

Letter to the Editor

Promiscuous T-cell responses to drugs and drug-haptens

To the Editor:

Activation of T cells is dependent on the binding of MHC-associated peptides to specific T-cell receptors. Peptide binding stimulates signal transduction events that ultimately lead to the T-cell response. The specific amino acid pattern of peptides determines MHC specificity and the nature of the response.¹ In the context of drug hypersensitivity, the drug (or product of metabolic activation) is postulated to interact with this MHC peptide T-cell receptor complex to trigger T cells that would not otherwise have been activated. The discovery of strong associations between certain HLA class I and class II alleles and different forms of drug hypersensitivity² has renewed interest in characterizing how the drug interacts with the MHC and T-cell receptors. Drug-responsive T cells are activated by (1) HLA-binding peptides derived from hapten-protein adducts,³ (2) peptide sequences displayed by MHC molecules with drugs bound directly to the antigen-binding cleft,⁴ and (3) drugs interacting directly with surface MHC peptide complexes.⁵

Recent studies have shown that T cells can be activated with certain drugs in an HLA allele-restricted manner even when the donor has no history of drug exposure. One example is flucloxacillin, for which pharmacogenetic studies identified a strong association between the expression of HLA-B*57:01 and susceptibility to liver injury.⁶ Willemin et al⁷ generated flucloxacillin-responsive CD8⁺ clones from healthy donors with and without HLA-B*57:01 to evaluate mechanisms of drug antigen presentation. Flucloxacillin was selected in part because it generates a hapten spontaneously in cell culture medium that binds covalently to lysine residues on protein.⁸ Hence, it is possible to differentiate between flucloxacillin and flucloxacillin hapten-responsive T cells through the use of (1) soluble drug and (2) antigen-presenting cells pulsed with flucloxacillin overnight to allow hapten binding, as sources of antigen. T cells from HLA-B*57:01[−] donors were preferentially

activated via a hapten mechanism involving antigen processing. In contrast, the parent drug was found to activate most T cells in HLA-B*57:01⁺ donors, without covalent binding. These data led the authors to conclude that the parent drug drives T-cell responses in HLA-B*57:01⁺ donors and presumably liver reactions in susceptible patients.

We similarly compared/contrasted β -lactam- and β -lactam hapten-specific T-cell responses, but importantly focused on cells from patients *and* healthy donors. In contrast to T cells from healthy donors, T cells from patients with drug-induced liver injury were activated via a hapten mechanism. The drug hapten-specific T-cell response was HLA allele-restricted, whereas the parent drug activated clones in the presence of antigen-presenting cells expressing multiple HLA alleles. Collectively, our data suggest that expansion of drug- and drug hapten-specific clones is dependent on the level of initial drug exposure: a high level of exposure *ex vivo* favors the expansion of HLA allele-unrestricted drug-specific T cells.

In initial experiments, more than 50 MHC class I-restricted CD8⁺ clones were generated from 3 HLA-B*57:01⁺ healthy donors after priming naive T cells with autologous dendritic cells and soluble flucloxacillin (1–2 mM; see this article's **Methods** section in the Online Repository at www.jacionline.org). A detailed description of the functionality of the clones generated using this method has been reported previously.⁹ In agreement with Willemin et al,⁷ most clones were activated to proliferate directly with high concentrations of soluble flucloxacillin (100 μ M and above [peak serum concentrations following therapeutic exposure are 19.4–32.0 μ M]; see **Fig E1** in this article's Online Repository at www.jacionline.org), but not antigen-presenting cells pulsed with flucloxacillin for 16 hours (**Fig 1**; left graph). Binding of flucloxacillin to lysine residues on the model protein human serum albumin was measured to confirm that hapten-protein adducts are formed during the 16-hour culture period (see **Fig E2** in this article's Online Repository at www.jacionline.org). Similar data were obtained on repeat testing with 5 additional clones selected from all 3 donors. Importantly, in this experiment, antigen-presenting

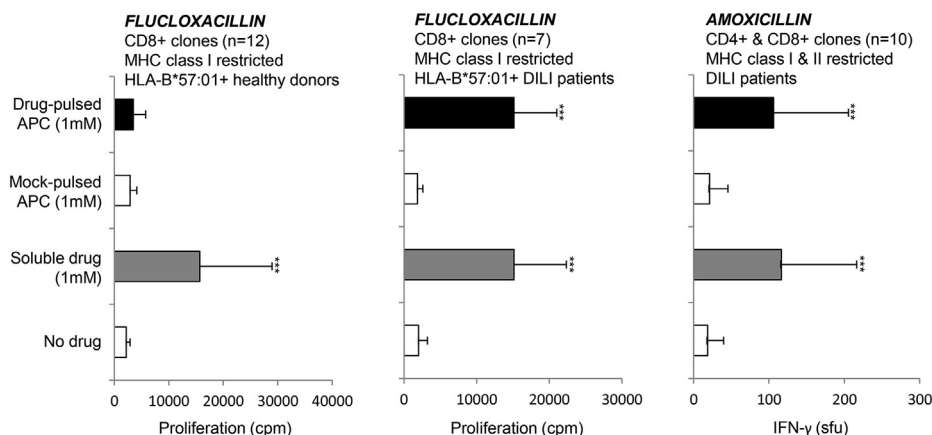


FIG 1. Activation of T-cell clones from healthy donors and patients with flucloxacillin-induced or amoxicillin-clavulanate-induced liver injury. Clones were cultured with soluble drug and autologous antigen-presenting cells (APCs) or drug-pulsed APCs. Proliferation and IFN- γ release were measured after 48 hours by the addition of [³H]thymidine and ELISPOT, respectively. Data presented as mean $\pm$ SD (Student *t* test was used to compare no drug to drug-treated and mock-pulsed to drug-pulsed data sets [****P* < .001]).

