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Characterization of amoxicillin and clavulanic acid specific T-cell clones from patients with immediate drug hypersensitivity

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Abstract

Background: Betalactam (BL) antibiotics are the most common cause of drug hypersensitivity. Amoxicillin (AX), which is often prescribed alongside clavulanic acid (Clav), is the most common elicitor. The aim of this study was to determine whether AX and Clav-responsive T-cells are detectable in patients with immediate hypersensitivity to AX-Clav, to assess whether these T-cells display the same specificity as that detected in skin and provocation testing, and to explore T-cell activation pathways.

Methods: Drug-specific T-cell clones were generated from immediate hypersensitive patients' blood by serial dilution and repetitive mitogen stimulation. Antigen specificity was assessed by measurement of proliferation and cytokine release. CD4⁺/CD8⁺ phenotype and chemokine receptor expression were analyzed by flow cytometry.

Results: 110 AX-specific and 96 Clav-specific T-cell clones were generated from seven patients with positive skin test to either AX or Clav. Proliferation of AX- and Clav-specific clones was dose-dependent, and no cross-reactivity was observed. AX- and Clav-specific clones required antigen-presenting cells to proliferate, and drugs were presented to CD4⁺ and CD8⁺ T-cells by MHC class-II and I, respectively. A higher secretion of IL-13 and IL-5 was detected in presence of the culprit drug compared with the alternative drug. Clones expressed CD69, CCR4, CXCR3, and CCR10.

Conclusions: Our study details the antigen specificity and phenotype of T-cell clones generated from patients with AX-Clav-induced immediate hypersensitivity diagnosed by positive skin test. AX- and Clav-specific clones were generated from patients irrespective of whether AX or Clav was the culprit, although differences in cytokine secretion were observed.

Abbreviations: APC, antigen-presenting cell; AX, amoxicillin; BL, β -lactam; BP, benzylpenicillin; BPO, benzylpenicilloyl; BP-OL, benzylpenicilloyl-octa-L-lysine; Clav, clavulanic acid; cpm, counts per minute; CR, cross-reactivity; DHR, drug hypersensitivity reaction; DPT, drug provocation test; EBV, Epstein-Barr virus; ENDA, European Network of Drug Allergy; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IDT, intradermal tests; IR, interquartile range; MD, minor determinant; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; ND, not done; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PHA, phytohemagglutinin; PPL, penicilloyl-polylysine; PV, penicillin V; SD, standard deviation; sfu, spot-forming units; SI, stimulation index; SPT, skin prick tests; SR, selective reaction; ST, skin test.

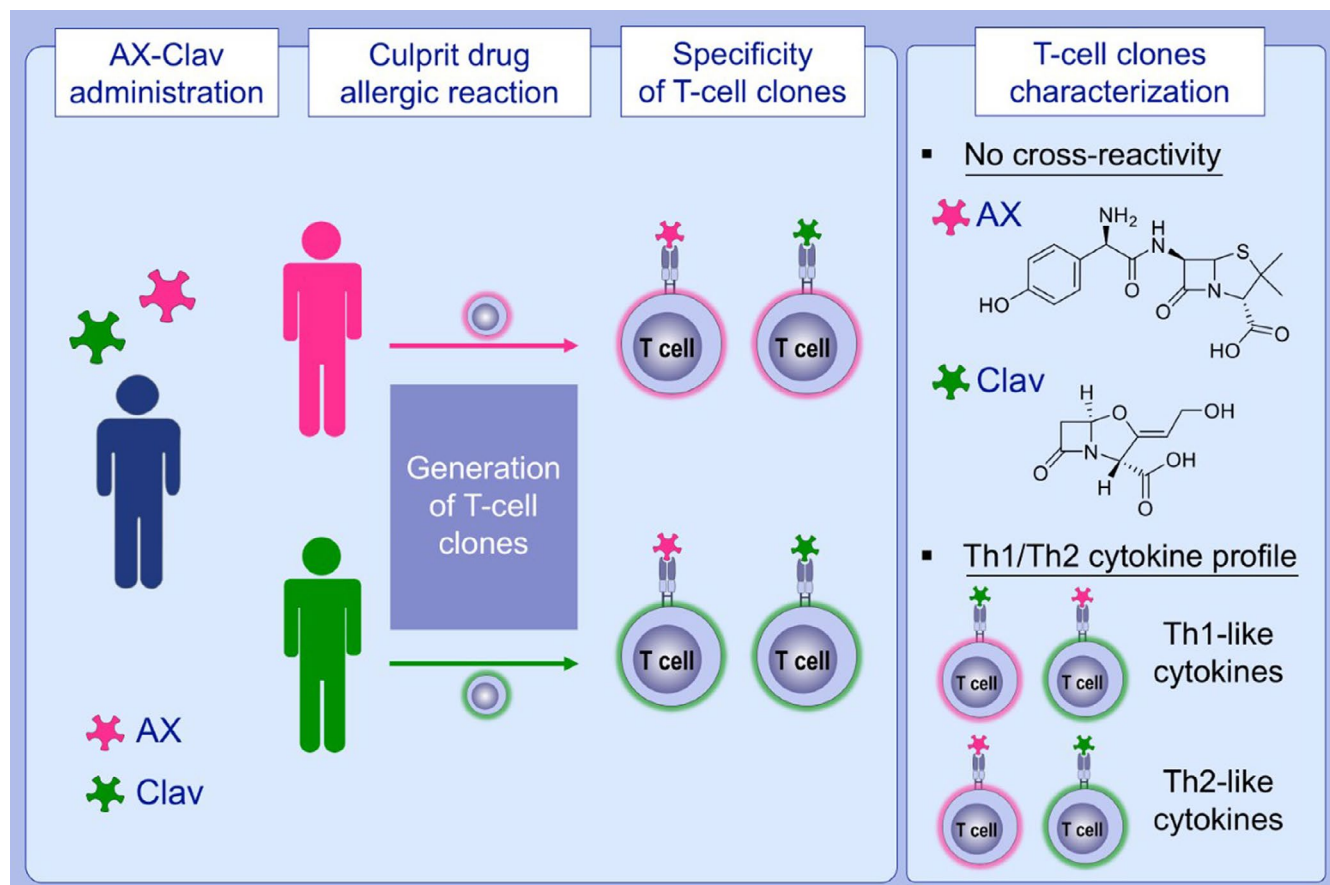
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KEYWORDS

amoxicillin, clavulanic acid, immediate hypersensitivity, phenotype, T-cell

**GRAPHICAL ABSTRACT**

AX- and Clav-specific T-cell clones can be generated from the same patient with immediate hypersensitivity, irrespective of whether AX or Clav is the culprit drug in the combination AX-Clav. No cross-reactivity between AX and Clav is detected. Differences in cytokine secretion are observed between T-cell clones generated against the culprit or the alternative drug in the combination AX-Clav. AX, amoxicillin; Clav, clavulanic acid.

1 | INTRODUCTION

Hypersensitivity reactions to β -lactam (BL) antibiotics are a major health problem in Europe, since they are the most frequently prescribed and also the most common culprits of drug hypersensitivity reactions (DHRs) mediated by a specific immunological mechanism.^{1,2} These reactions are clinically classified as immediate and nonimmediate, depending on their onset during treatment.³ Immediate reactions occur within 1–6 hours after drug administration, although they typically occur within the first hour and they are mainly mediated by an IgE-mechanism.⁴ Nonimmediate reactions occur at least 1 hour after drug administration, but they usually

appear from several hours to days after drug exposure and they are mediated by a T-cell mechanism.⁵

Prevalence and incidence values of DHRs have changed over time and across countries, possibly related to different patterns of prescription.² Nowadays, amoxicillin (AX) is the most common elicitor, in both children⁶ and adults.² Although AX can be prescribed alone, this antibiotic is often prescribed in combination with the β -lactamase inhibitor clavulanic acid (Clav)² to improve the efficacy of AX with the increase in bacterial resistance.⁷ Initially, hypersensitivity reactions in patients receiving AX-Clav were thought to be due to the AX, as animal studies models suggested that Clav did not activate specific components of the adaptive immune system.⁸

However, in 1995, two cases of selective allergic reactions to Clav were reported,⁹ and since then, the number of selective reactions to Clav has been increasingly described.¹⁰⁻¹² Although both drugs are BL structures, until now no cross-reactivity between them has been described. This may be due to differences in the chemical structure and the determinants derived from drug metabolism.¹⁰ Until now, only a few cases of patients sensitized independently to both AX and Clav have been reported, showing that specific reactions to both drugs can occur,^{13,14} even if cross-reactivity can be discounted. However, it is unclear why most hypersensitivity patients after the administration of AX-Clav develop selective hypersensitivity to AX while tolerating Clav and *vice versa*,¹⁰⁻¹² so further studies are necessary to clarify this.

In this work, we have generated and immunologically characterized AX- and Clav-specific T-cell clones from blood of patients that developed an immediate allergic reaction following exposure to AX-Clav. The diagnosis was confirmed in all cases by skin test (ST), and drug provocation test (DPT) was used to analyze the selectivity of the reaction. Previous studies utilizing peripheral blood mononuclear cells (PBMCs) from tolerant/naïve donors reported a large repertoire of naïve CD4⁺ and CD8⁺ T-cells that can be activated with the BL antibiotic benzylpenicillin (BP).¹⁵⁻¹⁷ This may be related to the fact that most of the general population in Europe has been exposed to BL antibiotics throughout their life. Similarly, T-cells with specificity for AX or Clav could be generated from healthy donor PBMCs; however, in this study we focused on patients with immediate reactions to (a) determine whether AX and Clav-specific T-cells are detectable, (b) assess whether T-cells display the same specificity as that detected in ST, and (c) explore pathways of T-cell activation.

