n/a
026	Tazocin	n/a		n/a	14	n/a
022	Tazocin	n/a		n/a	14	n/a
009	Tazocin	n/a		n/a	14	n/a
017	Tazocin	n/a		n/a	14	n/a
029	Tazocin	n/a		n/a	14	n/a
025	Tazocin	n/a		n/a	14	n/a

^aDay after commencement of therapy on which plasma HSA was analyzed by MS for piperacillin covalent binding.
FEV1, forced expiratory volume in 1 s.
n/a, not applicable.

PBMCs with a synthetic piperacillin–HSA conjugate. First, clones were incubated with APCs and titrated concentrations of soluble piperacillin or the piperacillin albumin adduct generated using a molar ratio of 250:1 (drug/protein) for 24 h (0.25–2 mg/ml). Unmodified albumin subjected to the same extraction protocol was used as a control. Second, clones were incubated with APCs and piperacillin albumin adducts generated using molar ratios of 2:1–250:1 (drug/protein) for 24 h (2 mg/ml). Third, APCs were fixed with glutaraldehyde to block protein processing and the generation of new MHC-associated peptide Ags. Finally, cross-reactivity was assessed by incubating clones with APCs and piperacillin, penicillin G, or amoxicillin albumin adducts generated using molar ratios of 100:1–250:1 (drug/protein) for 24 h (2 mg/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 analyze T cell responses. Experiments were conducted in duplicate or triplicate. Mean $\pm$ SD was calculated, and statistical analyses were performed using a 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 (Fig. 1A).

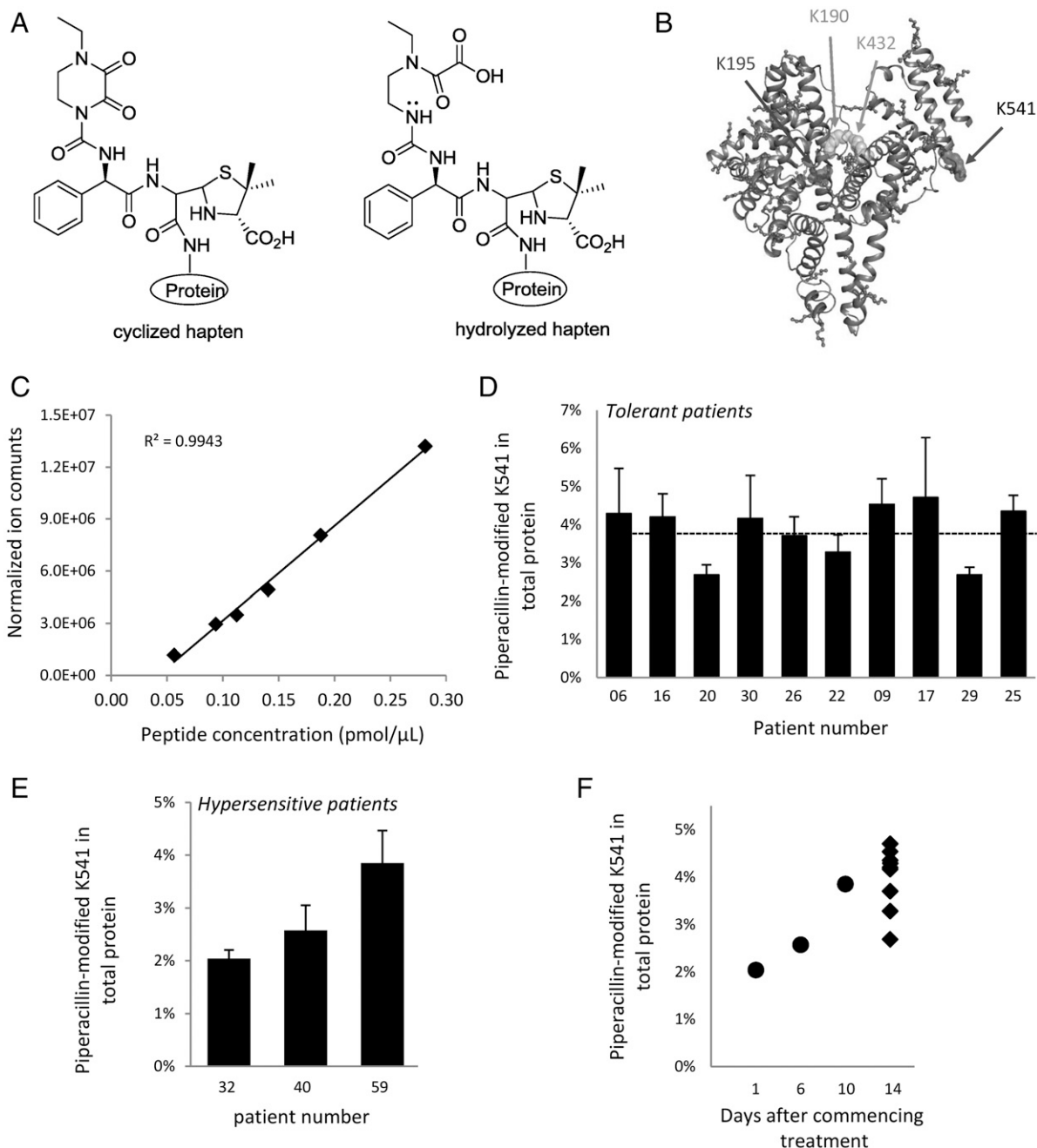


FIGURE 2. Piperacillin hapten structures and absolute quantification of piperacillin hapten albumin binding in tolerant and hypersensitive patient plasma. **(A)** Scheme showing the two potential piperacillin haptens bound covalently to protein. **(B)** Model of albumin showing piperacillin lysine binding sites in patients. **(C)** Standard curve constructed using synthetic piperacillin–modified Lys⁵⁴¹ peptide, with concentrations ranging from 0.05 to 1.5 μ M. Absolute level of piperacillin-modified Lys⁵⁴¹ peptide detected in plasma from tolerant **(D)** and hypersensitive **(E)** patients. MS analysis was repeated on three separate occasions. Dashed line in **(D)** indicates mean value. **(F)** Comparison of piperacillin Lys⁵⁴¹ binding in hypersensitive (●) and tolerant (◆) patients showing the day of analysis.