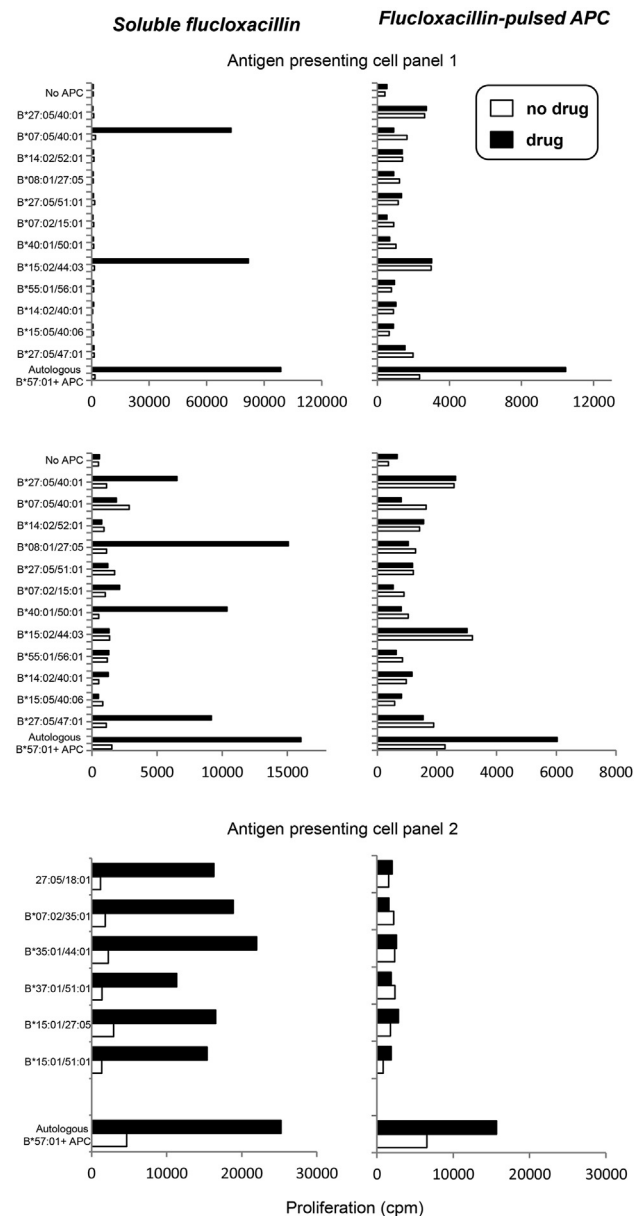


FIG 2. Activation of flucloxacillin-specific CD8⁺ T-cell patient clones with soluble drug (left-hand panel) and drug-pulsed autologous and HLA-mismatched antigen-presenting cells (APCs) (right-hand panel). Proliferation was measured by the addition of [³H]thymidine. Data presented as mean cpm of duplicate cultures. Results from 3 representative clones out of 11 are shown.

cells were pulsed with flucloxacillin for different durations (4–48 hours) and responses were not detected (see Fig E3 in this article's Online Repository at www.jacionline.org).

More than 30 MHC class I-restricted flucloxacillin-specific CD8⁺ T cells were also generated from PBMCs of 3 HLA-B*57:01+ patients with liver injury (clinical details provided in Table E1 in this article's Online Repository at www.jacionline.org; HLA typing is listed in Table E2 in this article's Online Repository at www.jacionline.org). Using the methodological approach outlined above, we obtained a different result with these clones. They were activated with soluble flucloxacillin and flucloxacillin-pulsed antigen-presenting

cells (Fig 1; middle graph). Additional experiments found that T-cell proliferative responses ($n = 5$ clones) were detected consistently when antigen-presenting cells were pulsed with flucloxacillin for 24 to 48 hours (and after 4 hours, to a lesser extent; Fig E3). Thus, flucloxacillin hapten-specific T cells predominate in patients with liver injury. The difference between patient and healthy donor T cells does not relate to a higher sensitivity of the patient clones because all clones responded at the same concentrations with similar dose-response curves (Fig E1).

