

# Characterization of Amoxicillin- and Clavulanic Acid-Specific T Cells in Patients With Amoxicillin-Clavulanate-Induced Liver Injury

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Drug-induced liver injury (DILI) frequently has a delayed onset with several human leukocyte antigen (HLA) genotypes affecting susceptibility, indicating a potential role for the adaptive immune system in the disease. The aim of this study was to investigate whether drug-responsive T lymphocytes are detectable in patients who developed DILI with the combination, antimicrobial amoxicillin-clavulanate. Lymphocytes from 6 of 7 patients were found to proliferate and/or secrete interferon-gamma (IFN- $\gamma$ ) when cultured with amoxicillin and/or clavulanic acid. Amoxicillin (n = 105) and clavulanic acid (n = 16) responsive CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones expressing CCR, chemokine (C-C motif) receptor 4, CCR9, and chemokine (C-X-C motif) receptor 3 were generated from patients with and without HLA risk alleles; no cross-reactivity was observed between the two drug antigens. Amoxicillin clones were found to secrete a heterogeneous panel of mediators, including IFN- $\gamma$ , interleukin-22 and cytolytic molecules. In contrast, cytokine secretion by the clavulanic acid clones was more restricted. CD4<sup>+</sup> and CD8<sup>+</sup> clones were major histocompatibility complex class II and I restricted, respectively, with the drug antigen being presented to CD4<sup>+</sup> clones in the context of HLA-DR molecules. Several pieces of evidence indicate that the clones were activated by a hapten mechanism: First, professional antigen-presenting cells (APCs) were required for optimal activation; second, pulsing APCs for 4-16 hours activated the clones; and third, inhibition of processing abrogated the proliferative response and cytokine release. **Conclusion:** Both amoxicillin- and clavulanic acid-specific T cells participate in the liver injury that develops in certain patients exposed to amoxicillin-clavulanate. (HEPATOLOGY 2015;62:887-899)

Drug-induced liver injury (DILI) is a major concern for public health as well as for drug development given that it is a leading cause of drug withdrawal. A recent study evaluating the short-term outcomes of 660 patients with DILI found that nearly 1 in 10 die or undergo liver transplantation within 6

months of DILI onset, and nearly 1 in 5 of the remaining patients have evidence of persistent liver injury at 6 months.<sup>1</sup> Liver injury tends to have a delayed onset, occurring 5-90 days after initial drug exposure. Furthermore, strong associations between expression of specific major histocompatibility complex (MHC; or human

*Abbreviations:* Abs, antibodies; ALP, alkaline phosphatase; ALT, alanine aminotransferase; APCs, antigen presenting cells; CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; cpm, counts per minute; CXCR, chemokine (C-X-C motif) receptor; DILI, drug-induced liver injury; EBV, Epstein-Barr virus; FasL, Fas ligand; IFN- $\gamma$ , interferon-gamma; HLA, human leukocyte antigen; IL, interleukin; MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; RUCAM, Roussel Uclaf Causality Assessment Method; SI, stimulation index; sfu, spot forming unit.

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leukocyte antigen [HLA]) class I and II genes and susceptibility to DILI has been reported for several drugs (e.g., flucloxacillin [HLA-B\*57:01<sup>2</sup>], ximelagatran [HLA-DRB1\*07:01<sup>3</sup>], lumiracoxib [HLA-DRB1\*15:01<sup>4</sup>], lapatinib [HLA-DRB1\*07:01<sup>5,6</sup>], amoxicillin-clavulanate [multiple alleles<sup>7,8</sup>]). Given that the proteins encoded by specific HLA alleles are responsible for antigen presentation to T lymphocytes, the data suggest that the adaptive immune system is involved in the development of liver injury.

Despite this, the underlying mechanisms of immunological DILI are poorly understood. Maria and Victorino<sup>9</sup> utilized the lymphocyte transformation test to study peripheral blood mononuclear cell (PBMC) responses to drugs in 95 DILI cases. Proliferative responses to a variety of drugs were detected in over 50% of patients, suggesting that drug-specific lymphocytes circulate in the periphery of patients with DILI. In contrast, PBMCs from controls with and without previous drug exposure were not stimulated to proliferate with the same panel of compounds. Unfortunately, assessment of the phenotypic properties and function of the drug-specific T cells was not undertaken.

Because flucloxacillin is known to bind covalently to selective lysine residues on proteins, including human serum albumin,<sup>10</sup> we recently utilized flucloxacillin-induced liver injury as a model to begin to characterize the nature of the drug-specific T-cell response and relate the genetic association (i.e., expression of HLA-B\*57:01) to the disease pathogenesis.<sup>11</sup> Flucloxacillin was found to preferentially activate cytotoxic CD8<sup>+</sup> T cells in an HLA-B\*57:01-restricted manner. Using cells from healthy donors, it is also possible to (1) prime naïve CD8<sup>+</sup> T cells to flucloxacillin with autologous dendritic cells expressing HLA-B\*57:01 and (2) drive flucloxacillin-specific T-cell responses in HLA-B\*57:01-positive and -negative individuals through long-term culture and repetitive rounds of drug stimulation.<sup>11,12</sup> Most recently, T-cell infiltrates have been detected in liver biopsy samples of patients with flucloxacillin-induced liver injury,<sup>13</sup> which suggests that the T cells described in the *ex vivo* studies play an active role in DILI.

The aim of this study was to investigate amoxicillin-clavulanate-induced liver injury, characterize the pheno-

type and function of T cells, and define antigen specificity. Amoxicillin-clavulanate, one of the most frequently prescribed antibiotics, is a combination therapy (amoxicillin and clavulanic acid) that provides broad-spectrum antimicrobial activity. The clavulanic acid component is believed to increase the risk of DILI given that fewer cases have been reported in patients administered amoxicillin alone.<sup>14</sup> Several independent studies have shown that amoxicillin-clavulanate-induced liver injury is observed more frequently in individuals expressing the HLA-class II alleles, DRB1\*15:01 and DQB1\*06:02, which form a common class II haplotype.<sup>15-17</sup> In addition, the HLA class I allele, HLA-A\*02:01, has been shown to be an additional risk factor independent of class II genotype with an increased risk of DILI in those positive for both the class I and II alleles.<sup>17</sup> Interestingly, a recent high-resolution HLA genotyping in amoxicillin-clavulanate DILI cases and controls from Spain indicated that phenotypic expression of DILI can be influenced by a specific HLA genotype; HLA-DRB1\*15:01-DQB1\*06:02 for cholestatic/mixed type, and HLA-A\*30:02 and B\*18:01 for hepatocellular type.<sup>8</sup> The B\*18:01 finding is consistent with an earlier report that this was a risk factor in Spanish cases only.<sup>17</sup> Given that it appears that multiple HLA alleles are a risk factor for amoxicillin-clavulanate-induced DILI, our study included patients with and without known risk alleles.

## Patients and Methods

**Human Subjects.** Seven patients with amoxicillin-clavulanate-induced liver injury were enrolled from the DILIGEN study.<sup>2</sup> Causality assessment utilized the Roussel-Uclaf Causality Assessment Method (RUCAM).<sup>18</sup> Clinical characteristics of the patients are described in Table 1. 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.

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Potential conflict of interest: Nothing to report.