2 | METHODS

2.1 | Human subjects

Seven patients with AX-Clav confirmed allergic reaction and ST positive were included in the study. Patients were diagnosed using the procedure described in the European Network of Drug Allergy (ENDA).^{3,18} Based on ST and DPT, patients were classified as allergic to AX (ST positive to AX and good tolerance to penicillin V in DPT [selective to AX] or ST positive to AX and DPT positive to penicillin V [cross-reactive with penicillin V]) and selective allergic reactors to Clav (ST positive to Clav and good tolerance to penicillin V and AX in DPT).

2.2 | Skin testing

Skin prick tests (SPT) were performed as recommended and if negative were followed by intradermal tests (IDT).³ The maximum concentration of penicillin derivatives used was as follows: benzylpenicilloyl-octa-L-lysine (BP-OL) 0.04 mg/mL (8.64×10^{-5} mol/L

benzylpenicilloyl [BPO] moiety); minor determinant (MD) 0.5 mg/mL (1.5×10^{-3} mol/L sodium benzylpenilloate); AX 20 mg/mL (5×10^{-2} mol/L), and Clav 20 mg/mL (8.4×10^{-2} mol/L) all from Diater-Ferrer. In the SPT, a wheal larger than 3 mm with a negative response to the control saline was considered positive. For IDT, the wheal area was marked at the beginning and 20 minutes after testing. An increase in the wheal diameter greater than 3 mm was considered positive.

2.3 | Drug provocation test

This test was carried out in a single-blind procedure.³ DPT consists of the administration of increasing doses of the drug at regular time intervals (30-60 minutes) to achieve the therapeutic dose, followed by the administration at home for two days. First, oral penicillin V (ERN) was administered at the clinical setting (400 mg) followed by two days of oral penicillin V (400 mg/12 h) at home. One week later, oral AX (GlaxoSmithKline) was administered at the clinical setting (500 mg) followed by two days of oral AX at home (500 mg/12 h).

2.4 | Biological samples/cell isolation

The Andalusian Public Health System Biobank has coordinated the collection, processing, management, and assignment of the biological samples used in this study, according to the standard procedures established for this purpose. Venous blood (70 mL) was collected from all the subjects, and PBMCs were isolated by density gradient separation.

2.5 | Generation of Epstein-Barr virus-transformed B-cell lines

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 fetal bovine serum (10%, v/v) (Invitrogen), L-glutamine (100 mmol/L), penicillin (100 µg/mL), and streptomycin (100 IU/mL).

2.6 | Medium for T-cell culture and cloning

RPMI 1640 supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES (25 mmol/L), L-glutamine (2 mmol/L), transferrin (25 mg/mL), penicillin (100 µg/mL), and streptomycin (100 IU/mL). IL-2 (100 IU/mL) was added to establish drug-specific T-cell clones. T-cell culture medium without antibiotics was used for testing the generated T-cell clones.

2.7 | Generation of T-cell clones

PBMCs from all the subjects (1×10^6 cells/well) were cultured with AX (0.5 and 1 mmol/L) or Clav (0.05 and 0.1 mmol/L) in T-cell culture medium without antibiotics for 14 days. To maintain antigen-specific proliferation, cultures were supplemented with recombinant human IL-2 (200 IU/mL) on days 6 and 9. On day 14, T-cells were serially diluted (0.3–3 cells/well) and subjected to phytohemagglutinin (PHA)-driven expansion (5 mg/mL) using well-established methodology described previously.^{19–21}

2.8 | Specificity of T-cell clones

T-cell clones were tested for AX and Clav specificity by measuring proliferation. T-cell clones (5×10^4 cells/well) were cultured with autologous irradiated EBV-transformed B-cells (1×10^4 cells/well) and AX (1 mmol/L) or Clav (0.1 mmol/L) for 48 hours. Proliferation was measured by the addition of [³H]thymidine (0.5 μ Ci/well, 5 Ci/mmol; Moravék Biochemicals) for the last 16 hours of the culture period. T-cell clones with a stimulation index (SI) greater than 1.5 were selected as potential specific T-cell clones and expanded by repetitive stimulation with irradiated allogeneic PBMCs (5×10^4 cells/well), PHA (10 μ g/mL), and IL-2 (700 IU/mL) for further experiments and specific response confirmation, as described below.

2.9 | Proliferative response and cross-reactivity studies

AX- and Clav-specific T-cell clones expanded after initial testing were tested for dose-response reactivity to AX (0.25–2 mmol/L) and Clav (0.025–0.2 mmol/L) for confirmation of specific drug response. Moreover, cross-reactivity between both drugs was also analyzed. Proliferation was measured by [³H]thymidine incorporation, and results were considered positive if SI higher than 2.0, as previously described.^{22,23} In addition, T-cell clones with SI lower than 2.0, but IFN- γ and/or IL-13 secretion tested by ELISpot (detailed below) in the presence of AX or Clav, were considered drug-specific T-cell clones.

2.10 | Release of cytokines and cytolytic molecules by T-cell clones

ELISpot was used to profile the cytokines (IFN- γ , IL-5, IL-10, IL-13, IL-17, and IL-22) and cytolytic molecules (granzyme B and perforin) released by specific T-cell clones. T-cell clones (5×10^4 cells/well) were cultured with autologous irradiated EBV-transformed B-cells (1×10^4 cells/well) and AX (1.5 mmol/L) or Clav (0.2 mmol/L) on plates precoated with capture antibody for 48 hours (Mabtech). Then, plates were washed and cytokine- or cytolytic molecules-secreting

cells were detected with biotin-conjugated antibodies and streptavidin-HRP, according to the manufacturer's instructions (Mabtech). Secreting cells were counted using an AID ELISpot reader.

2.11 | Phenotype of T-cell clones

CD phenotype of AX- and Clav-specific T-cell clones were characterized by flow cytometry. Antibodies used for flow cytometry staining were CD4-fluorescein isothiocyanate (FITC) and CD8-phycoerythrin (PE) (BD). Chemokine receptors were also analyzed by flow cytometry, using the antibodies CXCR3-allophycocyanin (APC), CCR2-APC, CCR9-APC, CCR6-APC, CXCR1-APC, CCR5-FITC, CCR3-FITC, CLA-FITC, E-cad-FITC, CD69FITC, CCR1-PE, CCR4-PE, CCR10-PE, CXCR6-PE, CCR8-PE. Chemokine receptor expression was presented as the mean fluorescence intensity (MFI) for the whole population of each analyzed T-cell clone, and data were normalized using the unstained cells.

2.12 | Functional studies of T-cell activation

To analyze the mechanistic basis of AX and Clav-specific T-cell activation, CD4⁺ and CD8⁺ T-cell clones were subjected to further experiments: (a) to explore the pathways of drug antigen presentation and intracellular processing for AX and Clav in the T-cell response, T-cell clones were cultured with AX or Clav in presence or absence of autologous irradiated APCs or irradiated and fixed APCs (fixation: 0.05% glutaraldehyde for 30 seconds, 1 mmol/L glycine for 45 seconds and extensive washing), (b) the importance of MHC class in the presentation of AX and Clav to specific T-cell clones was tested by the previous incubation of APC with MHC class-I and II blocking antibodies for 30 minutes at 37°C. Then, activation of T-cells was determined in both experiments by proliferation or IFN- γ and IL-13 secretion assays.

2.13 | Statistical analysis

Quantitative variables were expressed as mean and standard deviation (SD) or median and interquartile range (IR), and comparisons were carried out using the *t* test for mean values and the Mann-Whitney and Wilcoxon tests for median values of nonrelated and related samples, respectively. *P* values $\leq .05$ were considered statistically significant and represented as **P* $\leq .05$, ***P* $< .01$, or ****P* $< .001$.

2.14 | Ethical declaration

The study was conducted according to the Declaration of Helsinki principles and was approved by the local Ethics Committee (*Comité de Ética para la Investigación Provincial de Málaga*, Spain). All subjects

were informed orally about the study, and they signed the corresponding informed consent form.

3 | RESULTS

3.1 | Donor clinical characteristics

Patients 1-7 (five men and two women) were diagnosed with immediate hypersensitivity reaction after the administration of the combination AX-Clav (Table 1) (see Table S1). Clinical manifestations ranged from urticaria to anaphylactic shock. All patients were skin test positive in immediate reading to either AX (patients 1-4) or Clav (patients 5-7), one of them by SPT (patient 4) and the rest by IDT. Patients with positive skin test to AX tolerated (patients 2-4) or did not tolerate (patient 1) the administration of penicillin V. All patients with positive skin test to Clav tolerated the administration of AX.

3.2 | Generation of AX- and Clav-specific T-cell clones from blood

AX- and Clav-treated PBMCs from patients 1-7 were serially diluted and subjected to repetitive rounds of stimulation to generate over 3100 T-cell cultures originating from single precursor cells. A total of 110 and 97 T-cell clones displayed reactivity against AX and Clav, respectively, on initial testing and they were selected and expanded to be tested in further analysis (see Figure S1 and Table S2).