The availability of CD8⁺ clones from patients with liver injury that were activated with soluble flucloxacillin and flucloxacillin-pulsed antigen-presenting cells allowed us to probe the specificity of the drug HLA-binding interaction. Three panels of EBV-transformed B cells ($n = 5$ –12 antigen-presenting cells per panel) were generated from PBMCs of healthy donors selected from our HLA-typed cell bank. Donors were selected to obtain antigen-presenting cells that expressed a broad array of HLA-B alleles. In antigen-presenting cell pulsing experiments, the clones ($n = 11$, well-growing clones selected from all 3 patients) were activated in an HLA allele-restricted fashion; proliferative responses were seen only with antigen-presenting cells expressing HLA-B*57:01 (Fig 2) and in some instances the closely related HLA-B*58:01 (results not shown). In contrast, additional reactivity was detected with antigen-presenting cells expressing various HLA-B alleles when soluble drug was used as the antigen. Some clones were activated with flucloxacillin in a completely HLA allele-unrestricted fashion (Fig 2; clone 3); other clones were activated in the presence of 20% to 50% of the antigen-presenting cells expressing B alleles other than B*57:01 (Fig 2; clones 1 and 2). None of the clones was activated with flucloxacillin in the absence of antigen-presenting cells.

We have also generated (1) MHC class II, HLA-DR-restricted CD4⁺ and (2) MHC class I-restricted CD8⁺ amoxicillin-specific clones from 3 patients with amoxicillin-clavulanate-induced liver injury to investigate whether the phenomena outlined above with flucloxacillin is restricted to this one drug. In contrast to flucloxacillin, amoxicillin-clavulanate-induced liver injury is less strongly associated with particular HLA genotypes¹⁰ and the involvement of risk alleles in the drug-specific T-cell response has not been defined. All the antigen-specific clones ($n = 10$; 6 CD4⁺ and 4 CD8⁺) were activated with soluble amoxicillin and amoxicillin-pulsed antigen-presenting cells (Fig 1; right graph), suggesting a hapten mechanism. Two amoxicillin-responsive CD8⁺ clones were generated from a healthy donor following dendritic cell priming. In agreement with the flucloxacillin data, one clone was not activated with drug-pulsed antigen-presenting cells while with the other clone IFN- γ release was 50% lower (see Fig E4 in this article's Online Repository at www.jacionline.org; 8 clones from amoxicillin-clavulanate patients were used as a comparator). Although these data are preliminary, they (1) confirm that all patient clones are activated via a hapten mechanism and (2) reactivity against the parent drug is detectable when T cells from healthy donors are primed *in vitro*. HLA mismatch experiments revealed that the response of patient clones to pulsed antigen-presenting cells was again restricted to autologous cells. Amoxicillin-pulsed antigen-presenting cells expressing other HLA alleles did not activate the clones (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast, soluble amoxicillin stimulated IFN- γ release from clones cultured with most allogeneic antigen-presenting cells. Fig E5 shows results from 3 representative clones including

1 clone in which the additional allo-reactivity was detectable even though there was a degree of self-presentation (ie, IFN- γ release with amoxicillin in the absence of antigen-presenting cells; clone 1).

In conclusion, focusing on 2 forms of β -lactam-induced liver injury, we have shown that patients' T cells are activated via a hapten mechanism, whereas T cells from healthy donors are preferentially activated with the parent drug. Furthermore, the drug hapten-specific T-cell response is HLA allele-restricted. These data have important implications for studies focusing solely on drug-specific T cells derived from healthy human donors, in particular abacavir hypersensitivity in which theories have been developed solely on *ex vivo* studies using T cells from healthy donors.

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*Fiazia S. Yaseen, Mrs^a
Katy Saide, Mrs^a
Seung-Hyun Kim, PhD^{a,b}
Manal Monshi, PhD^a
Arun Tailor, Mrs^a
Sally Wood, BSc^a
Xiaoli Meng, PhD^a
Rosalind Jenkins, PhD^a
Lee Faulkner, PhD^a
Ann K. Daly, PhD^c
Munir Pirmohamed, FRCP^a
B. Kevin Park, PhD^a
Dean J. Naisbitt, PhD^a*

From ^athe Department of Clinical and Molecular Pharmacology, Medical Research Council Centre for Drug Safety Science, The University of Liverpool, Liverpool, United Kingdom; ^bthe Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon, Korea; and ^cthe Institute of Cellular Medicine, Newcastle University Medical School, Newcastle upon Tyne, United Kingdom. E-mail: dnes@liv.ac.uk.

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METHODS

Patient characteristics

Three patients with flucloxacillin-induced and amoxicillin-clavulanate-induced liver injury were enrolled from the DILIGEN (Pharmacogenetics of drug induced liver injury) study.^{E1} Causality assessment used the Roussel-Uclaf causality assessment method.^{E2} Clinical characteristics of the patients are described in Table E1. Approval for the study was acquired from the Liverpool local Research Ethics Committee, and informed written consent was obtained from each donor. Genomic DNA was extracted using Chemagic magnetic separation (Chemagen, Baesweiler, Germany), and high-resolution sequence-based HLA typing was performed by the Histogenetics laboratory (Histogenetics, Ossining, NY) at the following loci: HLA-A, -B, -C, -DRB1, -DQB1, and DQA1. Table E2 lists the HLA alleles expressed by the patients. Three healthy donors with no history of β -lactam hypersensitivity were selected as controls.