**Table 1. Clinical Details of Patients With Amoxicillin-Clavulanate-Induced Liver Injury**

ID	Age (Years)	Gender	Peak Liver Function Tests at Time of Liver Injury			Time to Onset (Days)	Disease Duration (Days)	RUCAM Score	Details of Reaction
			ALT* (U/L)	Bilirubin* ( $\mu\text{mol/L}$ )	ALP* (U/L)				
P1	74	Female	465	246	279	7	55	—	Sept 2009 Cholestatic/mixed
P2	65	Male	1,080	175	222	7	57	8	Aug 2012 Hepatocellular
P3	61	Male	202	551	331	39	71	10	June 2004 Cholestatic
P4†	70	Male	134	564	391	2	71	8	Jan 2006 Cholestatic
P5†	47	Female	420	456	897	3	35	8	April 2004 Cholestatic
P6	76	Male	50	445	762	17	22	8	Sept 2008 Cholestatic
P7	67	Female	120	234	520	11	66	7	Oct 1999 Cholestatic

\*Upper limit of normal range: ALT, 35; bilirubin, 21; ALP, 130.

†Time to onset is shorter than expected. Patient 4 did not have a history of previous amoxicillin-clavulanate exposure (1973-2006), but has been exposed to other  $\beta$ -lactam antibiotics. Patient 5 did not have a history of previous amoxicillin-clavulanate exposure (1999-2004), but has been exposed to other  $\beta$ -lactam antibiotics.

Supporting Table 1 lists the HLA alleles expressed by the patients. Four amoxicillin-clavulanate-exposed donors with no history of  $\beta$ -lactam hypersensitivity were selected as controls.

**Activation of Patient PBMCs in Response to Amoxicillin and/or Clavulanic Acid.** Proliferation of patient PBMCs ( $0.15 \times 10^6$  cells/well) on exposure to amoxicillin, clavulanic acid or amoxicillin-clavulanate (0.0625-2.0000 mM) was measured using the lymphocyte transformation test.<sup>19</sup> Proliferative responses were calculated as stimulation index (SI; counts per minute [cpm] in drug-treated cells/cpm in mock-treated cells) or expressed as unprocessed cpm values. Tetanus toxoid and medium alone served as positive and negative controls, respectively. Interferon-gamma (IFN- $\gamma$ ) secreting PBMCs were visualized using ELISpot (MabTech, Nacka Strand, Sweden) by culturing PBMCs ( $0.5 \times 10^6$  cells/well) with each drug or phytohemagglutinin (PHA; 5  $\mu\text{g/mL}$ ; positive control) for 48 hours.

**Generation of T-Cell Clones.** Patient 1, 2, and 3 PBMCs were selected for the cloning experiments. PBMC from patients 1 and 2 were stimulated to proliferate and/or secrete IFN- $\gamma$  when exposed to amoxicillin or clavulanic acid. Patient 3 was selected to determine whether it was possible to isolate drug-responsive clones from PBMCs displaying only a weak lymphocyte transformation test result. The 1 patient with a negative lymphocyte transformation test refused to donate a second blood sample; thus, it was not possible to study whether the negative data related to lack of sensitivity of the

PBMC assay or the absence of drug-specific T cells. PBMC ( $1 \times 10^6$  cells/well; 0.5 mL) were cultured with amoxicillin, clavulanic acid, or amoxicillin-clavulanate (0.1-1.0 mM) in RPMI 1640 supplemented with 10% human AB serum (Class A; Innovative Research Inc., Novi, MI), 25 mM of HEPES, 10 mM of L-glutamine, and 25  $\mu\text{g/mL}$  of transferrin (Sigma-Aldrich, Gillingham, UK). Cultures were supplemented with 200 IU/mL of recombinant human interleukin (IL)-2 (Pepro-Tech, London, UK) on days 6 and 9. On day 13, T cells were cloned by serial dilution, as described previously.<sup>20</sup> Epstein-Barr virus (EBV)-transformed B-cell lines were generated from PBMCs by transformation with supernatant from the EBV-producing cell line, B95.8. EBV-transformed B cells were used as a source of antigen-presenting cells (APCs) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100 mM L-glutamine, penicillin, and streptomycin.

**Specificity of T-Cell Clones.** Specificity of each T-cell clone was determined by measuring proliferation and/or IFN- $\gamma$  secretion after drug stimulation. Clones ( $5 \times 10^4$  cells/well) were cultured with autologous irradiated EBV-transformed B cells ( $1 \times 10^4$  cells/well) and amoxicillin or clavulanic acid for 48 hours. Proliferation was measured by the addition of [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci/well}$ , 5 Ci/mmol; Morovek Biochemicals, Brea, CA) for the last 16 hours of the culture period. IFN- $\gamma$  release was measured by ELISpot. Clones with a stimulation index of greater than 2 were expanded by repetitive

stimulation with irradiated allogeneic PBMCs ( $5 \times 10^4$  cells/well; 200  $\mu$ L) and 5 mg/mL of PHA in IL-2-containing medium and characterized in terms of CD phenotype and expression of chemokine receptors (chemokine [C-C motif] receptor [CCR]1-6, CCR8-10, chemokine [C-X-C motif] receptor [CXCR]1, CXCR3, and CXCR6) by flow cytometry. Twenty-four-well transwell chambers with 5- $\mu$ m pores were used to measure migration of clones in the presence and absence of chemokine (C-C motif) ligand (CCL)17 (CCR4 ligand) using an established protocol.<sup>11</sup>

**Cross-reactivity of T-Cell Clones.** Once clones were deemed responsive to either amoxicillin or clavulanic acid during initial testing, dose-response (0.0625-2.0000 mM) and cross-reactivity studies were conducted using proliferation and IFN- $\gamma$  release as markers of T-cell activation. Clones were tested against at least two (0.1 and 1 mM) stimulatory concentrations of the alternative drug antigen (i.e., amoxicillin or clavulanic acid). Well-growing clones were also tested for reactivity against the  $\beta$ -lactam antibiotics (penicillin G, piperacillin, ampicillin, and flucloxacillin; all 0.5-2.0 mM).

**Release of Cytokines and Cytolytic Molecules by T-Cell Clones.** ELISpot was used to profile the cytokines (IFN- $\gamma$ , IL-5, IL-13, IL-17, and IL-22) and cytolytic molecules (granzyme B, perforin, and Fas ligand [FasL]) released by amoxicillin- ( $n = 7$ ) and clavulanic acid-responsive ( $n = 4$ ) CD4<sup>+</sup> and CD8<sup>+</sup> clones. Clones ( $5 \times 10^4$  cells/well) were cultured with autologous irradiated EBV-transformed B cells ( $1 \times 10^4$  cells/well) and optimal stimulatory concentrations of amoxicillin or clavulanic acid (either 0.5 or 1 mM) on plates precoated with appropriate capture antibody (Ab) for 48 hours. After washing, cytokine-secreting cells were visualized according to the manufacturer's instructions (Mabtech) and counted using an AID ELISpot reader (Oxford Biosystems Cadama, Oxfordshire, UK).

**Functional Studies to Delineate Mechanisms of T-Cell Activation.** To investigate the mechanistic basis of amoxicillin- and clavulanic acid-specific T-cell activation, CD4<sup>+</sup> and CD8<sup>+</sup> clones were subjected to a series of analyses. First, clones were cultured with drug in the presence and absence of APCs. Second, MHC class I and II blocking (anti-DR, DP, and DQ) Abs (BD Biosciences, Oxford, UK) were added to proliferation and ELISpot assays containing clones and APCs, 30 minutes before the addition of drug. Third, APCs were pulsed with drug for 5 minutes-16 hours, before repeated washing and the addition of the APCs to proliferation and ELISpot assays in the absence of soluble drug. Fourth, APCs were fixed with glutaraldehyde (0.05%; Sigma-Aldrich) to prevent processing. Finally, the proteasome

inhibitor, bortezomib (1-100 nM), was added to ELISpot assays containing APCs, T cells, and soluble drug. Inhibitor concentrations selected did not inhibit spontaneous proliferation of EBV-transformed B cells.