We analyzed the percentage of responsive T-cell clones obtained and attempted to correlate the data obtained with time interval factors: (a) time interval between drug administration and reaction (min), (b) time interval between reaction and sample obtaining (days), but no significant correlation was observed for either. AX- and Clav-specific T-cell clones were detected in PBMCs of patients presenting AX- or Clav-diagnosed allergic reactions, with no significant difference in T-cell clone numbers when the patients were compared.

3.3 | Dose-response and cross-reactivity

AX- and Clav-responsive T-cell clones selected and expanded from initial testing were used in dose-response proliferation experiments. A dose-dependent increase in T-cell proliferation was obtained with both AX and Clav. No proliferation was detected when AX-responsive T-cell clones were cultured with Clav and *vice versa* (Figure 1).

3.4 | CD4⁺/CD8⁺ phenotype

T-cell clones displaying reactivity against AX or Clav were mainly CD4⁺ (see Figure S2 and Table S3), with a significant higher percentage of CD4⁺ clones compared with CD8⁺ and CD4⁺CD8⁺ clones. No significant differences in the CD4⁺/CD8⁺ phenotype were observed between AX- or Clav-specific T-cell clones as well as between T-cell clones generated against the culprit or the alternative drug.

3.5 | Profile of cytokines and cytolytic molecules secreted by specific T-cell clones

IFN- γ and IL-13 secretion was detected from CD4⁺ and CD8⁺ clones stimulated with either AX or Clav (Figure 2A). Differences between CD4⁺ and CD8⁺ were observed, with higher median values of IFN- γ and IL-13 secreted by CD8⁺ and CD4⁺ clones, respectively (Figure 2B). Focusing on the analysis of CD4⁺ T-cell clones, the main phenotype of specific T-cell clones detected in our study, the levels of secreted IFN- γ was significantly higher than secreted IL-13 for the T-cell clones specific to the alternative drug, with no significant differences for the specific to the culprit drug (Figure 2C).

A panel of 22 CD4⁺ T-cell clones was screened for release of additional cytokines and cytolytic molecules. The secretion of Th1 (IFN- γ) and Th2 (IL-5, IL-10, IL-13) cytokines was detected. Interestingly, IL-17 secretion was also detected with five T-cell clones (all of them specific to the alternative drug), and IL-22 secretion was detected for four T-cell clones (two of them also positive for IL-17 secretion). Significant differences for the mean values of cytokine secretion are showed for

TABLE 1 Clinical details of patients included in the study with confirmed hypersensitivity to AX or Clav after the administration of the combination AX-Clav

ID	Age	Gender	Drug-reaction interval (min)	React-study interval (days)	Reaction	Clinic diagnose
P1	47	M	30	20	Anaphylaxis	AX hypers (CR penicillin V)
P2	66	M	120	3650	Anaphylaxis	AX hypers (SR)
P3	51	M	30	730	Anaphylaxis	AX hypers (SR)
P4	66	M	10	2920	Anaphylactic shock	AX hypers (SR)
P5	44	F	20	240	Anaphylaxis	Clav hypers
P6	34	F	10	730	Urticaria	Clav hypers
P7	63	M	5	1460	Anaphylactic shock	Clav hypers

Abbreviations: AX, amoxicillin; Clav, clavulanic acid; CR, cross-reactivity; F, female; hypers, hypersensitivity; M, male; P, patient; SR, selective reaction.

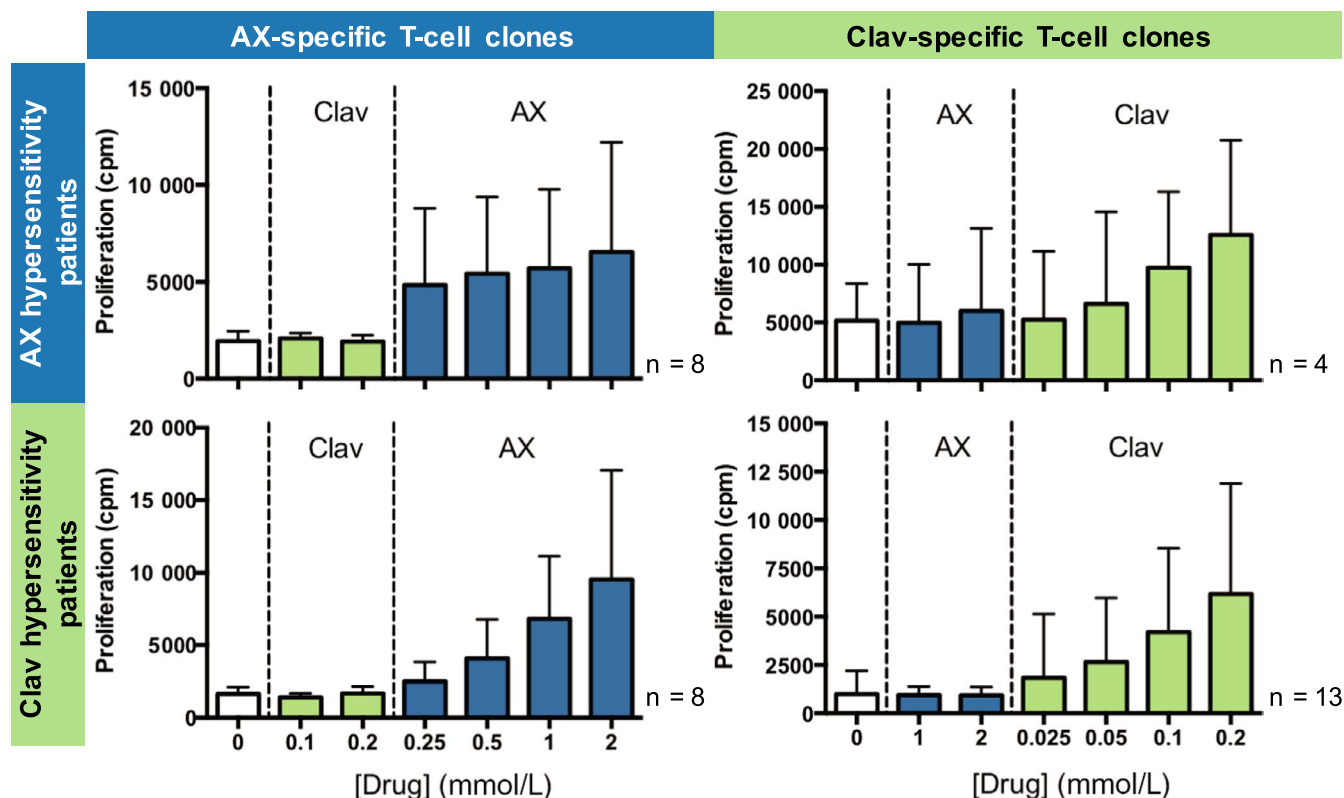


FIGURE 1 Dose-response and cross-reactivity proliferation assay with AX- and Clav-specific T-cell clones derived from blood. AX- and Clav-specific T-cell clones were cultured with APCs and either AX or Clav for 2 days before analysis of proliferation by the addition of [^{3} H]-thymidine. Results are expressed as mean + SD of counts per minute (cpm)

groups of specific T-cell clones generated from both AX and Clav hypersensitivity patients (Figure 3A, see Figure S3). Comparing levels of secreted cytokines by T-cell clones activated with the culprit or the alternative drug, significantly higher and lower levels of IL-5 and IFN- γ , respectively, were observed when activated with the culprit drug (AX or Clav) (Figure 3B). T-cell clones secreted low levels of the cytolytic molecules perforin and granzyme B (results not shown).

3.6 | CD4⁺ and CD8⁺ T-cell clones are activated via processing-dependent pathway in a MHC-restricted manner

Experiments to analyze the pathways of drug presentation and processing showed that CD4⁺ T-cell clones did not proliferate with the drug in absence of APCs or presence of fixed APCs (Figure 4, top panel). Similar results were observed for CD8⁺ T-cell clones (Figure 4, bottom panel). Although proliferation was detected for the CD8⁺ T-cell clone tested for the highest concentrations of drug, proliferation was only observed in the presence of APCs when drug concentration was reduced (Figure 4, left bottom panel). Moreover, proliferation assays using MHC blocking antibodies showed that AX and Clav were presented to CD4⁺ T-cell clones by MHC class-II and to CD8⁺ T-cell clones by MHC class-I (Figure 5).

3.7 | Chemokine receptor expression

Expression of 12 chemokine receptors, CLA, CD69, and E-cadherin was compared on AX- and Clav-specific T-cell clones. Both specific T-cell clones were found to express CD69, followed by CCR4, CXCR3, and CCR10 compared with the rest of tested chemokine receptors when flow cytometry data were analyzed (see Figure S4). Both CD69 and CCR4 were significantly highly expressed than CCR10 and CXCR3 in all the analyzed T-cell clones (see Figure S5A). When receptor expression was compared between reactive clones to the culprit and the alternative drug, higher expression of CCR10 was detected on T-cell clones displaying reactivity to the alternative drug (see Figure S5B).