Generation of T-cell clones from patients with drug-induced liver injury

PBMCs (1×10^6 /well; 0.5 mL) from patients with flucloxacillin-induced and amoxicillin-clavulanate-induced liver injury were cultured with flucloxacillin and amoxicillin, respectively (1–2 mM) in RPMI 1640 supplemented with 10% human AB serum (Class A; Innovative Research, Novi, Mich), 25 mM HEPES, 10 mM L-glutamine, and 25 μ g/mL transferrin (Sigma-Aldrich Co Ltd, Gillingham, United Kingdom). Cultures were supplemented with IL-2 (PeproTech EC Ltd, London, United Kingdom) on days 6 and 9. On day 14, individual T cells were cloned by serial dilution.^{E3}

Priming naive T cells from healthy donors

CD14⁺ cells were cultured in medium containing GM-CSF and IL-4 (PeproTech EC Ltd) for 8 days to generate dendritic cells. TNF- α and LPS (1 μ g/mL) were added for the last 16 hours of culture as a maturation cocktail. CD25 and CD45RO-depleted T cells (2×10^6 T cells/well; 2 mL) were cultured with dendritic cells (8×10^4 cells/well) in the presence of flucloxacillin or amoxicillin (1–2 mM) for 8 days using our recently established protocol.^{E4} The primed T cells were cloned by serial dilution.

Generation of antigen-presenting cells

EBV-transformed B-cell lines were created from PBMCs of patients and healthy donors selected from our HLA-typed cell bank by transformation with supernatant from the virus-producing cell line B9.58. Lines were maintained in RPMI 1640 supplemented with 10% FBS (Invitrogen, Paisley, United Kingdom), 100 mM L-glutamine, 100 μ g/mL penicillin, and 100 U/mL streptomycin and used as a source of autologous or allogeneic antigen-presenting cells.

Drug specificity assays

Drug specificity was initially assessed by culturing autologous irradiated EBV-transformed B cells (1×10^4 /well) and flucloxacillin or amoxicillin (1–2 mM) with T-cell clones (5×10^4 /well; 200 μ L) for 48 hours. Proliferation was measured through the addition of [³H]thymidine (0.5 μ Ci/well, 5 Ci/mmol, Morovek Biochemicals Ltd, Brea, Calif). Clones with a stimulation index of greater than 1.7 were expanded by repetitive stimulation with irradiated allogeneic PBMCs and PHA in medium containing IL-2.

CD phenotyping was conducted using flow cytometry. Dose-dependent T-cell responses were measured by culturing clones and autologous antigen-presenting cells with flucloxacillin or amoxicillin at concentrations between 0.1 and 2 mM for 2 days before analysis of [³H]thymidine incorporation or IFN- γ release by ELISPOT. Previous studies focusing exclusively on flucloxacillin-specific T-cell clones from healthy donors used antigen-presenting cell pulsing experiments to discriminate between hapten-responsive T cells and T cells activated with the parent drug through

a direct interaction with surface MHC molecules.^{E5} In this article, we used a similar method with 29 clones from healthy donors and patients with flucloxacillin-induced and amoxicillin-clavulanate-induced liver injury. Briefly, antigen-presenting cells (1×10^6 ; total volume 1 mL) were cultured with flucloxacillin or amoxicillin (1–2 mM) for 16 hours before 3 washing steps with medium to remove non-covalently bound drug. Clones (5×10^4) were then cultured with drug-pulsed antigen-presenting cells (1×10^4 ; for 48 hours). Proliferative responses were measured using [³H]thymidine and compared with responses obtained with soluble drug. An aliquot of supernatant was collected from the antigen-presenting cell drug culture after 16 hours to measure the lysine residues in human serum albumin modified with either flucloxacillin or amoxicillin after 16 hours using established methods.^{E6}

In repeat experiments, flucloxacillin-responsive clones ($n = 5$ from patients with liver injury and $n = 5$ from healthy donors) were cultured with antigen-presenting cells pulsed with flucloxacillin for 4 to 48 hours and proliferation was measured 48 hours later by incorporation of [³H]thymidine.

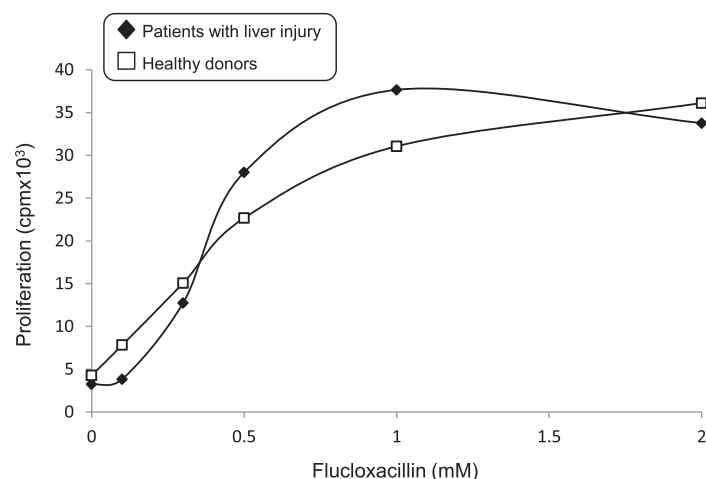
Drug specificity assays using autologous and allogeneic antigen-presenting cells

To investigate the importance of specific HLA alleles in the drug-specific T-cell response, flucloxacillin- and amoxicillin-responsive clones were cultured with the stimulatory drug and antigen-presenting cells expressing a range of different HLA alleles. To study flucloxacillin-specific T-cell responses, 3 panels of antigen-presenting cell (EBV-transformed B cells; $n = 5$ –12 antigen-presenting cells per panel) expressing different HLA-B alleles were generated and tested alongside the autologous HLA-B*57:01 + antigen-presenting cells in a standard proliferation assay using [³H]thymidine to detect specific responses. The number of antigen-presenting cells tested with each clone was dependent on the availability of T cells. In parallel experiments, activation of clones with flucloxacillin-pulsed autologous and HLA-mismatched antigen-presenting cells was examined using methods outlined above. In total, 11 CD8⁺ clones originating from different DILI patients were analyzed.