**Statistical Analysis.** Student *t* test was used to analyze proliferation and ELISpot data.

## Results

**Lymphocyte Proliferation Against Amoxicillin and Clavulanic Acid.** PBMCs from 6 of 7 patients cultured *in vitro* with amoxicillin or clavulanic acid showed an antigen-specific proliferative response and/or increased IFN- $\gamma$  secretion, visualized using ELISpot. Interestingly, responses to amoxicillin were detected by [<sup>3</sup>H]thymidine incorporation, whereas clavulanic acid responses were detected predominantly by IFN- $\gamma$  ELISpot, (Fig. 1). Several patients showing positive lymphocyte responses carried the class I risk allele HLA-A\*02:01 (one also carried the class II haplotype, HLA-DRB1\*15:01-DQB1\*06:02); however, it should be noted that the patient with the strongest ELISpot response to both amoxicillin and clavulanic acid (P4) was not positive for any of the HLA alleles previously reported to be associated with amoxicillin-clavulanate-induced liver injury.

Amoxicillin- and clavulanic acid-specific lymphocyte proliferative responses and/or IFN- $\gamma$  secretion were not detected with PBMCs from amoxicillin-clavulanate-tolerant controls with no previous history of  $\beta$ -lactam hypersensitivity ( $n = 4$ ; SI < 2).

**Generation of Amoxicillin and Clavulanic Acid-Responsive T-Cell Clones.** Amoxicillin- and clavulanic acid-treated PBMCs from patients 1-3 were serially diluted and subjected to repetitive rounds of stimulation to generate over 1,800 T-cell cultures originating from single precursor cells. T cells were then cultured with APCs and either amoxicillin or clavulanic acid and proliferation measured by incorporation of [<sup>3</sup>H]thymidine. A total of 105 and 16 clones displayed reactivity against amoxicillin and clavulanic acid, respectively, on initial testing (0 or 1 mM drug; Fig. 2A,B). After further expansion, IFN- $\gamma$  release was measured against titrated concentrations of the test compounds. Amoxicillin- and clavulanic acid-specific T-cell activation was dose dependent, with clones displaying different response profiles between 0.0625 and 1 mM (the highest concentration tested; Fig. 2C). After initial testing, most clavulanic acid-responsive clones became resistant to drug-specific proliferation; however, antigen specificity was readily detected with the IFN- $\gamma$  ELISpot kit. Thus, most subsequent experiments used IFN- $\gamma$



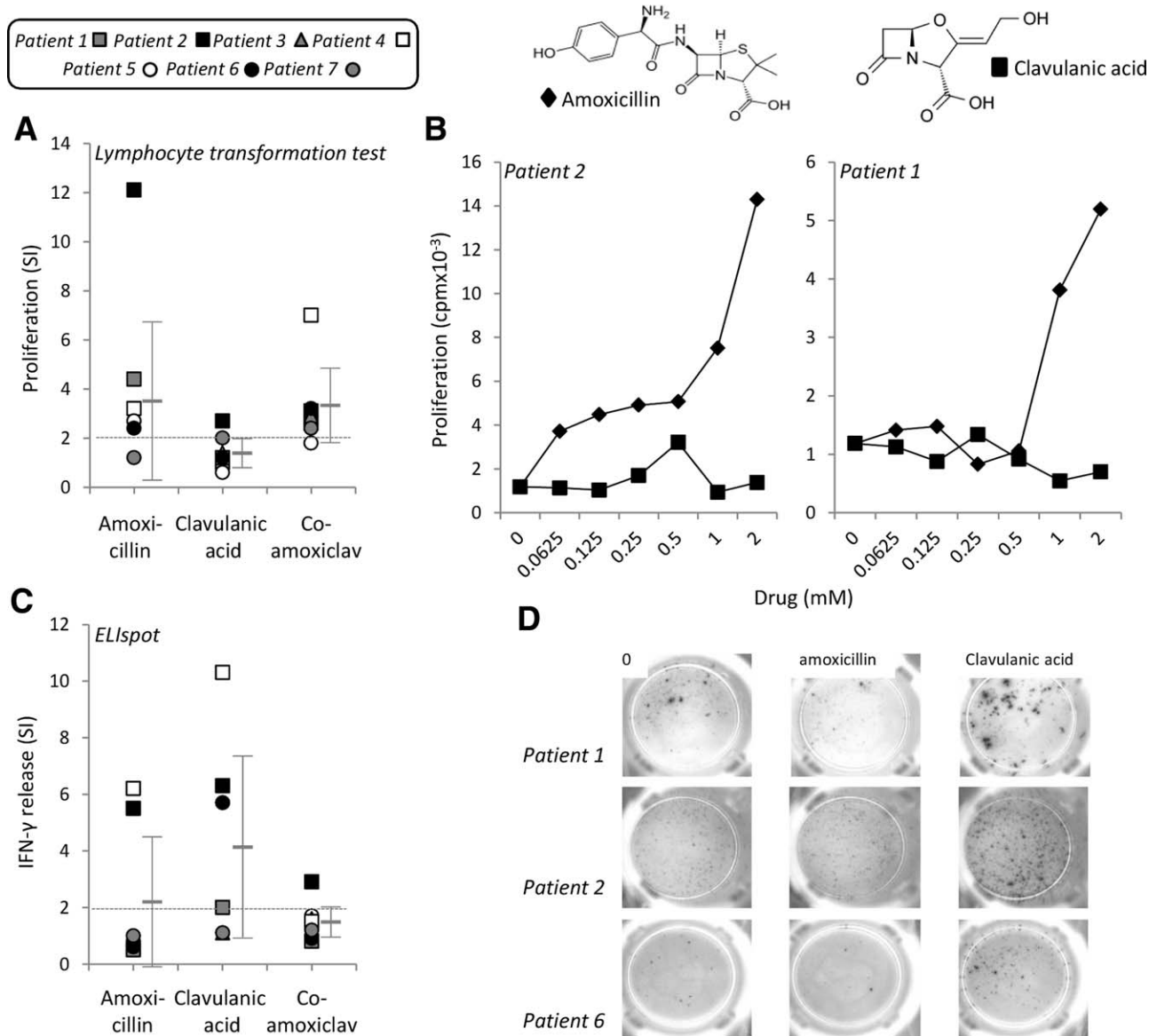


Fig. 1. Activation of DILI patient PBMCs with amoxicillin and/or clavulanic acid. (A and B) PBMCs from patients with DILI were cultured with titrated concentrations of amoxicillin, clavulanic acid, or amoxicillin-clavulanate, and drug-specific proliferative responses were measured by the addition of [<sup>3</sup>H]thymidine for the final 16 hours of the 6-day experiment. (A) Maximum stimulatory response detected with PBMCs from all 7 patients. Results are expressed as SI (cpm in test incubation/cpm in control incubations); values above 2 considered significant.<sup>34</sup> (B) Representative data showing concentration-dependent response with PBMCs from 2 patients. (C and D) Amoxicillin- and clavulanic acid-induced IFN- $\gamma$  release in PBMCs measured using ELISpot. (C) Maximum stimulatory response detected with PBMCs from all 7 patients. Results are expressed as SI (sfu in test incubation/sfu in control incubations); values above 2 considered significant. (D) Representative data showing ELISpot images from 3 patients.

secretion as a readout for the clavulanic acid-responsive T cells. This phenomenon has been noted previously with CD8<sup>+</sup> clones from patients hypersensitive to a range of drugs (e.g., carbamazepine and abacavir). However, drug-specific CD4<sup>+</sup> clones that secrete cytokines, but are largely resistant to proliferation, are rare and have not been seen previously in our laboratory.