4 | DISCUSSION

The combination of AX and Clav is widely used in Europe to control and prevent infections²⁴; however, nowadays it is also the most frequent cause of allergic drug reactions.^{2,25} Most patients suffering a reaction after AX-Clav intake present selective sensitization to either AX or Clav.^{10,12,26} Although both drugs are BL antibiotics, no cross-reactivity has been described, mainly due to differences in their chemical structure and degradation patterns.^{8,10,12,26-31} An accurate diagnosis of these patients is crucial, because Clav-allergic subjects can tolerate other BLs, including AX.²⁵ However, diagnosis is complex,

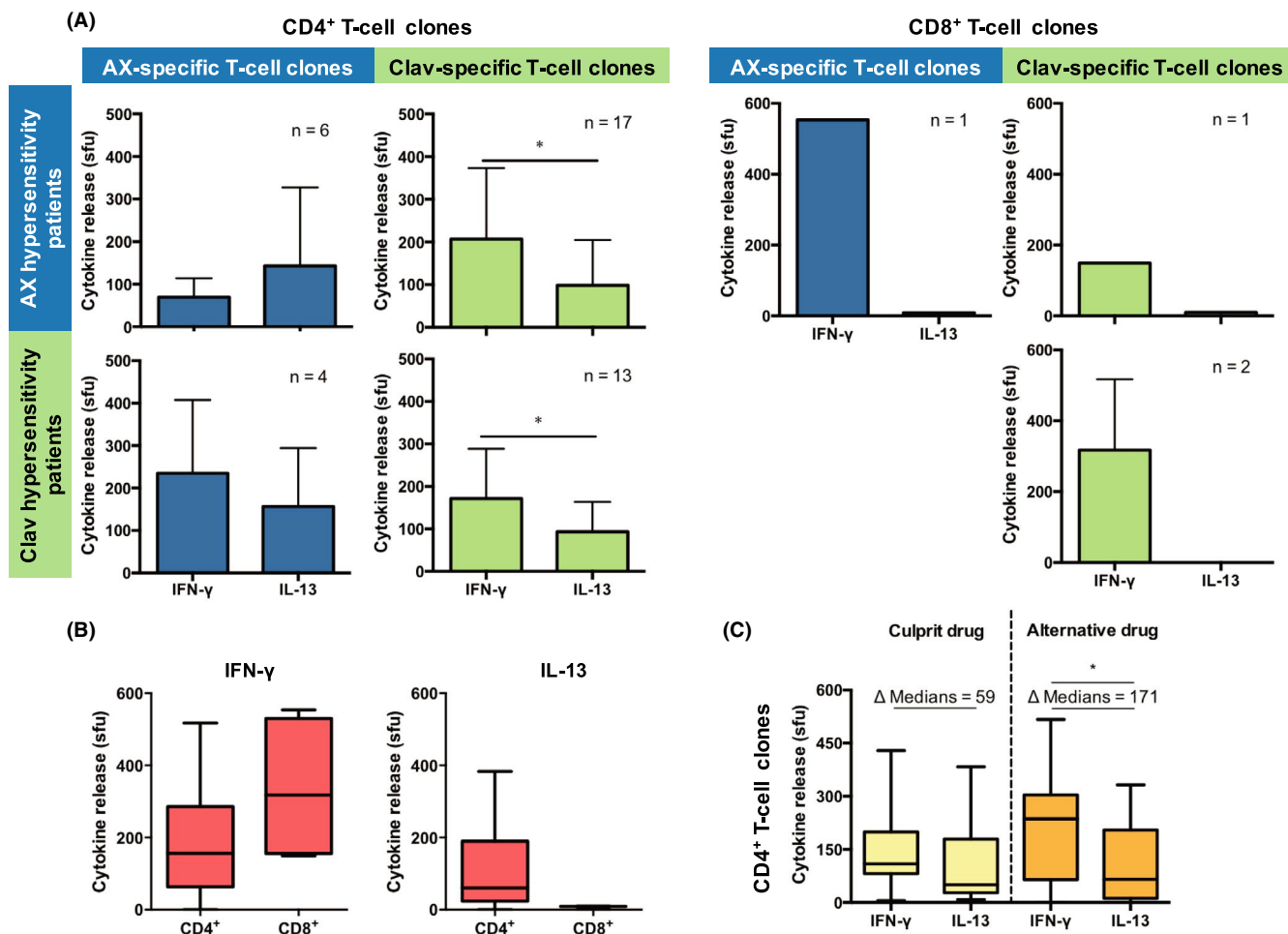


FIGURE 2 Levels of IFN- γ and IL-13 secreted by AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients. T-cell clones were cultured with irradiated APCs and AX or Clav, and cytokines secreted were detected by ELISpot. A, Level of IFN- γ and IL-13 secreted by T-cell clones derived from hypersensitivity patients. Results are expressed as mean + SD of spot-forming units (sfu). Statistical analysis was performed using *t* test (significant differences if $P \leq .05$). * $P \leq .05$. B, Comparison of IFN- γ and IL-13 secreted by CD4⁺ and CD8⁺ T-cell clones derived from hypersensitive patients. C, Comparison of IFN- γ and IL-13 secreted by CD4⁺ T-cell clones derived from hypersensitive patients that respond to the culprit or the alternative drug. Box plots show medians spot-forming units (sfu), with error bars showing 1st/99th percentiles. Statistical analysis was performed using Mann-Whitney test (significant differences if $P \leq .05$). * $P \leq .05$

including ST and DPT.^{32,33} In vitro tests can help as complementary diagnostic tool in the evaluation of these reactions, but the sensitivity is not optimal,³⁴ probably due to the use of chemical structures (parent drugs, metabolites, or synthetic drug-protein conjugates) that are not optimally recognized by patients' immune system. The main in vitro methods for the evaluation of IgE-mediated reactions are the determination of specific IgE by fluoroimmunoassay (ImmunoCAP, Phadia) with a low and variable sensitivity (0%-50%)³⁵⁻³⁸ or by radioimmunoassay (42.9%-75%),^{35,39,40} the basophil activation test with a sensitivity for penicillins ranged from 22% to 55% and for Clav up to 52%,^{38,41} and the histamine release test with a sensitivity lower than 60%.^{42,43} The aim of this study was to explore whether it was possible to detect T-cell responses to AX and Clav in patients with immediate hypersensitivity to the combination AX-Clav, where the culprit drug, either AX or Clav, had been defined through ST and DPT. It was possible to generate AX and Clav-specific T-cell clones from PBMCs isolated from all patients irrespective of whether AX or Clav was the

culprit drug. CD4⁺ and CD8⁺ T-cell clones were found to proliferate and/or secrete IFN- γ or IL-13 in the presence of either AX or Clav in a dose-dependent manner. The optimal concentration of AX and Clav required to activate the T-cell clones (1-2 and 0.1-0.2 mmol/L, respectively) was higher than circulating concentrations in plasma (about 0.1 mmol/L for the oral administration of 250/125 mg AX-Clav)⁴⁴; however, the percentage of drug bioavailability and drug binding on proteins does not correlate between in vivo and in vitro exposure. Furthermore, Meng et al⁴⁵ have shown using the BL piperacillin, that the levels of drug-albumin modification needed to activate T-cells in vitro are equivalent to the ones formed in allergic and tolerant patients, which indicates that threshold levels of drug-associated antigen are formed in all patients. Importantly, no cross-reactivity was observed between AX and Clav, as previously described with T-cells derived from AX-Clav-induced liver injury patients.²³

Until now, it is unclear why patients with hypersensitivity to the combination AX-Clav develop a selective reaction to AX while