In amoxicillin-clavulanate-induced liver injury, genetic studies^{E7} have identified several HLA alleles that predispose an individual to toxicity. However, to date, the specific HLA alleles that interact with amoxicillin to activate T cells have not been defined. Thus, antigen-presenting cells were generated from 10 HLA-mismatched donors expressing different HLA class I and HLA-DR alleles to patients with liver injury. These were used in an IFN- γ ELISPOT assay to study responses of amoxicillin-specific CD4⁺ ($n = 6$) and CD8⁺ ($n = 4$) clones to soluble drug and drug-pulsed antigen-presenting cells.

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Concentration-dependent proliferation of flucloxacillin-specific CD8⁺ clones from patients with liver injury and healthy donors

Concentration	Clone number							Mean	SD
	1	2	3	4	5	6	7		
	Clones from patients with flucloxacillin-induced liver injury								
0	4029.5 ^a	6516.5	6062.5	4796.5	1776.5	5029	1776.5	4283.857	1756.811
0.1	5254	22055.5	9152.5	6902.5	2234	6890	2234	7817.5	6269.231
0.3	12408	39987	21215.5	10065.5	6407	9007.5	6407	15071.07	11196.97
0.5	14939	49947	29911.5	13388.5	19134.5	12210	19134.5	22666.43	12402.76
1	23183.5	58043.5	32136	18027	36026	13947	36026	31055.57	13645.21
2	19695	75533	29899.5	25505	44547.5	13021.5	44547.5	36107	19457.36
	Clones from healthy donors								
0	2158	2280	4682	2981.5	3244.5	2608	4682	3233.714	979.5802
0.1	1586	2481	5242.5	2649.5	6399	3263.5	5242.5	3837.714	1654.41
0.3	4072.5	8040.5	16009	10485	30924.5	3740.5	16009	12754.43	8745.074
0.5	15873.5	35575	19633	34961	64675.5	5732	19633	28011.86	17864
1	36053.5	48784	30005	28214.5	80213.5	10297	30005	37653.21	20326.88
2	19024	51807	27004.5	14469	84931	12075.5	27004.5	33759.36	24186.62

^amean cpm from duplicate cultures shown

FIG E1. Activation of T-cell clones from healthy donors ($n = 7$) and patients with flucloxacillin-induced liver injury ($n = 7$) with titrated concentrations of soluble drug. Proliferation was measured after 48 hours through the addition of [³H]thymidine. Mean cpm values are shown in the figure. The table shows cpm values obtained with individual clones. Student t test was used to compare the response of clones from patient and healthy donors at each drug concentration. Significant differences were not detected.

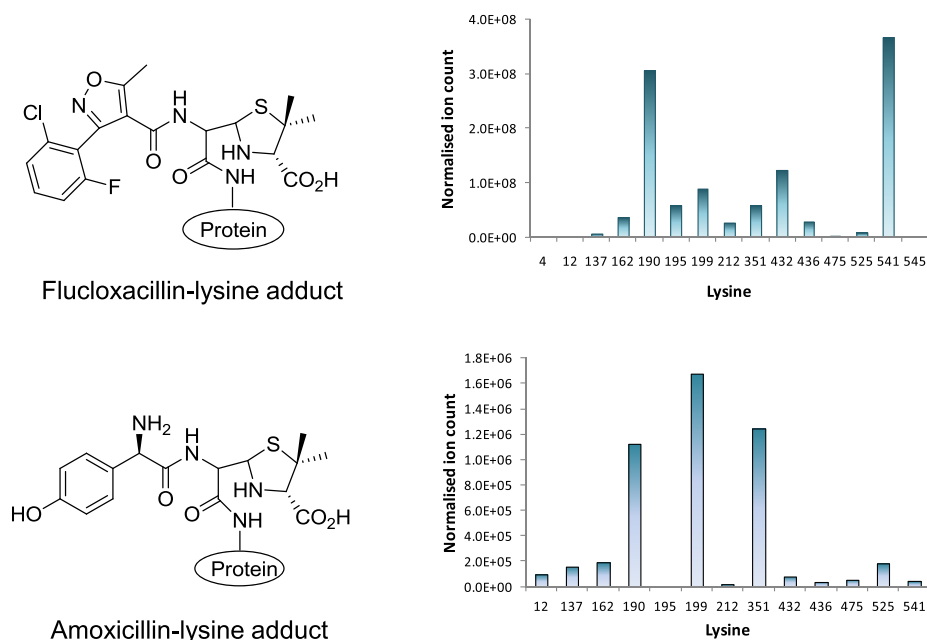


FIG E2. Binding of flucloxacillin and amoxicillin to lysine residues on human serum albumin. Mass spectrometry was used to measure the epitope profile of the lysine residues on albumin modified in antigen-presenting cell culture after 16 hours.