Clones displaying reactivity with clavulanic acid were, for the most part, CD4<sup>+</sup>. Similarly, most amoxicillin-responsive clones from patients 1 and 3 were CD4<sup>+</sup>.

However, 56 of 78 amoxicillin-responsive clones from patient 2, the patient with a hepatocellular pattern of damage, were identified as CD8<sup>+</sup>. In all subsequent experiments, a mixture of CD4<sup>+</sup> and CD8<sup>+</sup> clones were selected for analysis.

**Expression of Chemokine Receptors on Amoxicillin- and Clavulanic Acid-Responsive T-Cell Clones.** Twenty-three clones displaying activity against amoxicillin and clavulanic acid were screened for expression of 12 chemokine receptors. Clones expressed high levels of

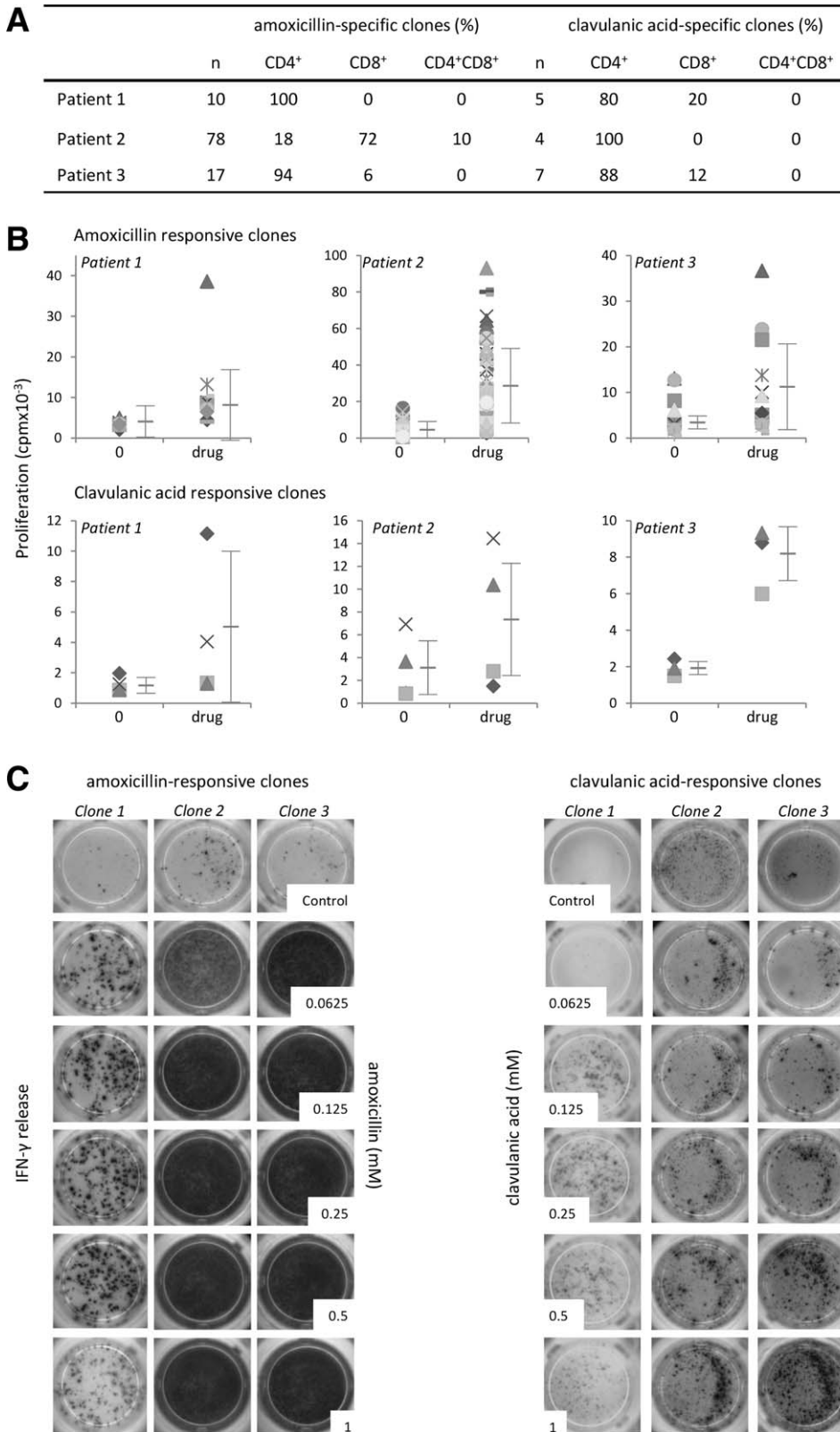


Fig. 2. Isolation of amoxicillin- and clavulanic acid-responsive CD4<sup>+</sup> and CD8<sup>+</sup> clones from patients with DILI. Approximately 1,800 individual T-cell clones were expand from patient PBMCs and cultured with autologous APCs and either amoxicillin or clavulanic acid for 2 days before analysis of proliferation by the addition of [<sup>3</sup>H]thymidine. (A) Table summarizing the phenotype of drug-responsive (SI 2 or above) clones. (B) Initial proliferative response of clones after amoxicillin or clavulanic acid stimulation (four clavulanic acid-responsive clones identified by ELISpot are not shown). (C) Dose-dependent IFN- $\gamma$  release in response to amoxicillin or clavulanic acid from six representative clones.

CCR4, CCR9, and CXCR3 (Supporting Table 2). No significant difference in receptor expression was observed when amoxicillin- and clavulanic acid-respon-

sive clones were compared. A migration assay using transwell plates and the CCR4 ligand, CCL17, was used to show that receptor expression was functionally

relevant. The chemokine promoted migration of all four clones studied ( $4.7 \pm 2.6$ -fold increase in number of migrating cells).

***Amoxicillin- and Clavulanic Acid-Responsive Clones Display No Cross-Reactivity.*** Amoxicillin-specific clones ( $n = 59$ ) did not proliferate when stimulated with clavulanic acid (Fig. 3A). Similarly, clavulanic acid-specific clones displayed no proliferative activity or IFN- $\gamma$  release when incubated with amoxicillin (Fig. 3B).

***Reactivity of T-Cell Clones With Other  $\beta$ -Lactam Antibiotics.*** Amoxicillin-specific T-cell clones showed a strong proliferative response and IFN- $\gamma$  release in the presence of ampicillin, but not flucloxacillin, piperacillin, or the closely related benzyl penicillin (Fig. 3C,D). A single clavulanic acid-responsive clone was cultured with ampicillin, benzyl penicillin, piperacillin, and flucloxacillin, but antigen-specific IFN- $\gamma$  release was not detected (Fig. 3D).

***Profile of Cytokines/Cytolytic Molecules Secreted by Amoxicillin- and Clavulanic Acid-Responsive Clones.*** A panel of 11 clones were screened for release of cytokines and cytolytic molecules. Amoxicillin-responsive CD4<sup>+</sup> and CD8<sup>+</sup> clones secreted high levels of IFN- $\gamma$  and the cytolytic molecules, perforin, granzyme B, and FasL. IL-22 secretion was also detected with three clones. Interestingly, both CD4<sup>+</sup> and CD8<sup>+</sup> clones secreted IL-22. Four clavulanic acid-responsive CD4<sup>+</sup> clones were available for the analysis. The profile of cytokines secreted was much more restricted. Three clones secreted IFN- $\gamma$  and granzyme B in the absence of additional cytokines, whereas one clone secreted IL-17 and IL-22 (Fig. 4).