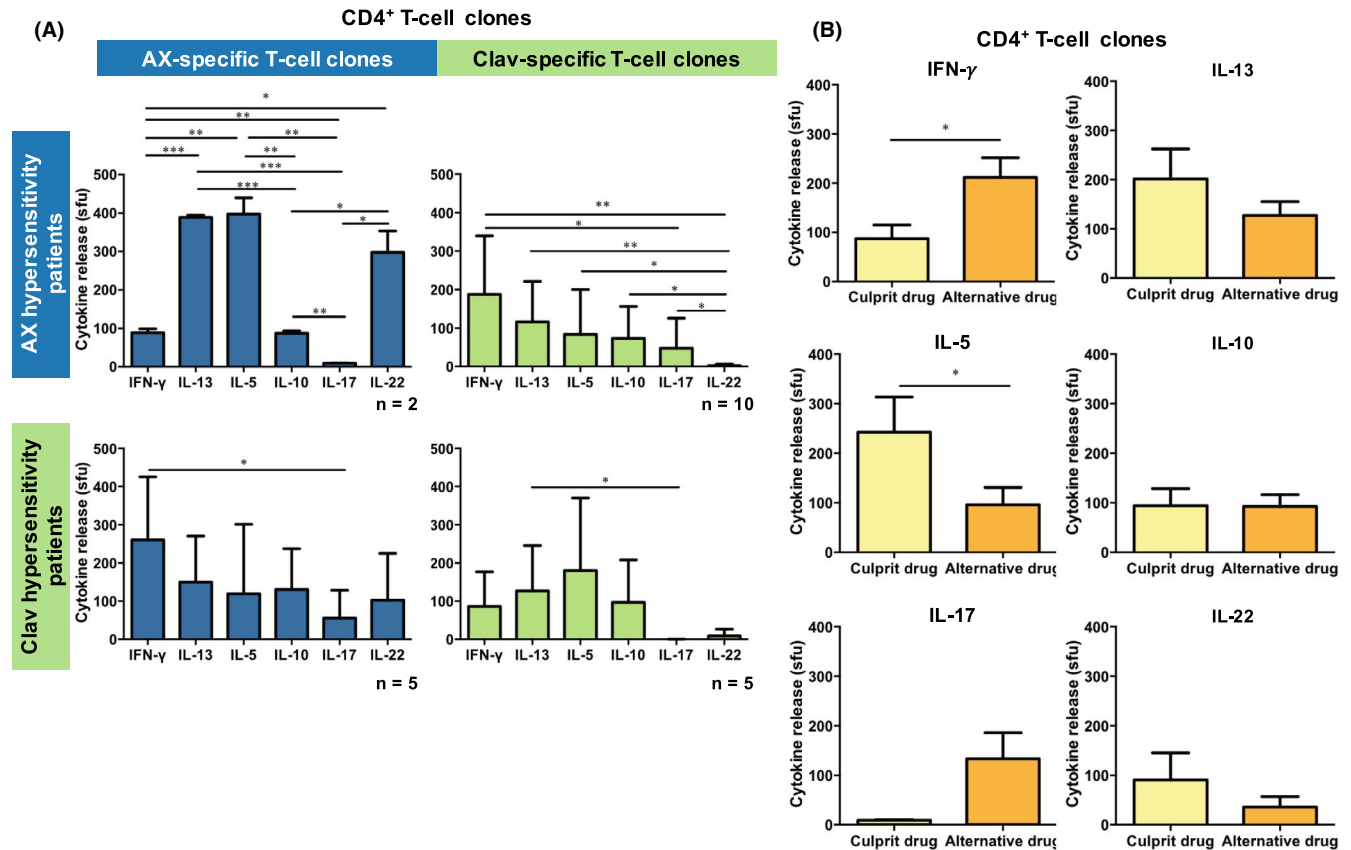


FIGURE 3 Profile of cytokines secreted by CD4⁺ AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients. T-cell clones were cultured with irradiated APCs and AX or Clav, and secreted cytokines were quantified by ELISpot. A, Profile of cytokines secreted by CD4⁺ AX- and Clav-specific T-cell clones. Results are expressed as mean $\pm$ SD spot-forming units (sfu). B, Comparison of levels of cytokines molecules secreted by T-cell clones derived from hypersensitivity patients that respond to the culprit or alternative drug. Results are shown as mean $\pm$ SD spot-forming units (sfu). Statistical analysis was performed using t test (significant differences if $P \leq .05$) * $P \leq .05$; ** $P < .01$; *** $P < .001$

tolerating Clav and *vice versa*.¹⁰⁻¹² In this study, we were able to generate specific T-cell clones to both drugs using PBMCs from a same donor that developed allergic reactions selectively to either AX or Clav. Previous studies with PBMCs from tolerant donors have reported a repertoire of naïve CD4⁺ and CD8⁺ T-cells that can be activated with benzylpenicillin.¹⁵⁻¹⁷ This observation may be related to the fact that most of the general population in Europe has been exposed to BL antibiotics throughout their life. This exposure could also explain the detection of AX-specific T-cell clones in Clav-allergic patients and *vice versa*, that could be reinforced by the fact that both drugs are administered simultaneously in therapeutical treatments. However, when we analyzed cytokines secreted from drug-responsive CD4⁺ T-cell clones (the most abundant clones), a higher secretion of IL-13 and IL-5 (Th2 cytokines)⁴⁶ was detected when clones were stimulated with the culprit drug (AX or Clav). Furthermore, T-cell clones activated with the alternative drug secreted higher levels of IFN- γ (Th1 cytokine).⁴⁷ These results suggest that the ratio of Th2/Th1 secreting T-cell clones is higher with T-cell clones displaying reactivity toward the culprit drug. Patients included in the present study developed an immediate hypersensitivity reaction after the administration of the combination AX-Clav, with diagnostic

confirmation of the culprit component (AX or Clav) by positive ST, and tolerance confirmation of the other component by negative DPT. Th2 immune response is described to be involved in immediate or IgE-mediated reactions, and the secretion of Th2-type cytokines (IL-4, IL-5, IL-9, and IL-13) to be entailed in the modulation of the response of effector cells and the activation of allergen-specific B-cells as well as the induction of class switching to IgE.^{48,49} On the other hand, Th1 cells and Th1-type cytokines (IFN- γ) have been reported to induce B-cell class switching to IgG isotypes and inhibit IgE production.⁵⁰ Furthermore, IFN- γ has been described to inhibit Th2-mediated allergic inflammatory responses.^{49,51,52} This reported information could explain the secretion of higher levels on IFN- γ by specific T-cell clones to the alternative drug, whose administration is tolerated by patients up to therapeutical doses. The AX- and Clav-specific T-cell clones were also found to secrete IL-17 and IL-22. IL-17 is involved in autoimmune diseases and immune responses against extracellular bacteria and fungi⁵³ and helps to maintain immune responses including allergic inflammation.⁵⁴⁻⁵⁶ IL-22 increases the innate immunity of tissue cells, protects tissues from damage, and enhances tissue regeneration, targeting specifically the skin, the digestive tract, the lungs, and kidney.⁵⁷ In our study, IL-17 secretion was only observed

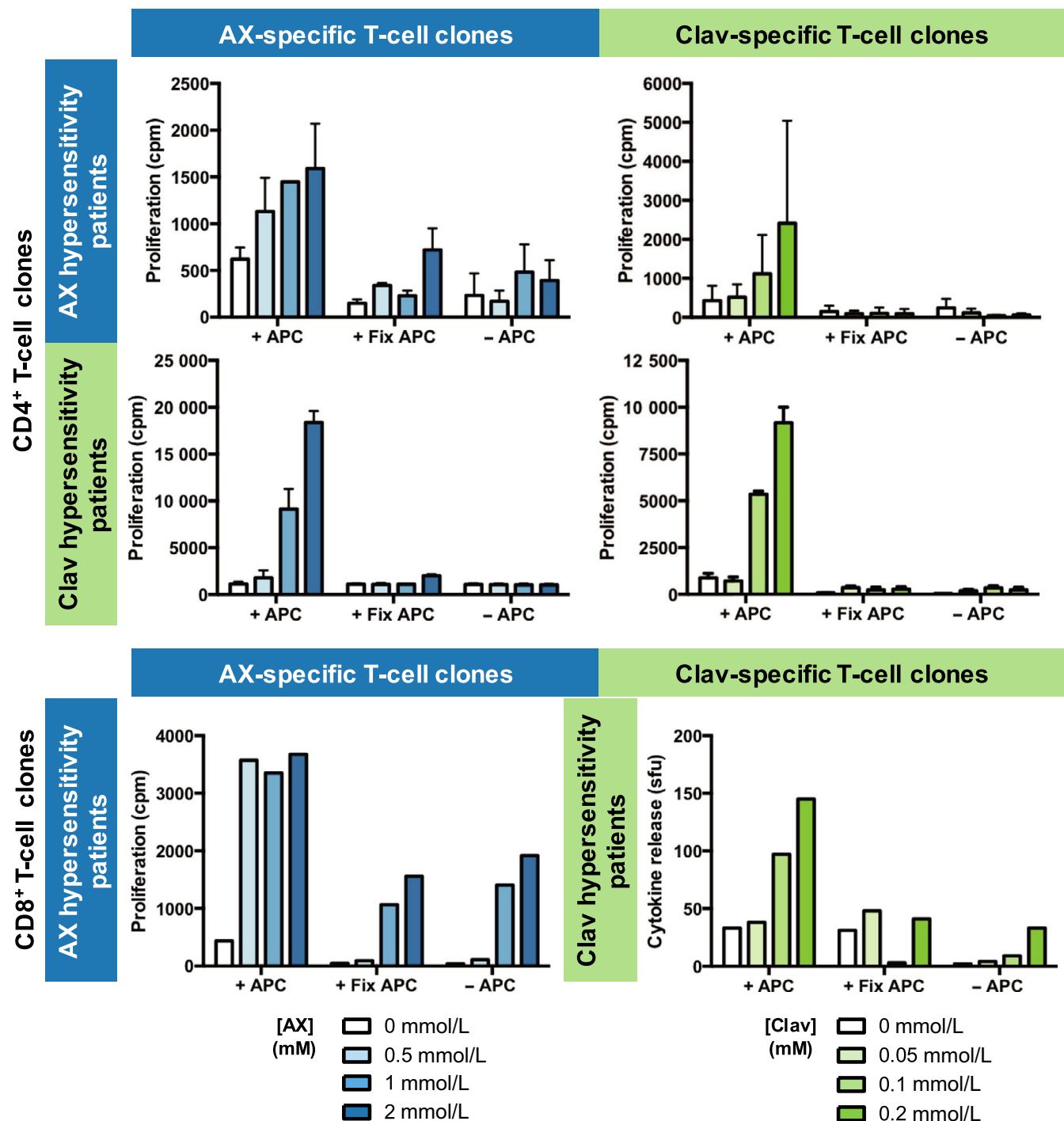


FIGURE 4 Proliferation or cytokine secretion assay to analyze the antigen processing-dependent or independent pathway for the activation of T-cell clones. AX- and Clav-specific T-cell clones generated from hypersensitivity patients were co-cultured with APCs (+APC), fixed APC (+Fix APC), or non-APC (–APC) for 2 days before the proliferation analysis by the addition of [³H]thymidine or cytokine secretion by ELISpot. Results are expressed as mean + SD of counts per minute (cpm) or spot-forming unit (sfu)

in a small number of T-cell clones, all of them responsive toward the alternative drug. IL-22 secretion was also detected with several T-cell clones together with other cytokines.