The presence of a doubly charged ion at m/z 676.8 on the MS/MS spectrum (Fig. 1B), corresponding to the peptide ATK(E)QLK with an additional mass of 535 amu, demonstrated that a hydrolyzed 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 Lys⁵⁴¹ (Fig. 1B, Table I). Purification of crude peptide products by HPLC afforded peptide ATK(piperacillin)EQLK at >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 (cyclized and hydrolyzed, Fig. 2A), the hydrolyzed form was the only hapten detected on albumin lysine residues in tolerant patient plasma, with tryptic peptides incorporating Lys⁵⁴¹ and Lys¹⁹⁰ being the dominant sites of modification (results not shown). Four lysine residues (Lys¹⁹⁰, Lys⁴³², Lys⁵²⁵, and Lys⁵⁴¹) were detected in all patients (Fig. 2B). A calibration curve was constructed using the synthetic piperacillin-Lys⁵⁴¹ peptide to quantify piperacillin albumin binding in tolerant patient plasma after a 14-d treatment course (Fig. 2C); the analyses were conducted on three separate occasions. The level of modification of Lys⁵⁴¹ in total HSA ranged from 2.7 to 4.7% (mean 3.86%), with six patients displaying modification levels $\geq$ 4% (Fig. 2D).

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

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 three LTT+ patients with a history of piperacillin-mediated maculopapular exanthema (Tables I, II). The majority of clones were CD4⁺; four clones expressed CD8, whereas two clones expressed CD4 and CD8 receptors. Fifteen distinct V β TCRs were detected on 53 of 64 clones analyzed. TCR V β 5.1, 13.1, and 17 were detected with the greatest frequency (Fig. 3A). As described recently (22), clones expressed several chemokine receptors, including CCR4, CCR9, CCR10, and CXCR3 (Fig. 3B). Soluble piperacillin, which forms conjugates with multiple proteins in culture medium, activated the clones in a concentration-dependent manner (0.5–4 mM, Fig. 3C).

Twenty CD4⁺ clones were used to study the relationship between piperacillin hapten protein binding and T cell activation. Because 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 five of nine clones at a concentration of 0.01 mM (Fig. 3D, 3E). However, statistical significance was only reached at concentrations $\geq$ 0.1 mM (Fig. 3D). Coinciding with the T cell response at 0.1 mM piperacillin, $\sim$ 1% of piperacillin-modified Lys⁵⁴¹ was detectable after 48 h. Increasing the concentration of piperacillin resulted in higher levels of piperacillin hapten–modified Lys⁵⁴¹, with 4.7% modification detected at 2 mM piperacillin. Moreover, piperacillin hapten albumin binding was detected at earlier time points (4–24 h) with piperacillin concentrations ranging from 0.5 to 4 mM (Fig. 3F). As described previously, fixation of APCs, which blocks protein processing (23–26), prevented the piperacillin-specific activation of all clones (results not shown).

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

Patient Identification	Age (y)/Gender	Clinical Features	Drug	Reaction	Delay Between Course Initiation and Reaction (d)	Time Since Reaction (y)	Delayed ID Readings	Piperacillin LTT
A	26/M	Chronic <i>Pseudomonas</i> infection; FEV1 2.87 l (68% predicted); BMI = 23; CF-related diabetes; 5 d of i.v. antibiotics over last 12 mo	Piperacillin	MPE/fevers	9	12	—	++
			Aztreonam	MPE	4	10	—	
			Ceftazidime	Delayed angioedema	3	10	—	
B	28/M	Chronic <i>Pseudomonas</i> infection; FEV1 0.61 l (15% predicted); BMI = 16; osteoporosis; 197 d of i.v. antibiotics over past 12 mo	Meropenem	MPE	5	10	—	
			Piperacillin	MPE	11	9	+ve at 24 h	+++
C	27/M	Chronic <i>Pseudomonas</i> infection; FEV1 1.1 l (28% predicted); BMI = 17; osteoporosis; 96 d of i.v. antibiotics over past 12 mo	Ceftazidime	MPE	5	9	—	++++
			Piperacillin	MPE/fever	2	5	+ve at 48 h	

—, negative response; ++, stimulation index (SI) 5–10; +++, SI 10–20; +++, SI >20; BMI, body mass index; ID, intradermal; M, male; MPE, maculopapular exanthema; +ve, positive.