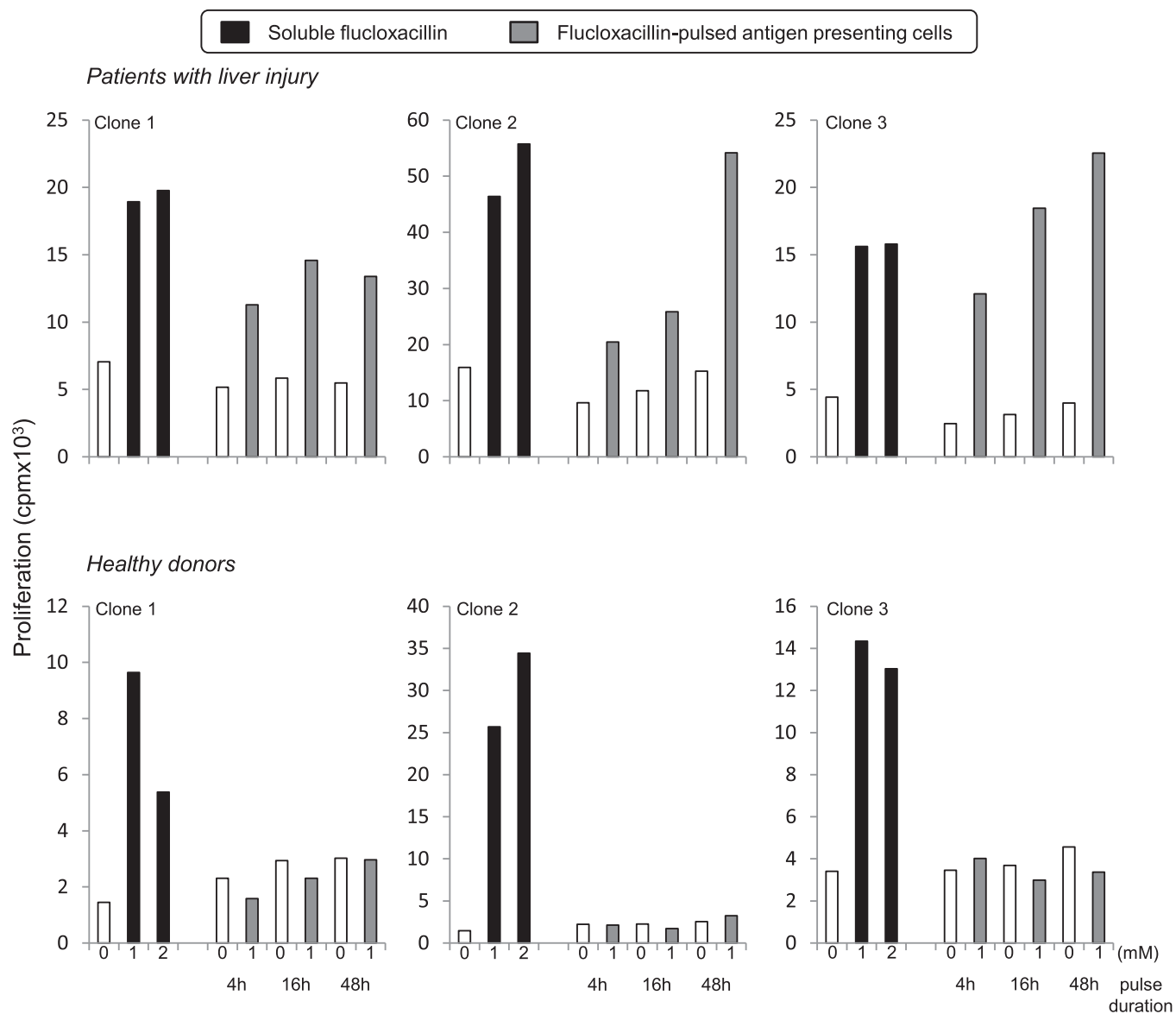


FIG E3. Activation of flucloxacillin-specific CD8⁺ T-cell clones from patients with liver injury and healthy donors with soluble drug and antigen-presenting cells pulsed with drug for 4 to 48 hours. Proliferation was measured by the addition of [³H]thymidine.