All the T-cell clones expressed the chemokine receptors CCR4 (highly expressed on Th2 cells),⁵⁸ CXCR3 (highly expressed on Th1 cells),⁵⁹ CCR10, and the activation marker CD69. CCR4 and CXCR3

have been also reported to be expressed on AX- and Clav-specific T-cell clones from patients with AX-Clav-induced liver injury.²³ Interestingly, a statistically significant lower expression of CCR10 was detected for T-cell clones specific to the culprit drug. CCR10 is a homeostatic receptor expressed under basal conditions, but overexpressed in inflammatory states.⁶⁰

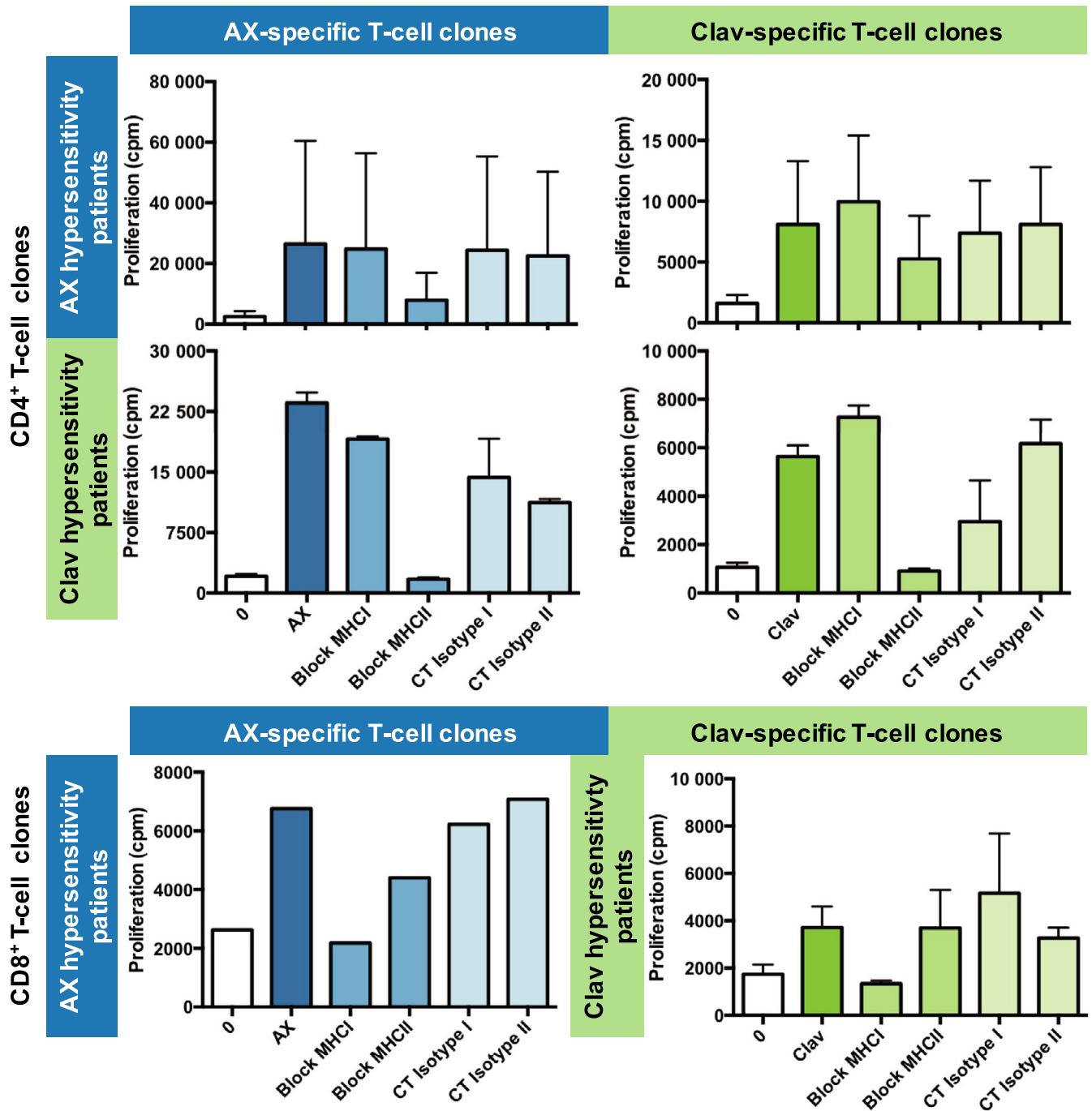


FIGURE 5 Proliferation assay to analyze the antigen presentation to T-cell clones by MHC. APCs were incubated for 30 min at 37°C with MHC class-I and II blocking antibodies previously to carry on with the culture with T-cell clones and AX or Clav for 2 days before the proliferation analysis by the addition of [³H]thymidine. Results are expressed as mean + SD of counts per minute (cpm)

Activation of T-cell clones with either AX or Clav was dependent on APCs, since chemical fixation or absence of APCs inhibited the activation of both CD4⁺ and CD8⁺ T-cell clones. Thus, processing of protein adducts seems to be the principal pathway involved in T-cell activation by AX and Clav in patients with immediate allergic reactions. Although some response was detected for AX-specific CD4⁺ and CD8⁺ clones in the presence of 1-2 mmol/L AX and fixed APCs, no response was observed at the lower AX concentration for which we observed a clear response in the presence of APCs. Moreover,

no response was detected in the absence of APCs, suggesting that AX should be processed and presented by APC to induce a proliferation response. The relatively weak response with high concentrations and fixed APC likely relates to the AX hapten binding directly to HLA peptides expressed on the surface of APCs. This supports the hapten hypothesis for the recognition and presentation of BL antibiotics.⁶¹ Moreover, activation of the clones with AX or Clav was MHC restricted by class-I to CD8⁺ and by class-II to CD4⁺ clones.

In summary, our study details the antigen specificity and phenotype of T-cell clones generated from patients with AX-Clav-induced immediate hypersensitivity reactions. Differences in cytokine secretion were observed between T-cell clones generated against the culprit drug (AX or Clav) to which the patients selectively responded or the alternative drug in the combination AX-Clav. The availability of well-characterized AX- and Clav-responsive T-cell clones will allow testing the immunological recognition of a wide panel of chemical structures derived from AX and Clav that nowadays are not included in diagnostic tests, to go further in the improvement of the sensitivity of the in vitro tests for the evaluation of drug hypersensitivity reactions.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

MJT and DJN designed the study and coordinated the work of the rest of the authors. MS and GB recruited the study individuals and conducted the clinical procedures. AA, RFS, XM, MOO, and AT performed the in vitro experiments. AA, MJT, and DJN analyzed the experimental results and wrote the manuscript, which was reviewed by the rest of the authors.