***CD4<sup>+</sup> and CD8<sup>+</sup> Clones Are Activated in an MHC-Restricted Manner by a Hapten Mechanism.*** To explore pathways of drug antigen presentation, clones were initially cultured with drug in the presence or absence of APCs. In the absence of APCs, amoxicillin-specific clones were stimulated to release IFN- $\gamma$  with high concentrations of drug (250  $\mu$ M and above). The number of sfu detected was significantly higher in the presence of APCs and the drug-specific response was detected over a much broader range of concentrations (30  $\mu$ M and above; Fig. 5A). Clavulanic acid clones were only stimulated to secrete IFN- $\gamma$  when cultured with drug and APCs (Fig. 5A). Ab-blocking experiments revealed that activation of CD4<sup>+</sup> amoxicillin- and clavulanic acid-specific clones was MHC class II restricted and the drug-derived antigens were presented in the context of HLA-DR molecules. In contrast, activation of CD8<sup>+</sup> clones was reduced with an MHC class I blocking Ab (Fig. 5B-E).

APC pulsing experiments have recently been used to differentiate flucloxacillin-specific T-cell clones activated with drug protein adducts from those that are activated with the parent drug.<sup>11,12</sup> Herein, APCs were pulsed with amoxicillin (clavulanic acid clones had been consumed in the experiments outlined above) for 5 minutes-16 hours to characterize the mechanism of antigen presentation and also to relate drug covalent protein adduct formation to T-cell response. All 10 (five CD4<sup>+</sup> and five CD8<sup>+</sup>) clones were activated with amoxicillin-pulsed APCs in a time-dependent fashion (Fig. 6A,B). A 4- to 16-hour incubation period was optimal for activation of clones. IFN- $\gamma$  release was not detected when APCs pulsed with amoxicillin for 5 minutes were added to clones. These data confirm that clones are not activated by residual soluble drug that may be present after washing.

Activation of CD4<sup>+</sup> and CD8<sup>+</sup> clones in response to amoxicillin was blocked through fixation of APCs with glutaraldehyde, which blocks antigen processing (Fig. 7A). Interestingly, the proteosomal inhibitor, bortezomib, also blocked activation of both CD4<sup>+</sup> and CD8<sup>+</sup> clones with amoxicillin at inhibitor concentrations between 10 and 100 nM (Fig. 7B). In control experiments, these bortezomib concentrations did not inhibit nonspecific proliferation of EBV-transformed B-cell lines (results not shown).

## Discussion

Pathogenesis of DILI is multifaceted, involving, to different extents, stress signaling, mitochondrial impairment, altered bile acid transport, the innate immune system, and specific patient factors (e.g., infections). However, the most intriguing contributory element and least well studied is the adaptive immune system. Genomic studies identifying drug-specific HLA alleles as susceptibility factors provide indirect evidence to support a role for the adaptive immune system and, specifically, HLA-restricted, antigen-specific T cells.<sup>2-5,7,8,15</sup> However, to date, flucloxacillin is the only example where drug-specific T cells have been isolated from DILI patients and characterized in terms of phenotype and function.<sup>11</sup> We chose to focus on a second example, amoxicillin-clavulanate, primarily because it is one of the most common causes of DILI worldwide with a well-established HLA association involving several different alleles. Because amoxicillin-clavulanate is a combination therapy, it is also important to delineate whether both drugs participate in adaptive immune response.

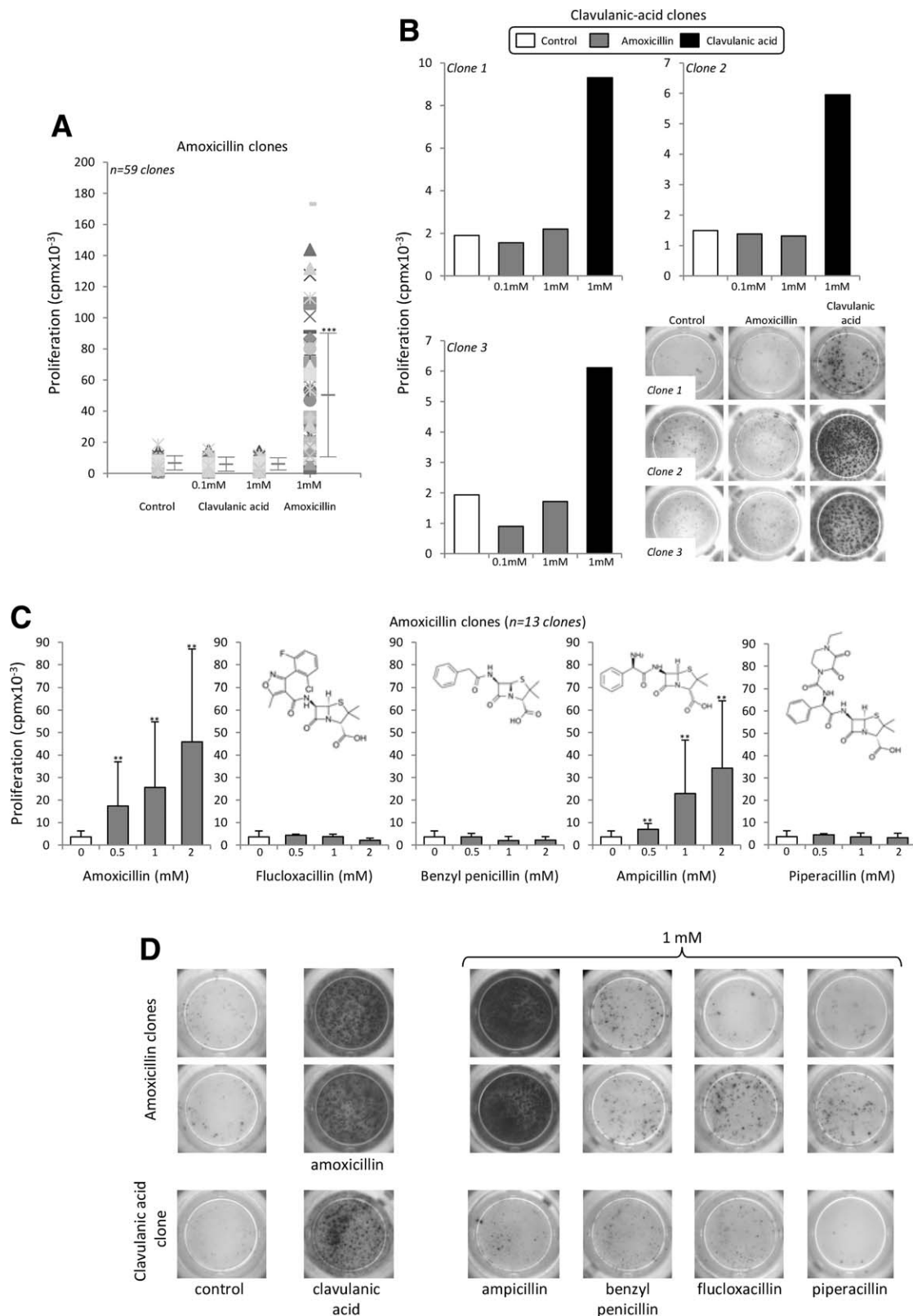


Fig. 3. Cross-reactivity of amoxicillin- and clavulanic acid-responsive CD4<sup>+</sup> and CD8<sup>+</sup> clones. (A and B) Amoxicillin- (n = 59) and clavulanic acid-responsive (n = 3) clones are not activated with the alternative drug. Amoxicillin- and clavulanic acid-responsive clones were incubated with APCs and at least two stimulatory concentrations of the alternative drug. [<sup>3</sup>H]Thymidine was added for the final 16 hours of the experiment to measure proliferation. Clavulanic acid-responsive clones were also analyzed using ELISPOT to measure IFN- $\gamma$  release. (C and D) Amoxicillin-responsive clones are activated with ampicillin, but not other  $\beta$ -lactam antibiotics. Clavulanic acid-responsive clones were not activated with the structurally related drugs. [<sup>3</sup>H]Thymidine was added for the final 16 hours of the experiment to measure proliferation. ELISPOT was used to measure IFN- $\gamma$  release. Statistical analysis compared proliferation in the presence and absence of drug.