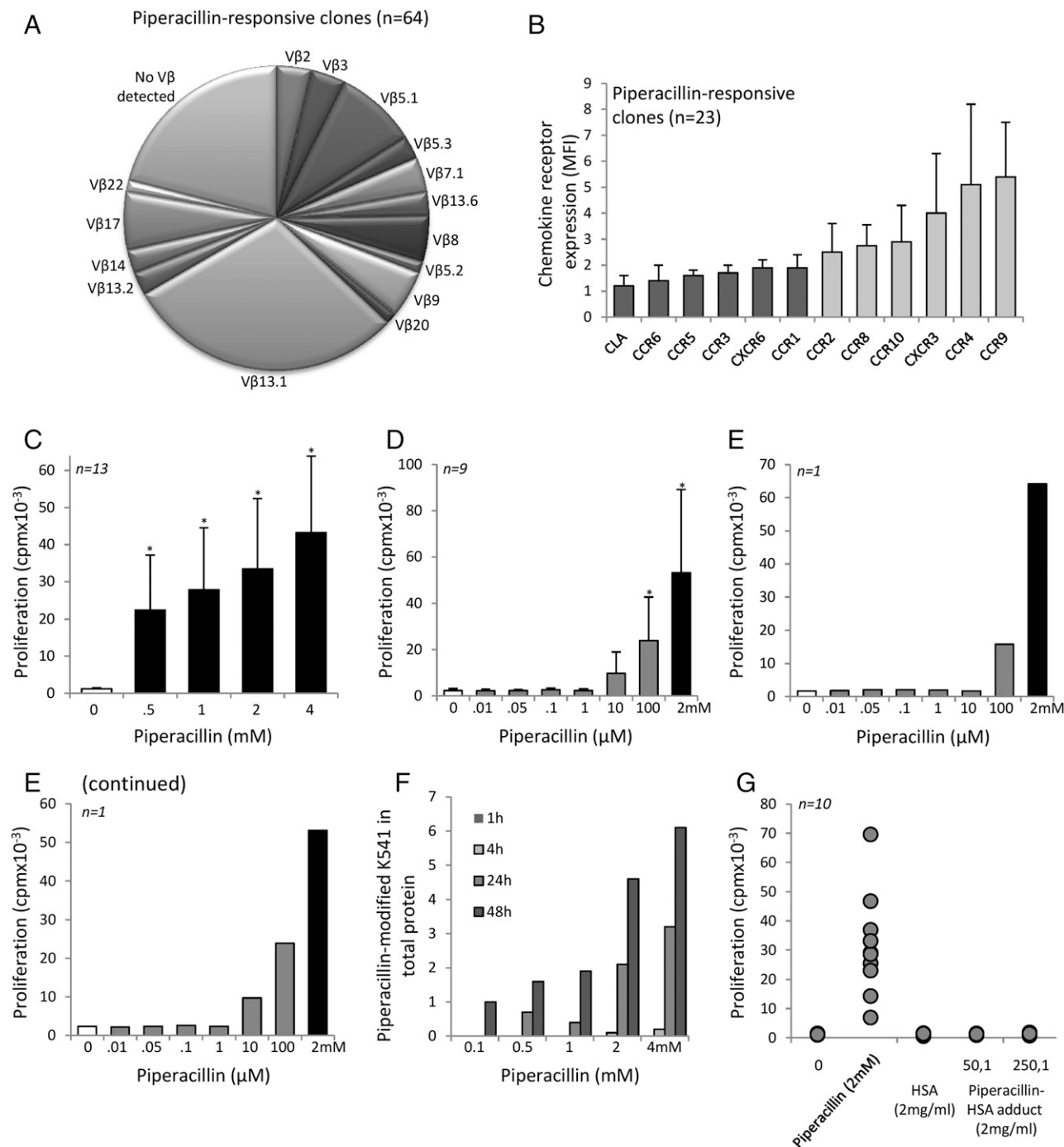


FIGURE 3. Processing-dependent activation of piperacillin-responsive CD4⁺ clones from hypersensitive patients and quantification of piperacillin albumin binding. Flow cytometric analysis of TCR Vβ (A) and chemokine receptor (B) expression on piperacillin-responsive clones. T cell clones were cultured with irradiated APCs and piperacillin [0.5–4 mM (C); 0.01–100 μM (D)], and proliferative responses were measured by [³H]thymidine uptake. (E) Representative proliferative response of two clones shown in (D). (F) Absolute level of time-dependent piperacillin-modified Lys⁵⁴¹ peptide detected in culture supernatant. (G) T cell clones were cultured with irradiated APCs and piperacillin or HSA adducts, and proliferative responses were measured by [³H]thymidine uptake. **p* < 0.05.

In contrast to our previous study using a piperacillin–albumin conjugate purified through solvent precipitation (free piperacillin concentration, 150 nM) (7), clones were not activated with the albumin conjugate used in this study (Fig. 3G). As described above, free piperacillin was removed from the conjugate by repeated ultrafiltration, and the noncovalently bound drug concentration was <2.5 nM.

The level of piperacillin hapten adduct required for triggering T cells was determined by pulsing APCs with piperacillin (2 mM) for 1, 4, 24, and 48 h prior to washing and exposure of APCs to

clones. APCs pulsed with piperacillin for 1 and 4 h did not stimulate a proliferative response, and only low levels of piperacillin-modified Lys⁵⁴¹ were detected (Fig. 4). In contrast, APCs pulsed for 24 or 48 h stimulated all clones to proliferate, and the strength of the induced response was equivalent to or stronger than that seen with the soluble drug (Fig. 4). From these 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.

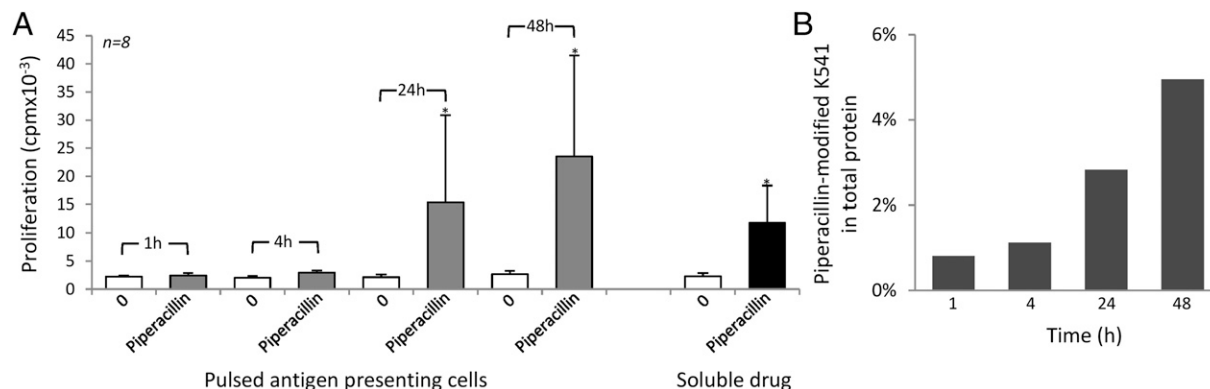


FIGURE 4. Time-dependent activation of CD4⁺ clones with piperacillin-pulsed APCs and quantification of piperacillin albumin binding. **(A)** T cell clones ($n = 8$) were cultured with APCs pulsed with piperacillin (2 mM, 1–48 h), and proliferative responses were measured by [³H]thymidine uptake. APCs were subjected to repeated washing with drug-free medium prior to exposure to clones. **(B)** Absolute level of time-dependent piperacillin-modified Lys⁵⁴¹ peptide detected in APC culture supernatant.