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REFERENCES

- Adriaenssens N, Coenen S, Versporten A, et al. European Surveillance of Antimicrobial Consumption (ESAC): outpatient antibiotic use in Europe (1997–2009). *J Antimicrob Chemother*. 2011;66(suppl_6):vi3–vi12.
- Dona I, Blanca-Lopez N, Torres MJ, et al. Drug hypersensitivity reactions: response patterns, drug involved, and temporal variations in a large series of patients. *J Invest Allergol Clin Immunol*. 2012;22(5):363–371.
- Blanca M, Romano A, Torres MJ, et al. Update on the evaluation of hypersensitivity reactions to betalactams. *Allergy*. 2009;64(2):183–193.
- Bircher AJ, Scherer Hofmeier K. Drug hypersensitivity reactions: inconsistency in the use of the classification of immediate and non-immediate reactions. *J Allergy Clin Immunol*. 2012;129(1):263–264; author reply 5–6.
- Torres MJ, Salas M, Ariza A, Fernandez TD. Understanding the mechanisms in accelerated drug reactions. *Curr Opin Allergy Clin Immunol*. 2016;16(4):308–314.
- Zambonino MA, Corzo JL, Muñoz C, et al. Diagnostic evaluation of hypersensitivity reactions to beta-lactam antibiotics in a large population of children. *Pediatric Allergy Immunol*. 2014;25(1):80–87.
- Solensky R. Penicillin allergy as a public health measure. *J Allergy Clin Immunol*. 2014;133(3):797–798.
- Edwards RG, Dewdney JM, Dobrzanski RJ, Lee D. Immunogenicity and allergenicity studies on two beta-lactam structures, a clavam, clavulanic acid, and a carbapenem: structure-activity relationships. *Int Arch Allergy Appl Immunol*. 1988;85(2):184–189.
- Fernandez-Rivas M, Perez Carral C, Cuevas M, Marti C, Moral A, Senent CJ. Selective allergic reactions to clavulanic acid. *J Allergy Clin Immunol*. 1995;95(3):748–750.
- Blanca-Lopez N, Perez-Alzate D, Ruano F, et al. Selective immediate responders to amoxicillin and clavulanic acid tolerate penicillin derivative administration after confirming the diagnosis. *Allergy*. 2015;70(8):1013–1019.
- Sanchez-Morillas L, Perez-Ezquerro PR, Reano-Martos M, Laguna-Martinez JJ, Sanz ML, Martinez LM. Selective allergic reactions to clavulanic acid: a report of 9 cases. *J Allergy Clin Immunol*. 2010;126(1):177–179.
- Torres MJ, Ariza A, Mayorga C, et al. Clavulanic acid can be the component in amoxicillin-clavulanic acid responsible for immediate hypersensitivity reactions. *J Allergy Clin Immunol*. 2010;125(2):502–505.e2.
- Cahen YD, Wuthrich B. Drug allergy to the beta-lactam antibiotics clavulanic acid and amoxicillin. *Allergy*. 1997;52(1):117–118.
- Salas M, Laguna JJ, Dona I, et al. Patients taking amoxicillin-clavulanic can become simultaneously sensitized to both drugs. *J Allergy Clin Immunol Pract*. 2017;5(3):694–702.e3.
- Azoury ME, Fili L, Bechara R, et al. Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy. *Allergy*. 2018;73(8):1662–1672.
- Bechara R, Maillere B, Joseph D, Weaver RJ, Pallardy M. Identification and characterization of a naive CD8+ T cell repertoire for benzylpenicillin. *Clin Exp Allergy*. 2019;49(5):636–643.
- Nhim C, Delluc S, Halgand F, et al. Identification and frequency of circulating CD4(+) T lymphocytes specific to Benzylpenicillin in healthy donors. *Allergy*. 2013;68(7):899–905.
- Dona I, Romano A, Torres MJ. Algorithm for betalactam allergy diagnosis. *Allergy*. 2019;74(9):1817–1819.
- Wu Y, Farrell J, Pirmohamed M, Park BK, Naisbitt DJ. Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. *J Allergy Clin Immunol*. 2007;119(4):973–981.
- Mauri-Hellweg D, Bettens F, Mauri D, Brander C, Hunziker T, Pichler WJ. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol*. 1995;155(1):462–472.
- Schnyder B, Burkhardt C, Schnyder-Frutig K, et al. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J Immunol*. 2000;164(12):6647–6654.
- Sullivan TJ, Wedner HJ, Shatz GS, Yecies LD, Parker CW. Skin testing to detect penicillin allergy. *J Allergy Clin Immunol*. 1981;68(3):171–180.
- Kim SH, Saide K, Farrell J, et al. Characterization of amoxicillin- and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate-induced liver injury. *Hepatology*. 2015;62(3):887–899.

24. Adriaenssens N, Coenen S, Kroes AC, et al. European Surveillance of Antimicrobial Consumption (ESAC): systemic antiviral use in Europe. *J Antimicrob Chemother.* 2011;66:9.
25. Torres MJ, Montanez MI, Ariza A, et al. The role of IgE recognition in allergic reactions to amoxicillin and clavulanic acid. *Clin Exp Allergy.* 2016;46(2):264-274.
26. Sanchez-Morillas L, Rojas Perez-Ezquerro P, Reano-Martos M, Laguna-Martinez JJ, Sanz ML, Morales Martinez L. Selective allergic reactions to clavulanic acid: a report of 9 cases. *J Allergy Clin Immunol.* 2010;126:177-179.
27. Meng X, Earnshaw CJ, Tailor A, et al. Amoxicillin and clavulanate form chemically and immunologically distinct multiple haptenic structures in patients. *Chem Res Toxicol.* 2016;29(10):1762-1772.
28. Barbero N, Fernandez-Santamaria R, Mayorga C, et al. Identification of an Antigenic determinant of clavulanic acid responsible for IgE-mediated reactions. *Allergy.* 2019;74(8):1490-1501.
29. Levine BB, Ovary Z. 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.* 1961;114:875-904.
30. Levine BB, Redmond AP. Minor haptenic determinant-specific reagins of penicillin hypersensitivity in man. *Int Arch Allergy Appl Immunol.* 1969;35(5):445-455.
31. Moreno F, Blanca M, Mayorga C, et al. Studies of the specificities of IgE antibodies found in sera from subjects with allergic reactions to penicillins. *Int Arch Allergy Immunol.* 1995;108(1):74-81.
32. Torres MJ, Blanca M, Fernandez J, et al. Diagnosis of immediate allergic reactions to beta-lactam antibiotics. *Allergy.* 2003;58(10):961-972.
33. Romano A, Blanca M, Torres MJ, et al. Diagnosis of nonimmediate reactions to beta-lactam antibiotics. *Allergy.* 2004;59(11):1153-1160.
34. Mayorga C, Celik G, Rouzaire P, et al. In vitro tests for drug hypersensitivity reactions: an ENDA/EAACI Drug Allergy Interest Group position paper. *Allergy.* 2016;71(8):1103-1134.
35. Fontaine C, Mayorga C, Bousquet PJ, et al. Relevance of the determination of serum-specific IgE antibodies in the diagnosis of immediate beta-lactam allergy. *Allergy.* 2007;62(1):47-52.
36. Blanca M, Mayorga C, Torres MJ, et al. Clinical evaluation of pharmac CAP system RAST FEIA amoxicilloyl and benzylpenicilloyl in patients with penicillin allergy. *Allergy.* 2001;56(9):862-870.
37. Torres MJ, Romano A, Mayorga C, et al. Diagnostic evaluation of a large group of patients with immediate allergy to penicillins: the role of skin testing. *Allergy.* 2001;56(9):850-856.
38. Sanz ML, Gamboa PM, Antepara I, et al. Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics. *Clin Exp Allergy.* 2002;32(2):277-286.
39. Romano A, Mayorga C, Torres MJ, et al. Immediate allergic reactions to cephalosporins: cross-reactivity and selective responses. *J Allergy Clin Immunol.* 2000;106(6):1177-1183.
40. Antunez C, Blanca-Lopez N, Torres MJ, et al. Immediate allergic reactions to cephalosporins: evaluation of cross-reactivity with a panel of penicillins and cephalosporins. *J Allergy Clin Immunol.* 2006;117(2):404-410.
41. Torres MJ, Padial A, Mayorga C, et al. The diagnostic interpretation of basophil activation test in immediate allergic reactions to betalactams. *Clin Exp Allergy.* 2004;34(11):1768-1775.
42. Demoly P, Lebel B, Messaad D, et al. Predictive capacity of histamine release for the diagnosis of drug allergy. *Allergy.* 1999;54(5):500-506.
43. Pineda F, Ariza A, Mayorga C, et al. Role of histamine release test for the evaluation of patients with immediate hypersensitivity reactions to clavulanic acid. *Int Arch Allergy Immunol.* 2015;168(4):233-240.
44. O'Neill PM, Willock DJ, Hawley SR, et al. Synthesis, antimalarial activity, and molecular modeling of tebuquine analogues. *J Med Chem.* 1997;40(4):437-448.
45. Meng X, Al-Attar Z, Yaseen FS, et al. Definition of the nature and hapten threshold of the beta-lactam antigen required for T cell activation in vitro and in patients. *J Immunol.* 2017;198(11):4217-4227.
46. Ansel KM, Djuretic I, Tanasa B, Rao A. Regulation of Th2 differentiation and IL4 locus accessibility. *Annu Rev Immunol.* 2006;24:607-656.
47. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. Molecular mechanisms regulating Th1 immune responses. *Annu Rev Immunol.* 2003;21:713-758.
48. Akdis M, Burgler S, Cramer R, et al. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *J Allergy Clin Immunol.* 2011;127(3):701-721 e1-70.
49. Smarr CB, Bryce PJ, Miller SD. Antigen-specific tolerance in immunotherapy of Th2-associated allergic diseases. *Crit Rev Immunol.* 2013;33(5):389-414.
50. de Boer BA, Kruize YC, Rotmans PJ, Yazdanbakhsh M. Interleukin-12 suppresses immunoglobulin E production but enhances immunoglobulin G4 production by human peripheral blood mononuclear cells. *Infect Immun.* 1997;65(3):1122-1125.
51. Tang C, Inman MD, van Rooijen N, et al. Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism. *J Immunol.* 2001;166(3):1471-1481.
52. Nakagome K, Okunishi K, Imamura M, et al. IFN-gamma attenuates antigen-induced overall immune response in the airway as a Th1-type immune regulatory cytokine. *J Immunol.* 2009;183(1):209-220.
53. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol.* 2007;25:821-852.
54. Bhakta NR, Woodruff PG. Human asthma phenotypes: from the clinic, to cytokines, and back again. *Immunol Rev.* 2011;242(1):220-232.
55. Ishioka T, Yamada Y, Kimura H, et al. Elevated macrophage inflammatory protein 1alpha and interleukin-17 production in an experimental asthma model infected with respiratory syncytial virus. *Int Arch Allergy Immunol.* 2013;161(Suppl 2):129-137.
56. Akhmaltdinova LL, Gazalieva MA, Akhmetova SB. Levels of cytokines in drug hypersensitivity. *Cent Eur J Immunol.* 2017;42(4):354-357.
57. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity.* 2004;21(2):241-254.
58. Yoshie O, Matsushima K. CCR4 and its ligands: from bench to bedside. *Int Immunol.* 2015;27(1):11-20.
59. Groom JR, Luster AD. CXCR3 in T cell function. *Exp Cell Res.* 2011;317(5):620-631.
60. Pease JE. Asthma, allergy and chemokines. *Curr Drug Targets.* 2006;7(1):3-12.
61. Meng X, Ariza A, Waddington J, Park K, Naisbitt D. Immunological mechanisms of drug hypersensitivity. *Curr Pharm Des.* 2016;22(45):6734-6747.