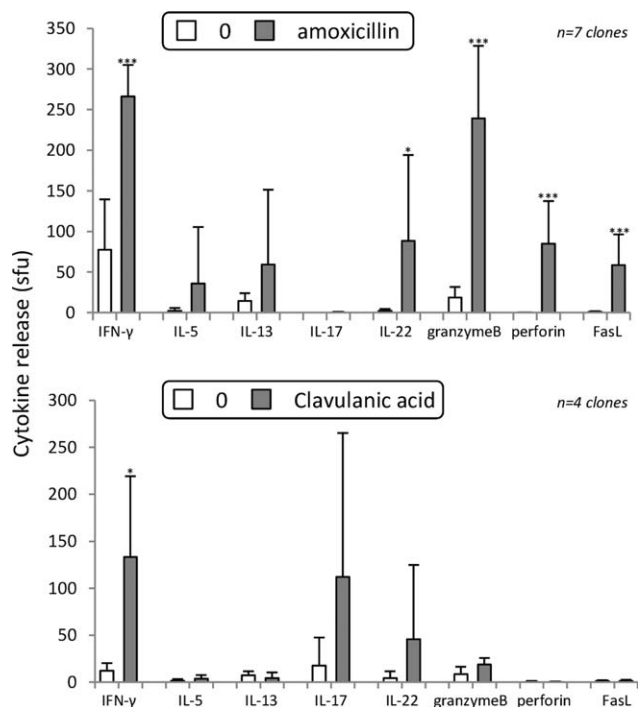


Fig. 4. Profile of cytokines secreted by amoxicillin- and clavulanic acid-responsive T-cell clones. Clones were cultured with APCs and either amoxicillin or clavulanic acid for 48 hours. Release of cytokines/cytolytic molecules was visualized by ELISpot. Statistical analysis compared cytokine release in the presence and absence of drug. Supporting Fig. 1 shows the ELISpot images.

Lymphocyte proliferative responses and IFN- $\gamma$  secretion in response to amoxicillin was observed in 6 of 7 and 2 of 7 DILI patients, respectively. In contrast, clavulanic acid-specific responses were more readily detected using the IFN- $\gamma$  ELISpot. In line with the genetic association studies,<sup>17</sup> 5 DILI patients carried HLA-A\*02:01 and 1 was also positive for the class II alleles, DRB1\*15:01 and DQB1\*06:02. However, it must be noted that amoxicillin- and clavulanic acid-specific PBMC responses were also detected in patients that did not express known amoxicillin-clavulanate HLA risk alleles.

To study antigen specificity and the phenotype of the drug-responsive T-cells, clones were generated from patients 1-3. Patient 3 was selected for the cloning experiments to determine whether it was possible to isolate drug-responsive clones from PBMCs displaying only a weak lymphocyte transformation test result. Amoxicillin- and clavulanic acid-responsive clones were isolated from all 3 patients. On initial testing, CD4<sup>+</sup> and CD8<sup>+</sup> clones were found to proliferate and secrete IFN- $\gamma$  in the presence of either amoxicillin or clavulanic acid in a dose-dependent fashion. The concentration of amoxicillin and clavulanic acid required to activate the clones (60  $\mu$ M-1 mM; Fig. 2C) was higher than circulating concentrations in patient plasma (less than

30  $\mu$ M); however, the concentration of free drug does not relate directly to *in vivo* and *in vitro* exposure to protein adducts. Thus, there is a need to develop quantitative mass spectrometry methods to accurately measure drug protein adducts in each system. There was no cross-reactivity between the drug antigens, despite the fact that they are both  $\beta$ -lactam-containing drugs that likely form covalent adducts with similar lysine residues on protein. Although our results do not clarify why the addition of clavulanic acid to amoxicillin increases the risk of DILI, we do show that (1) both drugs contribute to the T-cell response that develops in patients and (2) all clones express the chemokine receptors, CCR4, CCR9, and CXCR3, which are expressed on clones from patients with flucloxacillin-induced liver injury,<sup>11</sup> but not on clones from patients with cutaneous hypersensitivity reactions.<sup>21</sup> There are several possible explanations for the increased DILI risk with amoxicillin-clavulanate. First, clavulanic acid might form hepatic antigens that redirect the amoxicillin-specific T-cell response. Second, mediators released by clavulanic acid-specific T cells might draw amoxicillin-specific T cells to the liver or regulate T-cell-mediated cytotoxicity. Finally, clavulanic acid might activate hepatic stress-signaling pathways, which promote hepatic recruitment of T cells. Each of these possibilities should be addressed in future studies.

Activation of clones with amoxicillin and clavulanic acid was (1) dependent on, or enhanced with, professional APCs and (2) MHC restricted, with anti-class I and II Abs blocking activation of CD8<sup>+</sup> and CD4<sup>+</sup> clones, respectively. Activation of CD4<sup>+</sup> clones was blocked with an Ab against HLA-DR, but not with Abs against HLA-DQ or -DP. Although we have not characterized all of the possible DR alleles involved in antigen presentation, our findings show that amoxicillin- and clavulanic acid-derived antigens interact with several HLA-DR molecules given that DILI patients each expressed different HLA-DR alleles. Genetic association studies have already shown that expression of HLA risk alleles are neither necessary (i.e., certain DILI patients do not express known risk alleles) nor sufficient (i.e., many patients expressing risk alleles do not develop DILI) to lead to DILI. Our studies expand on this to show that expression of risk alleles is not required for activation of T cells. Nevertheless, it is possible that cooperativity with a combination of HLA alleles (risk and protective alleles and alleles displaying a high degree of linkage disequilibrium) might make an individual more or less likely to generate an antigen-specific T-cell response with the potential to cause DILI. This could be studied in the future after optimization of existing cell-culture