Generation of piperacillin HSA adduct-responsive T cell clones

Because clones generated through the culture of PBMCs 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/HSA at a ratio of 250:1 for 24 h, prior to purification by ultracentrifugation, was used to activate patient PBMCs. After 2 wk in culture, T cells were cloned by serial dilution. Twenty-two CD4⁺ clones and one 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 V β receptor expression; V β 9 was expressed on 15 clones, whereas other clones expressed V β 2, V β 17, and V β 20 (Fig. 5A). Thirteen piperacillin HSA adduct-responsive clones were analyzed for chemokine receptor expression, and high expression of CCR2, CCR4, CCR8, CCR9, CCR10, and CXCR3 was detected (Fig. 5B).

Clones were stimulated to proliferate with titrated concentrations of the piperacillin HSA adduct [0.25–2 mg/ml (Fig. 5C), concentrations ≤ 0.1 mg/ml did not activate clones] via a pathway that is dependent on protein processing (i.e., glutaraldehyde fixation of APCs inhibited the Ag-specific proliferative response [results not shown]). Importantly, unmodified HSA processed in the same way as the piperacillin HSA adduct did not activate the clones (Fig. 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 stimulated proliferation of the clones (Fig. 5E); the strength of the response increased 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 (Fig. 5F, 5G). Despite this, the concentration of piperacillin required to activate the clones and the kinetics for T cell activation (i.e., APC pulse duration) were the same as described with clones depicted in Figs. 3 and 4.

The synthetic piperacillin–HSA conjugates were characterized by MS and Western blot. Epitope profiling showed that cyclized and hydrolyzed hapten were formed; piperacillin-modified Lys⁵⁴¹

was the major site of binding (Fig. 5H, 5I). The levels of hydrolyzed piperacillin-modified Lys⁵⁴¹ 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 (Fig. 5J). The concentration-dependent binding of piperacillin was also demonstrated using Western blotting (Fig. 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 24 h. Epitope profiles showed that the same subset of lysine residues was targeted by all three drugs; however, the relative level of binding differed at the individual sites of modification (Fig. 6A, 6B). Piperacillin HSA adduct-responsive clones were not stimulated to proliferate with penicillin G or amoxicillin HSA adducts (Fig. 6C, 6D).

Discussion

It is 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 Ags to the TCR. In vitro analyses have revealed that certain drugs bind directly to the MHC peptide binding cleft and/or prebound peptides to activate T cells (25–30). However, the dominant pathway for drugs such as the β -lactam antibiotics (12, 25, 31–33) and sulfonamides (34–36) 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 APCs liberating peptides that associate with MHC molecules to activate T cells.

Our previous studies have demonstrated that the β -lactam antibiotic piperacillin covalently modifies lysine 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 150 nM piperacillin bound noncovalently to the protein. We repeated these experiments and found comparable results (data not shown). However, in the current study using a fully characterized and highly purified HSA adduct, for which we used low m.w. MS to exclude the presence of noncovalently