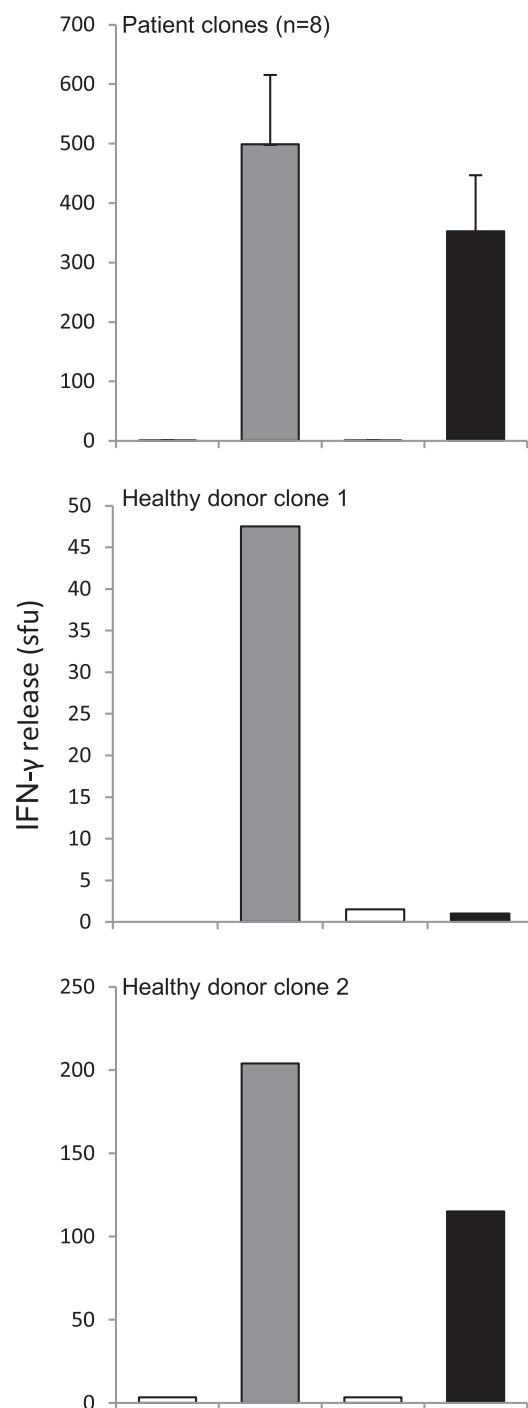
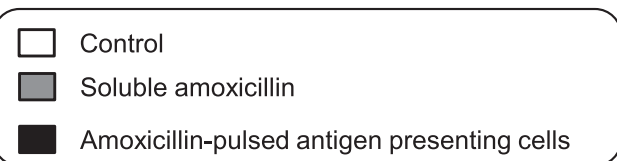
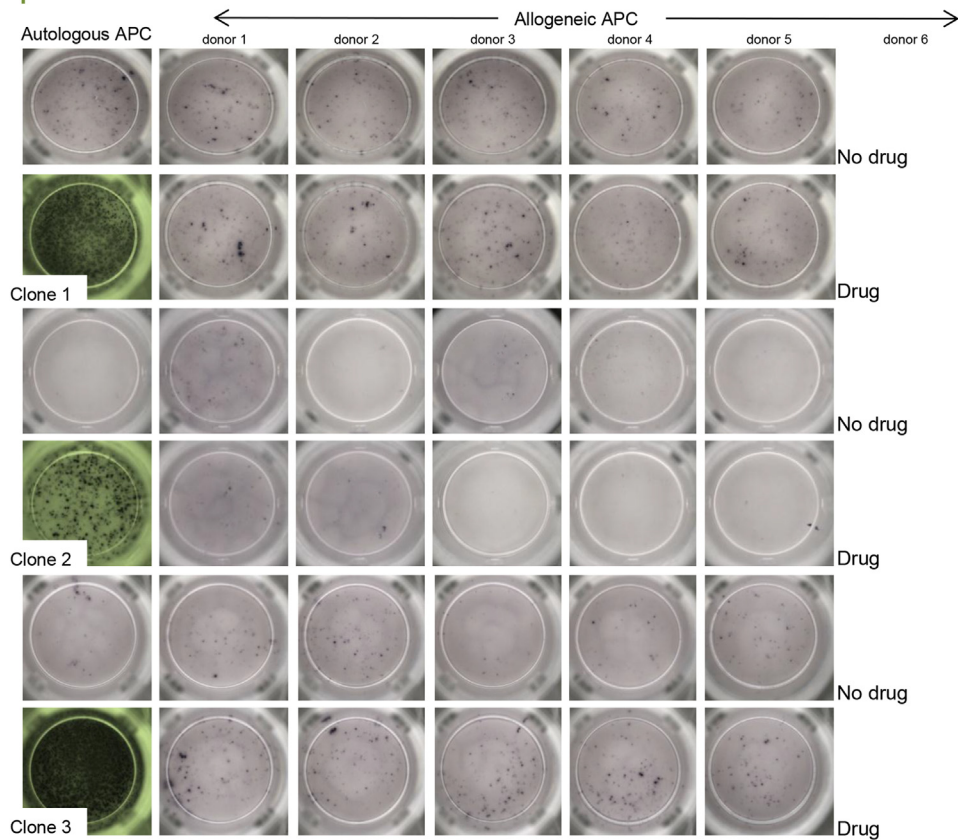


FIG E4. Activation of T-cell clones from healthy donors and patients with amoxicillin-clavulanate-induced liver injury ($n = 8$). Clones were cultured with soluble drug and autologous antigen-presenting cells or drug-pulsed antigen-presenting cells. IFN- γ release was measured after 48 hours by ELISpot. Data presented as mean $\pm$ SD for the patient clones.