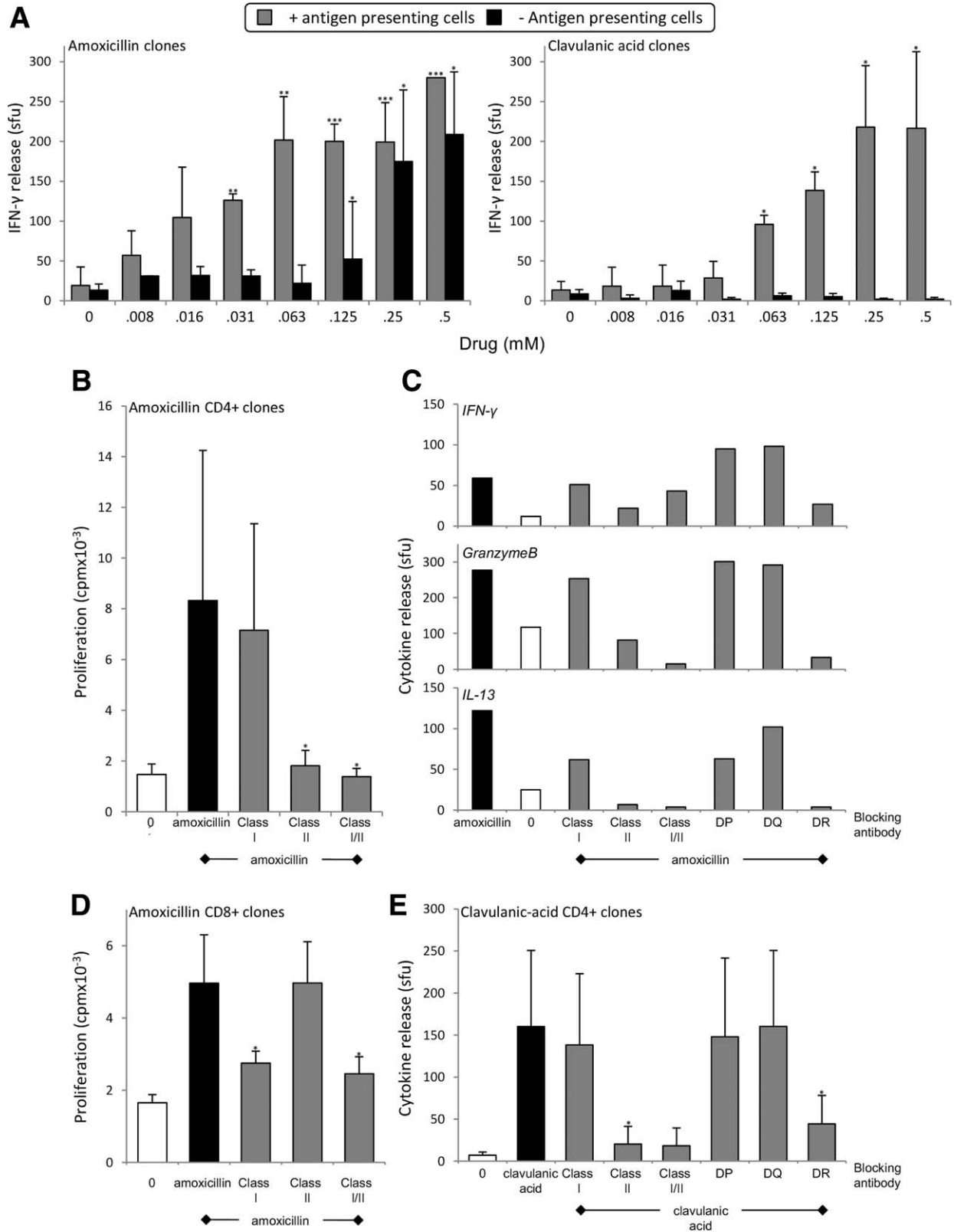


Fig. 5. Amoxicillin- and clavulanic acid-specific T-cell responses are MHC restricted and dependent on (or enhanced by) the professional APCs. (A) Stimulation of amoxicillin- or clavulanic acid-responsive T-cell clones with drug in the presence or absence of APCs. IFN- $\gamma$  release was visualized by ELISpot. Statistical analysis compares cytokine release in the presence and absence of drug. (B-E) Inhibition of amoxicillin- or clavulanic acid-specific proliferation and cytokine release with HLA-class I/II blocking Abs. Anti-HLA blocking Abs were added to the assays 30 minutes before the drug. (B and C) Amoxicillin-responsive CD4<sup>+</sup> clones. (D) Amoxicillin-responsive CD8<sup>+</sup> clones. (E) Clavulanic acid-responsive CD4<sup>+</sup> clones.

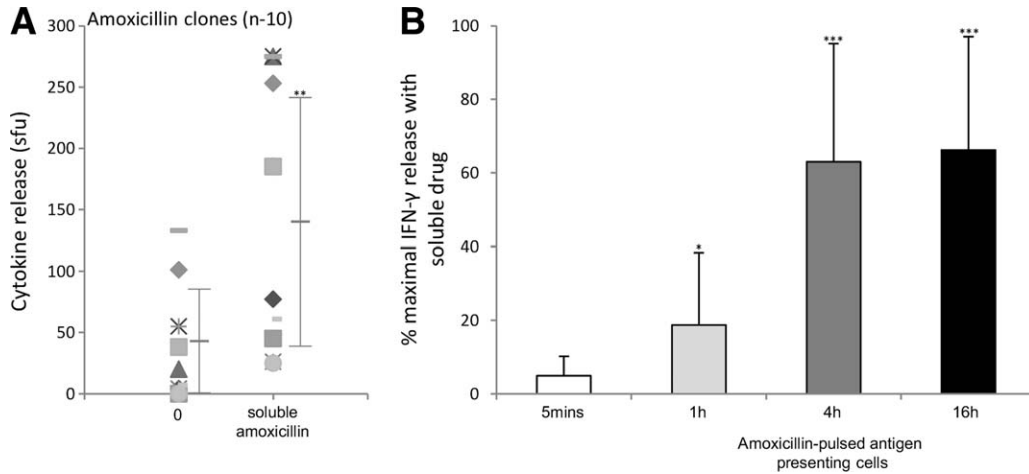


Fig. 6. Amoxicillin binds irreversibly to protein to stimulate CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones. Stimulation of a panel of 10 clones with (A) soluble amoxicillin and (B) amoxicillin-pulsed APCs. APCs were pulsed for 5 minutes-16 hours, then washed repeatedly after pulsing to remove unbound drug. Statistical analysis compares T-cell IFN-γ release postexposure to drug- and mock-pulsed APCs.

methods by screening a large cohort of drug-naïve healthy donors for T-cell responses to amoxicillin and clavulanic acid.

Fine specificity of the drug-specific response was studied by culturing clones with four additional β-lactam antibiotics (piperacillin, flucloxacillin, benzyl penicillin,