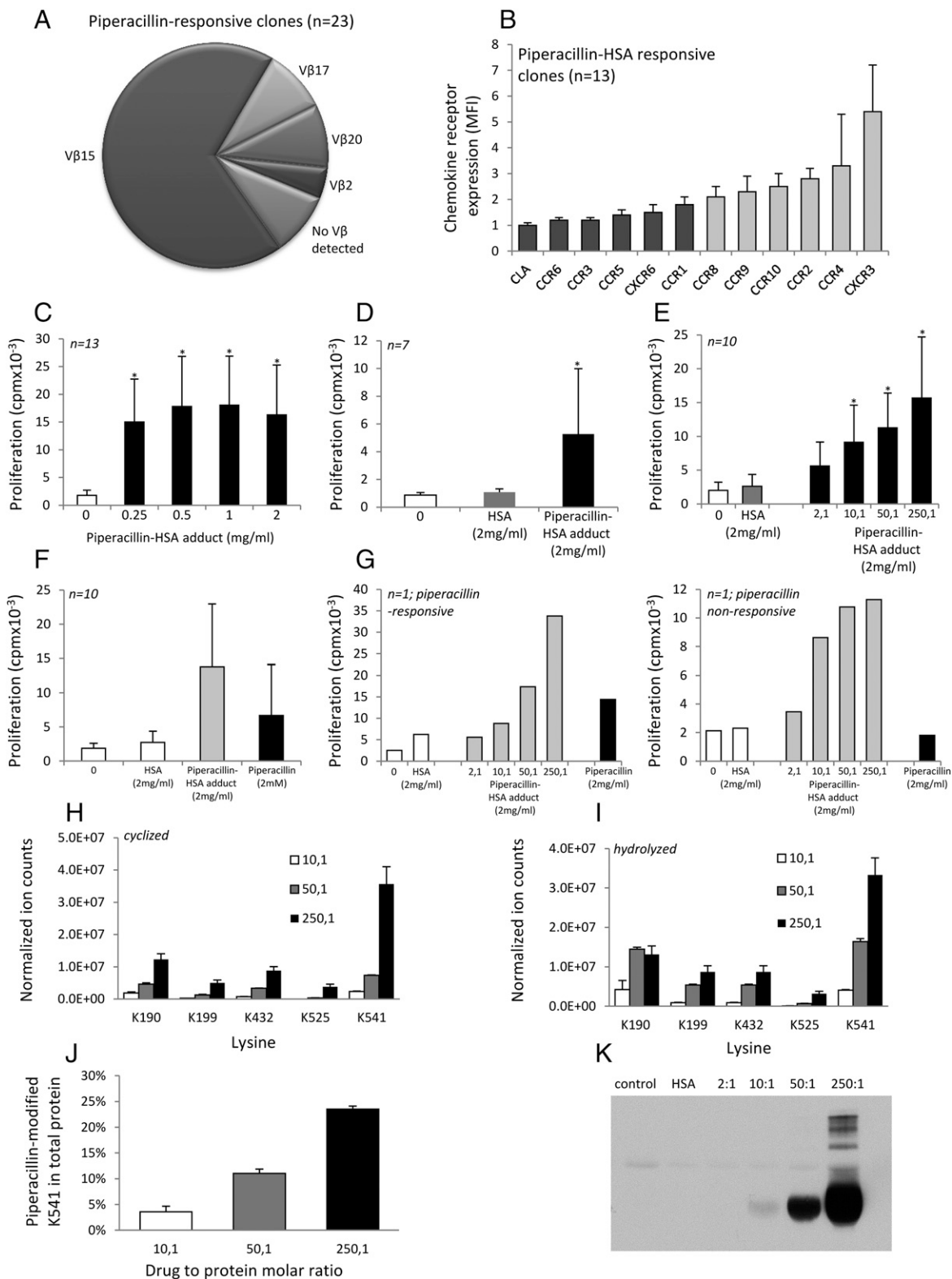


FIGURE 5. Processing-dependent activation of piperacillin-modified albumin-responsive CD4⁺ clones from hypersensitive patients and quantification of piperacillin albumin binding. Flow cytometric analysis of TCR Vβ (A) and chemokine receptor expression (B) on piperacillin-responsive clones. (C) T cell clones were cultured with irradiated APCs and piperacillin-modified HSA (generated using a molar ratio of 250:1 drug/protein for 24 h), and proliferative responses were measured by [³H]thymidine uptake. (D) Proliferative response of T cell clones cultured with unmodified and piperacillin-modified HSA and APCs. (E) Proliferative response of T cell clones cultured with APCs and piperacillin-modified HSA adducts generated using different molar ratios of drug/protein. Proliferative response of T cell clones cultured with APCs and piperacillin-modified HSA adducts or piperacillin [combined data (F); representative clones (G)]. Relative quantification of cyclized (H) and hydrolyzed (I) forms of the piperacillin hapten generated at different lysine residues on the piperacillin albumin adduct (drug/protein ratios of 10:1–250:1). (J) Absolute level of piperacillin-modified Lys⁵⁴¹ peptide detected on the piperacillin albumin adduct generated at drug/protein ratios of 10:1–250:1. (K) Western blot analysis of the piperacillin albumin adducts. **p* < 0.05.