Amoxicillin-pulsed APC



Soluble amoxicillin

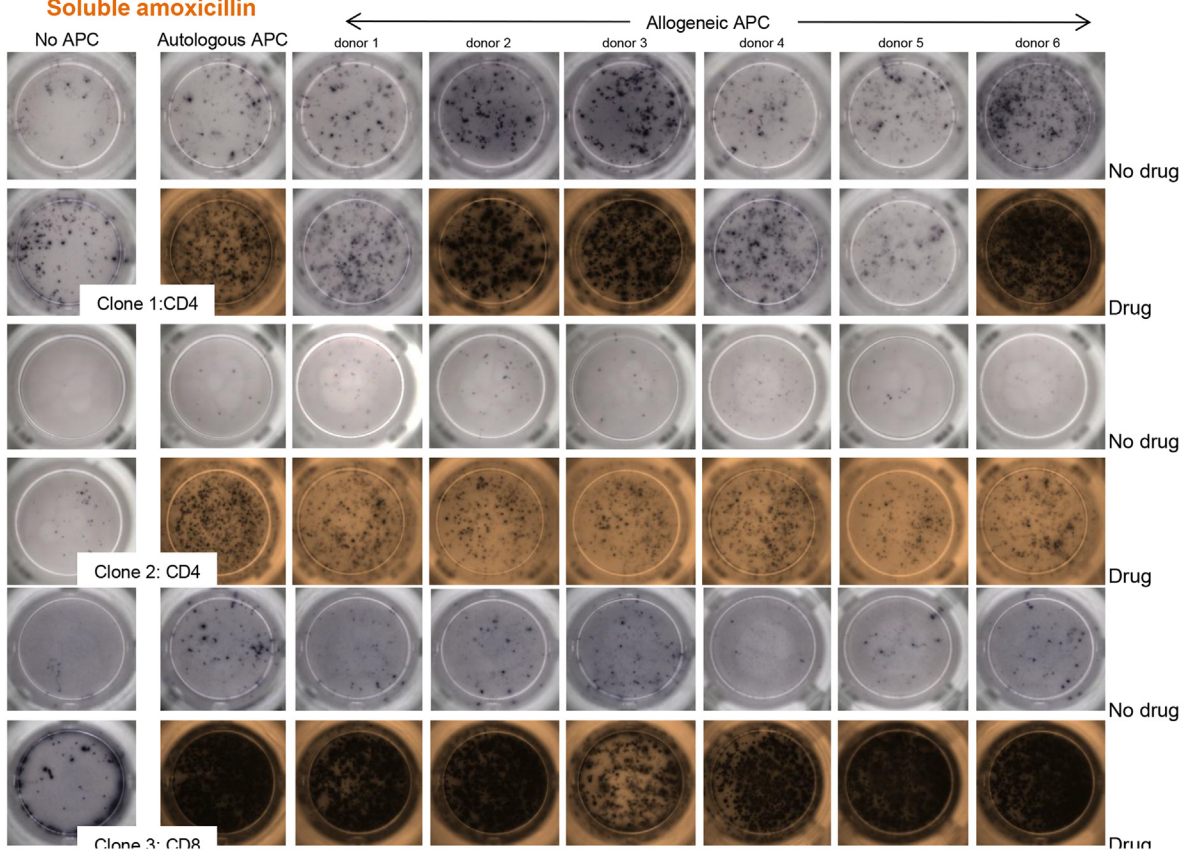


FIG E5. Activation of amoxicillin-specific T-cell clones from patients with liver injury with soluble drug (*bottom* component) and drug-pulsed antigen-presenting cells (APCs) (*top* component) using a panel of HLA-mismatched APCs. ELISPOT was used to measure IFN- γ release. Three representative clones are shown. A no APCs control was not performed in the pulsing experiments because this would equate to T cells alone. Thus, the no drug autologous APC wells were used as a relevant control.

TABLE E1. Clinical details of patients with liver injury

ID	Culprit drug	Age (y)	Sex	Peak liver function tests at time of liver injury			Time to onset (wk)	RUCAM score	Since reaction (y)
				ALT	Bilirubin	ALP			
P1	Amoxicillin-clavulanate	74	Female	13× ULN	12× ULN	2× ULN	1	—	6
P2	Amoxicillin-clavulanate	65	Male	30× ULN	8× ULN	2× ULN	1	8	3
P3	Amoxicillin-clavulanate	61	Male	6× ULN	26× ULN	3× ULN	5	10	11
P4	Flucloxacillin	63	Male	23× ULN	3× ULN	9× ULN	3	7	13
P5	Flucloxacillin	90	Female	11 × ULN	13 × ULN	4.5 × ULN	3	6	8
P6	Flucloxacillin	67	Female	4 × ULN	36 × ULN	2 × ULN	3	5	7

ALP, Alkaline phosphatase; ALT, alanine transaminase; RUCAM, Roussel Uclaf Causality Assessment Method; ULN, upper limit of normal.

TABLE E2. HLA type of patients

ID	HLA profile				
	HLA-A	HLA-B	HLA-DRB1	HLA-DQB1	HLA-DQA1
Patients with flucloxacillin-induced liver injury					
P1	02:01/03:01	07:02/57:01	15:01/07:01	03:03/06:02	01:02/02:01
P2	—*	—	—	—	—
P3	02:01/24:02	15:01/27:05	04:01/08:01	03:01/04:02	03:01/04:01
P4	01:01/02:01	05:01/06:02	01:01/07:01	03:03/05:01	01:01/02:01
P5	01:01/29:02	06:02/06:02	03:01/07:01	02:01/03:03	02:01/05:01
P6	01:01/29:02	06:02/07:01	03:01/07:01	02:01/03:03	02:01/05:01

*Genotyping data not available.