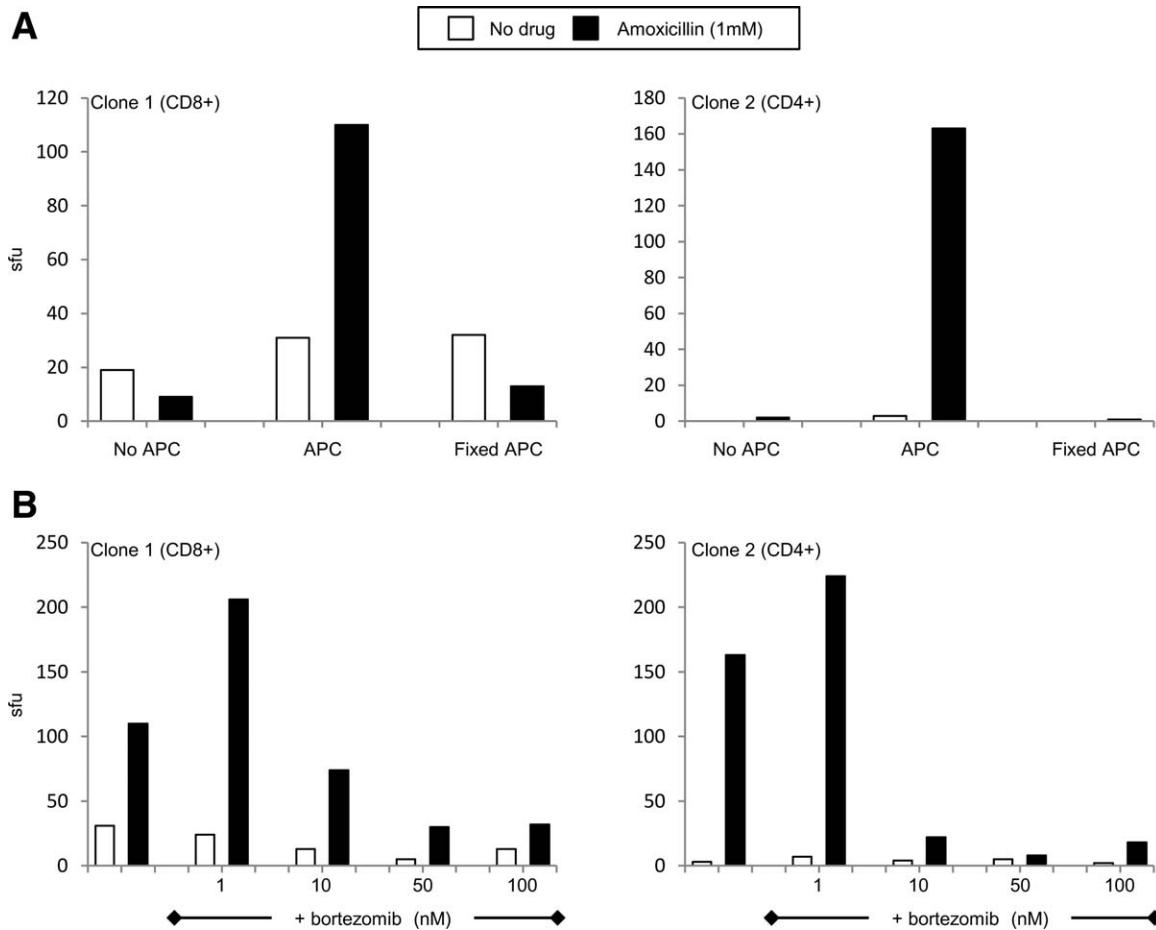


Fig. 7. Inhibition of antigen processing blocks amoxicillin-specific T-cell responses. (A) Clones were cultured in the presence of amoxicillin and irradiated or glutaraldehyde-fixed APCs. A no antigen-presenting cell condition was included as a control. (B) Clones were cultured in the presence of amoxicillin, irradiated APCs, and titrated concentrations of the proteosomal inhibitor, bortezomib. IFN-γ secretion was quantified using ELISpot.

and ampicillin). Clones were activated with amoxicillin and ampicillin, which indicates that the aromatic hydroxyl group of amoxicillin is not required for MHC T-cell receptor binding. In contrast, clones were not activated when the aliphatic amine moiety of amoxicillin was removed (i.e., benzyl penicillin) or modified with a bulky substituent (i.e., piperacillin). Detailed analyses of  $\beta$ -lactam T-cell cross reactivity using PBMCs from patients with cutaneous reactions has shown responses against amoxicillin and ampicillin.<sup>22</sup> However, these clones were generally detected alongside clones displaying reactivity with a large number of drugs, which presumably contribute to the multiple skin test positivity observed in patients with skin conditions.<sup>22</sup> A recent report describing a case of acute generalized exanthematous pustulosis—a skin condition involving IL-8-secreting, drug-specific T cells<sup>23</sup>—resulting from amoxicillin-clavulanate similarly reports multiple skin test positivity to drugs, including amoxicillin and benzyl penicillin.<sup>24</sup> Our data showing that all amoxicillin-responsive T cells are selectively activated with just two drugs (amoxicillin and ampicillin) suggest that the T-cell response is more restricted in patients with liver injury. As expected, owing to its differing structure, clavulanic acid clones were not activated with other  $\beta$ -lactam antibiotics.

Through characterization of the phenotype and profile of cytokines secreted by drug-specific T cells, it has been possible to develop a classification system for cutaneous adverse drug reactions.<sup>25,26</sup> For example, severe bullous reactions involve cytotoxic CD8<sup>+</sup> T cells that secrete effector molecules, such as perforin, FasL, and granzyme B, whereas CD4<sup>+</sup> T-cell responses predominate in patients with milder reactions, such as maculopapular exanthema. In this study, activation of amoxicillin-specific CD4<sup>+</sup> and CD8<sup>+</sup> clones was associated with secretion of high levels of IFN- $\gamma$  and the cytolytic molecules, granzyme B, perforin, and FasL. Several clones also secreted IL-22, but not IL-17. A more-restricted profile was observed with the clavulanic acid-specific clones; three secreted IFN- $\gamma$  and low levels of granzyme B, whereas one clone secreted IL-17 and IL-22 in the absence of other cytokines. Together, these data show that both CD4<sup>+</sup> and CD8<sup>+</sup> clones have the capacity to kill target cells and hence might contribute directly to tissue injury. Furthermore, they align with the recent demonstration that flucloxacillin-responsive CD8<sup>+</sup> T cells (1) from human donors kill liver cell lines transfected with HLA-B\*57:01<sup>13</sup> and (2) from a mouse model of flucloxacillin sensitization secrete IFN- $\gamma$  and granzyme B and promote hepatocyte apoptosis.<sup>27</sup> Generation of an HLA-transfected cell line to study amoxicillin-/clavulanic acid-specific T-cell-mediated

cytotoxicity is not possible owing to the fact that the drug antigens are presented to T-cells by multiple patient-specific HLA class I and II molecules. Thus, the approach we have initiated is to attempt to generate autologous induced pluripotent stem-derived hepatocytes from PBMCs of patients to study drug disposition in hepatocytes, formation of hepatocyte protein adducts, and ability of T cells to kill target cells. The role of IL-17 and IL-22 in the disease pathogenesis is intriguing. IL-17 is known to participate directly in several autoimmune diseases.<sup>28</sup> Serum IL-17 levels are increased in certain patients with acute liver failure,<sup>29</sup> whereas a recent study has reported that isoniazid-induced liver injury is associated with an increase in the number of IL-17-secreting cells.<sup>30</sup> In contrast, IL-22 is thought to be hepatoprotective through increasing expression of mitogenic and antiapoptotic proteins.<sup>31</sup> Obviously, further work is required to fully delineate the role of these cytokines in human DILI.

Despite several decades of research, the mechanisms of drug-specific T-cell activation remain an area of debate. All of the clones tested were activated with APCs pulsed with amoxicillin for 1-16 hours, suggesting that drug-protein adducts activate the clones. A rapid APC pulse, which does not lead to significant levels of adduct formation (results not shown), did not activate the clones. Hence, residual free drug does not activate T cells. Inhibition of antigen processing, either through chemical fixation of APCs or the addition of a specific proteasomal enzyme inhibitor, bortezomib, blocked activation of CD4<sup>+</sup> and CD8<sup>+</sup> clones; thus, processing of protein adducts seems to be the principal pathway involved in T-cell activation. Inhibition of CD4<sup>+</sup> clones with bortezomib was somewhat surprising; however, a parallel decrease in CD4<sup>+</sup> and CD8<sup>+</sup> activity has been reported previously in several *in vivo* models.<sup>32-34</sup>

Collectively, these data detail the nature of the drug-specific T-cell response induced in patients with amoxicillin-clavulanate-induced liver injury. This is the second example of human DILI where we have confirmed findings from genetic association studies using direct cell-based assays. Similar to flucloxacillin, drug hapten-responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells were detectable in most DILI patients. On the other hand, amoxicillin- and clavulanic acid-specific responses were not restricted to one major HLA allele, as is the case with flucloxacillin. To conclude, the adaptive immune system should now be considered an important contributory element in the development of human DILI.

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## Supporting Information

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep.27912/supinfo](http://onlinelibrary.wiley.com/doi/10.1002/hep.27912/supinfo).