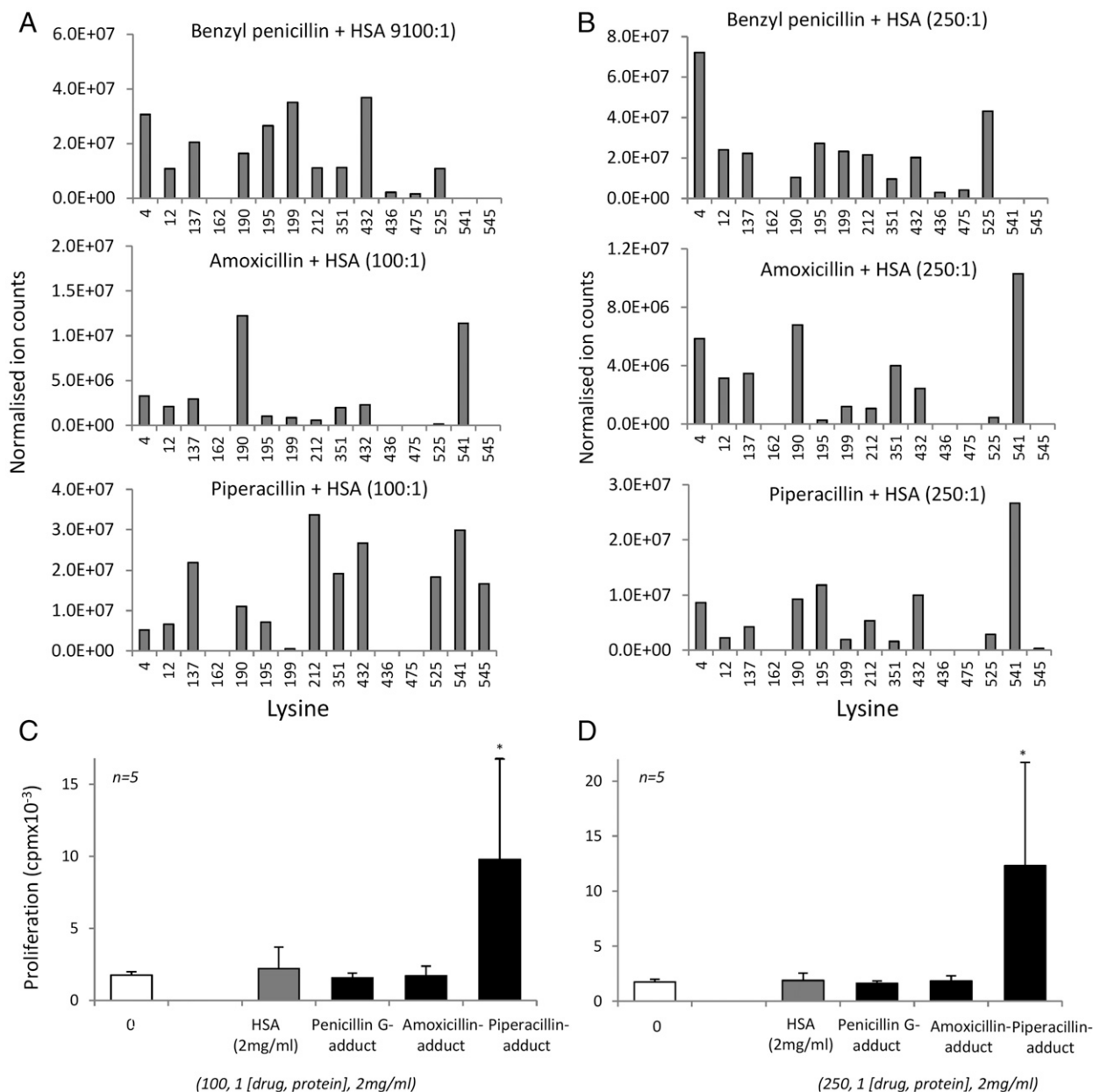


FIGURE 6. Piperacillin albumin adduct-responsive CD4⁺ clones are not activated with alternative β -lactam albumin adducts. Relative MS quantification of benzyl penicillin, amoxicillin, and piperacillin haptens formed at different lysine residues in vitro when the drug was incubated at drug/protein ratios of 100:1 (**A**) and 250:1 (**B**). T cell clones ($n = 5$) were cultured with irradiated APCs and benzyl penicillin, amoxicillin, or piperacillin-modified HSA [generated using a molar ratio of drug/protein of 100:1 (**C**) or 250:1 (**D**) for 24 h], and proliferative responses were measured by [³H]thymidine uptake. * $p < 0.05$.

associated piperacillin above an analytical limit of 2.5 nM, we can now rigorously demonstrate two classes of clones from the same patient: clones stimulated by a single protein (albumin) adduct per se and clones stimulated as a result of modification of proteins present in the incubation by addition of the parent drug. CD4⁺ T cells expressing skin-homing receptors, such as CCR4 and CCR10, were selectively activated with the piperacillin albumin adduct or the parent drug, via a hapten mechanism. All clones were activated with piperacillin-pulsed APCs. Furthermore, fixation of APCs, which blocks protein processing, inhibited the activation of T cells with piperacillin. Thus, we did not identify clones that were activated with the parent drug bound directly to MHC via a pharmacological interaction mechanism. The piperacillin albumin adduct-responsive clones displayed a more restricted profile of V β receptors, which may relate to the limited number of epitopes available for MHC TCR binding (~10–12)

compared with the parent drug, which, in theory, could modify thousands of serum and/or cellular proteins within the culture medium. Analysis of piperacillin hapten binding revealed that piperacillin binds to HSA within 24 h at comparable levels in patient plasma and in vitro. Thus, antigenic determinants with the potential to activate T cells and cause tissue injury are formed in all patients exposed to the drug.

The availability of piperacillin hapten-specific clones with specificity for albumin adducts led us to investigate whether the level of piperacillin HSA modification differs in tolerant and hypersensitive individuals exposed to the same treatment regimen. To do this, a synthetic drug hapten peptide standard incorporating amino acid residues found in the native protein (37, 38) was generated. A total of 3.9% of Lys⁵⁴¹ in HSA isolated from plasma of tolerant patients exposed to piperacillin for 14 d was modified with the piperacillin hapten. The level of Lys⁵⁴¹ binding ranged

from 2.7 to 4.7%, indicating that there is a 2-fold difference in exposure to piperacillin HSA adducts in patients exposed to the same treatment regimen. Somewhat surprisingly, the piperacillin hapten with a hydrolyzed 4-ethyl-2,3-dioxopiperazine ring was the only moiety bound to HSA in patient plasma, which indicates that this form of the hapten almost certainly interacts with TCRs expressed on Ag-specific T cells.

The levels of Lys⁵⁴¹ modification on albumin from patients sampled on the day of hypersensitivity diagnosis (days 1, 6, and 10) matched those circulating in tolerant patients. This indicates that the level of covalent binding in all patients exposed to piperacillin is sufficient to activate T cells; however, only a portion of patients develop hypersensitivity. Thus, the propensity to develop hypersensitivity may be dependent 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. Interestingly, piperacillin albumin adducts were formed rapidly after commencement of piperacillin therapy. After day 1 of treatment, the level of Lys⁵⁴¹ modification exceeded 2%.

These data quantifying piperacillin protein adducts in plasma led us to measure the threshold level of HSA modification using piperacillin concentrations required to activate T cells. CD4⁺ T cell clones were cultured with APCs and titrated concentrations of piperacillin to ascertain the lowest drug concentration associated with a significant proliferative response. Piperacillin-modified Lys⁵⁴¹ was detectable at 0.1 mM, the lowest concentration associated with T cell-proliferative responses. A dose-dependent increase in the level of modification was observed with piperacillin concentrations associated with a 20-fold increase in the proliferation of T cells (i.e., 0.5–4 mM). Importantly, equivalent levels of Lys⁵⁴¹ modification were detected in patient plasma and in vitro with piperacillin concentrations that stimulated a T cell response. To estimate the absolute levels of HSA binding at the earliest possible time that T cells are activated, APCs were pulsed with piperacillin (2 mM) for 1–48 h prior to washing and exposure of clones to the pulsed cells. APCs pulsed with piperacillin for 24 h stimulated all clones to proliferate, and the response was stronger than that seen with the soluble drug. At this time point, 2.8% of Lys⁵⁴¹ was modified with the piperacillin hapten.

Because 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 assess the relationship between the level of hapten binding and the T cell response, as well as to 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 glutaraldehyde 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; 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 noncovalent interaction, which positions the drug favorably to facilitate covalent binding. Therefore, the three-dimensional shape of the drug, as well as its inherent chemical reactivity, determines selectivity of covalent binding and, as demonstrated in Fig. 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 MS 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 drug Ag-specific T cell response and clinical manifestations of hypersensitivity. To do this, we have recently initiated a prospective investigation of piperacillin hypersensitivity. Blood samples are being collected during repeated drug courses and when patients develop an adverse event to define the quantitative relationship between Ag formation and the factors that control the balance between immune tolerance and activation.

Acknowledgments

We thank the patients and volunteers for agreeing to donate blood and tissue samples.

Disclosures

The authors have no financial conflicts of interest.

References

- Uetrecht, J., and D. J. Naisbitt. 2013. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol. Rev.* 65: 779–808.
- Park, B. K., A. Boobis, S. Clarke, C. E. Goldring, D. Jones, J. G. Kenna, C. Lambert, H. G. Lavery, D. J. Naisbitt, S. Nelson, et al. 2011. Managing the challenge of chemically reactive metabolites in drug development. *Nat. Rev. Drug Discov.* 10: 292–306.
- Levine, B. B., and Z. Ovary. 1961. Studies on the mechanism of the formation of the penicillin antigen. III. The N-(D-alpha-benzylpenicilloyl) group as an antigenic determinant responsible for hypersensitivity to penicillin G. *J. Exp. Med.* 114: 875–904.
- Ariza, A., D. Collado, Y. Vida, M. I. Montañez, E. Pérez-Inestrosa, M. Blanca, M. J. Torres, F. J. Cañada, and D. Pérez-Sala. 2014. Study of protein haptation by amoxicillin through the use of a biotinylated antibiotic. *PLoS One* 9: e90891.
- Ariza, A., D. Garzon, D. R. Abánades, V. de los Ríos, G. Vistoli, M. J. Torres, M. Carini, G. Aldini, and D. Pérez-Sala. 2012. Protein haptation by amoxicillin: high resolution mass spectrometry analysis and identification of target proteins in serum. *J. Proteomics* 77: 504–520.
- Jenkins, R. E., F. S. Yaseen, M. M. Monshi, P. Whitaker, X. Meng, J. Farrell, J. Hamlett, J. P. Sanderson, S. El-Ghaiesh, D. Peckham, et al. 2013. β -Lactam antibiotics form distinct haptenic structures on albumin and activate drug-specific T-lymphocyte responses in multiallergic patients with cystic fibrosis. *Chem. Res. Toxicol.* 26: 963–975.
- El-Ghaiesh, S., M. M. Monshi, P. Whitaker, R. Jenkins, X. Meng, J. Farrell, A. Elsheikh, D. Peckham, N. French, M. Pirmohamed, et al. 2012. Characterization of the antigen specificity of T-cell clones from piperacillin-hypersensitive patients with cystic fibrosis. *J. Pharmacol. Exp. Ther.* 341: 597–610.
- Whitaker, P., X. Meng, S. N. Laverne, S. El-Ghaiesh, M. Monshi, C. Earnshaw, D. Peckham, J. Gooi, S. Conway, M. Pirmohamed, et al. 2011. Mass spectrometric characterization of circulating and functional antigens derived from piperacillin in patients with cystic fibrosis. *J. Immunol.* 187: 200–211.
- Meng, X., R. E. Jenkins, N. G. Berry, J. L. Maggs, J. Farrell, C. S. Lane, A. V. Stachulski, N. S. French, D. J. Naisbitt, M. Pirmohamed, and B. K. Park. 2011. Direct evidence for the formation of diastereoisomeric benzylpenicilloyl haptens from benzylpenicillin and benzylpenicillenic acid in patients. *J. Pharmacol. Exp. Ther.* 338: 841–849.
- Sudlow, G., D. J. Birkett, and D. N. Wade. 1975. Spectroscopic techniques in the study of protein binding. A fluorescence technique for the evaluation of the albumin binding and displacement of warfarin and warfarin-alcohol. *Clin. Exp. Pharmacol. Physiol.* 2: 129–140.
- Pan, J., Z. Ye, X. Cai, L. Wang, and Z. Cao. 2012. Biophysical study on the interaction of ceftriaxone sodium with bovine serum albumin using spectroscopic methods. *J. Biochem. Mol. Toxicol.* 26: 487–492.
- Brander, C., D. Mauri-Hellweg, F. Bettens, H. Rolli, M. Goldman, and W. J. Pichler. 1995. Heterogeneous T cell responses to beta-lactam-modified self-structures are observed in penicillin-allergic individuals. *J. Immunol.* 155: 2670–2678.
- Padovan, E., D. Mauri-Hellweg, W. J. Pichler, and H. U. Weltzien. 1996. T cell recognition of penicillin G: structural features determining antigenic specificity. *Eur. J. Immunol.* 26: 42–48.
- Aguilar-Pimentel, J. A., F. Alessandrini, K. M. Huster, T. Jakob, H. Schulz, H. Behrendt, J. Ring, M. H. de Angelis, D. H. Busch, M. Mempel, and M. Ollert. 2010. Specific CD8 T cells in IgE-mediated allergy correlate with allergen dose and allergic phenotype. *Am. J. Respir. Crit. Care Med.* 181: 7–16.
- Long, S. A., M. Rieck, M. Tatum, P. L. Bollyky, R. P. Wu, I. Muller, J. C. Ho, H. G. Shilling, and J. H. Buckner. 2011. Low-dose antigen promotes induction of FOXP3 in human CD4⁺ T cells. *J. Immunol.* 187: 3511–3520.
- Oling, V., K. Geubtner, J. Ilonen, and H. Reijonen. 2010. A low antigen dose selectively promotes expansion of high-avidity autoreactive T cells with distinct phenotypic characteristics: a study of human autoreactive CD4⁺T cells specific for GAD65. *Autoimmunity* 43: 573–582.

17. Kim, M., H. B. Moon, K. Kim, and K. Y. Lee. 2006. Antigen dose governs the shaping of CTL repertoires in vitro and in vivo. *Int. Immunol.* 18: 435–444.
18. Levine, B. B. 1962. N(alpha-D-penicilloyl) amines as univalent hapten inhibitors of antibody-dependent allergic reactions to penicillin. *J. Med. Pharm. Chem.* 91: 1025–1034.
19. Sashidhar, R. B., A. K. Capoor, and D. Ramana. 1994. Quantitation of epsilon-amino group using amino acids as reference standards by trinitrobenzene sulfonic acid. A simple spectrophotometric method for the estimation of hapten to carrier protein ratio. *J. Immunol. Methods* 167: 121–127.
20. Sánchez-Gómez, F. J., J. M. González-Morena, Y. Vida, E. Pérez, M. Inestrosa, M. J. Blanca, Torres, and D. Pérez-Sala. 2017. Amoxicillin haptens intracellular proteins that can be transported in exosomes to target cells. *Allergy* 72: 385–396.
21. Whitaker, P., D. Naisbitt, and D. Peckham. 2012. Nonimmediate β -lactam reactions in patients with cystic fibrosis. *Curr. Opin. Allergy Clin. Immunol.* 12: 369–375.
22. Sullivan, A., E. Wang, J. Farrell, P. Whitaker, L. Faulkner, D. Peckham, B. K. Park, and D. J. Naisbitt. 2017. β -Lactam hypersensitivity involves expansion of circulating and skin-resident TH22 cells. *J. Allergy Clin. Immunol.* DOI: 10.1016/j.jaci.2017.01.020
23. Zanni, M. P., S. von Greyserz, B. Schnyder, K. A. Brander, K. Frutig, Y. Hari, S. Valitutti, and W. J. Pichler. 1998. HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J. Clin. Invest.* 102: 1591–1598.
24. Yaseen, F. S., K. Saide, S. H. Kim, M. Monshi, A. Tailor, S. Wood, X. Meng, R. Jenkins, L. Faulkner, A. K. Daly, et al. 2015. Promiscuous T-cell responses to drugs and drug-haptens. *J. Allergy Clin. Immunol.* 136: 474–6.e8.
25. Yun, J., M. J. Marcaida, K. K. Eriksson, H. Jamin, S. Fontana, W. J. Pichler, and D. Yerly. 2014. Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B*58:01. *J. Immunol.* 192: 2984–2993.
26. Adam, J., N. Willemin, S. Watkins, H. Jamin, K. K. Eriksson, P. Villiger, S. Fontana, W. J. Pichler, and D. Yerly. 2014. Abacavir induced T cell reactivity from drug naïve individuals shares features of allo-immune responses. *PLoS One* 9: e95339.
27. Illing, P. T., J. P. Vivian, N. L. Dudek, L. Kostenko, Z. Chen, M. Bharadwaj, J. J. Miles, L. Kjer-Nielsen, S. Gras, N. A. Williamson, et al. 2012. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature* 486: 554–558.
28. Ko, T. M., W. H. Chung, C. Y. Wei, H. Y. Shih, J. K. Chen, C. H. Lin, Y. T. Chen, and S. I. Hung. 2011. Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome. *J. Allergy Clin. Immunol.* 128:1266–76.e11.
29. Ostrov, D. A., B. J. Grant, Y. A. Pompeu, J. Sidney, M. Harndahl, S. Southwood, C. Oseroff, S. Lu, J. Jakoncic, C. A. de Oliveira, et al. 2012. Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proc. Natl. Acad. Sci. USA* 109: 9959–9964.
30. Chung, W. H., W. C. Chang, S. L. Stocker, C. G. Juo, G. G. Graham, M. H. Lee, K. M. Williams, Y. C. Tian, K. C. Juan, Y. J. Jan Wu, et al. 2015. Insights into the poor prognosis of allopurinol-induced severe cutaneous adverse reactions: the impact of renal insufficiency, high plasma levels of oxypurinol and granulysin. *Ann. Rheum. Dis.* 74: 2157–2164.
31. Monshi, M. M., L. Faulkner, A. Gibson, R. E. Jenkins, J. Farrell, C. J. Earnshaw, A. Alfirevic, K. Cederbrant, A. K. Daly, N. French, et al. 2013. Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury. *Hepatology* 57: 727–739.
32. Kim, S. H., K. Saide, J. Farrell, L. Faulkner, A. Tailor, M. Ogese, A. K. Daly, M. Pirmohamed, B. K. Park, and D. J. Naisbitt. 2015. Characterization of amoxicillin- and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate-induced liver injury. *Hepatology* 62: 887–899.
33. Padovan, E., T. Bauer, M. M. Tongio, H. Kalbacher, and H. U. Weltzien. 1997. Penicilloyl peptides are recognized as T cell antigenic determinants in penicillin allergy. *Eur. J. Immunol.* 27: 1303–1307.
34. Elsheikh, A., L. Castrejon, S. N. Laverne, P. Whitaker, M. Monshi, H. Callan, S. El-Ghaiesh, J. Farrell, W. J. Pichler, D. Peckham, et al. 2011. Enhanced antigenicity leads to altered immunogenicity in sulfamethoxazole-hypersensitive patients with cystic fibrosis. *J. Allergy Clin. Immunol.* 127: 1543–1551.e3.
35. Castrejon, J. L., N. Berry, S. El-Ghaiesh, B. Gerber, W. J. Pichler, B. K. Park, and D. J. Naisbitt. 2010. Stimulation of human T cells with sulfonamides and sulfonamide metabolites. *J. Allergy Clin. Immunol.* 125: 411–418.e4.
36. Schnyder, B., C. Burkhardt, K. Schnyder-Frutig, S. von Greyserz, D. J. Naisbitt, M. Pirmohamed, B. K. Park, and W. J. Pichler. 2000. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J. Immunol.* 164: 6647–6654.
37. Lange, V., P. Picotti, B. Domon, and R. Aebersold. 2008. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* 4: 222.
38. Gallien, S., E. Duriez, and B. Domon. 2011. Selected reaction monitoring applied to proteomics. *J. Mass Spectrom.* 46: 298–312.