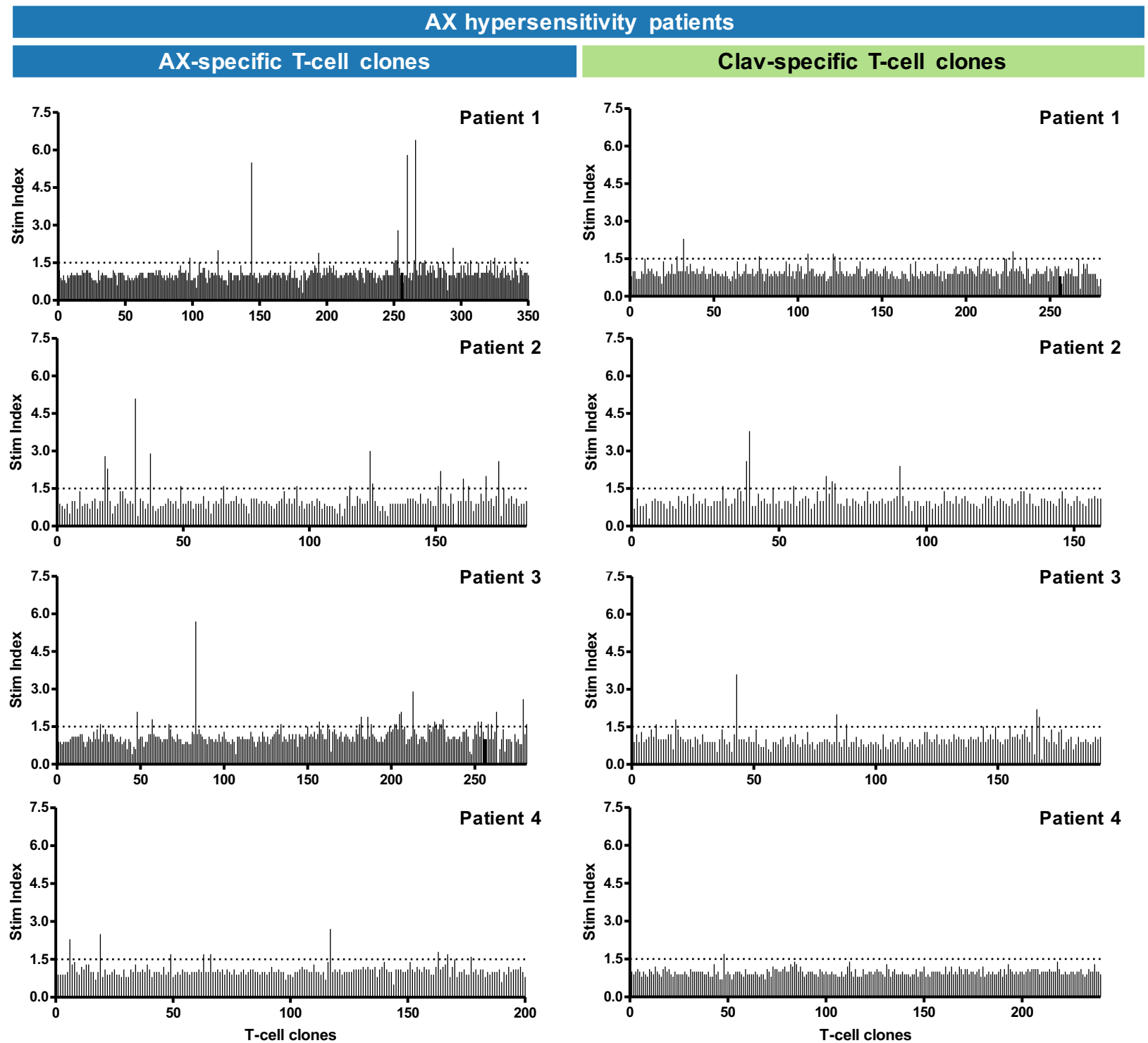
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Figure S1

A



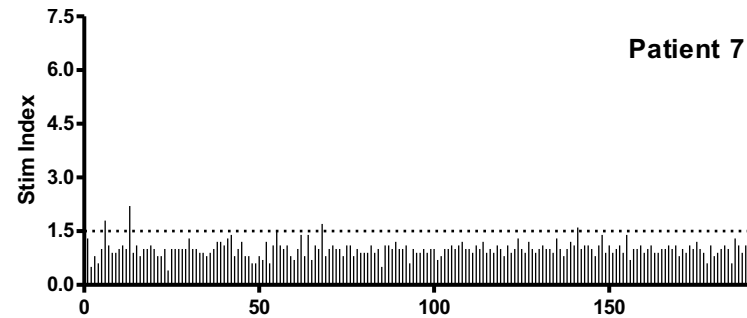
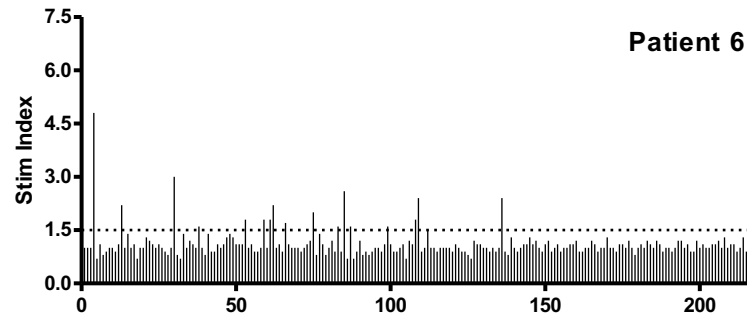
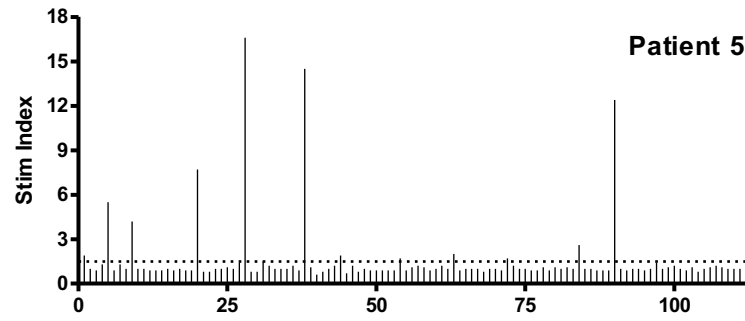
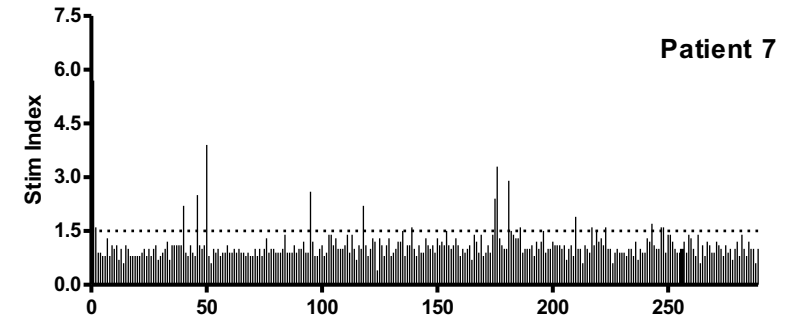
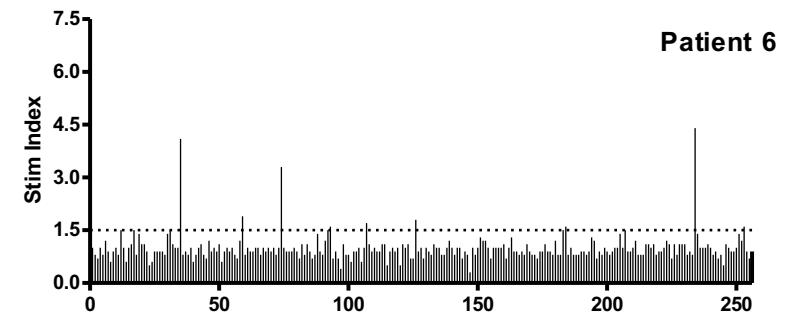
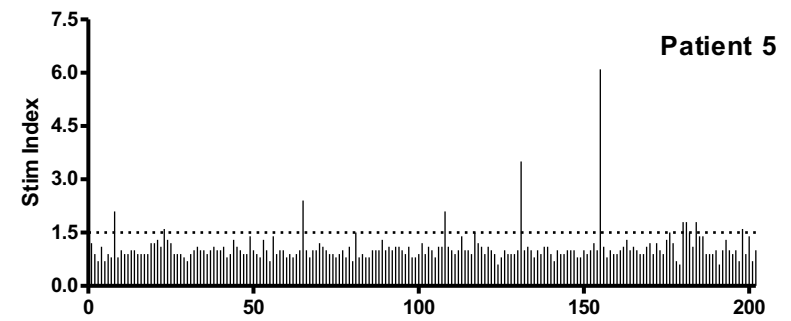
B**Clav hypersensitivity patients****AX-specific T-cell clones****Clav-specific T-cell clones**

Figure S2

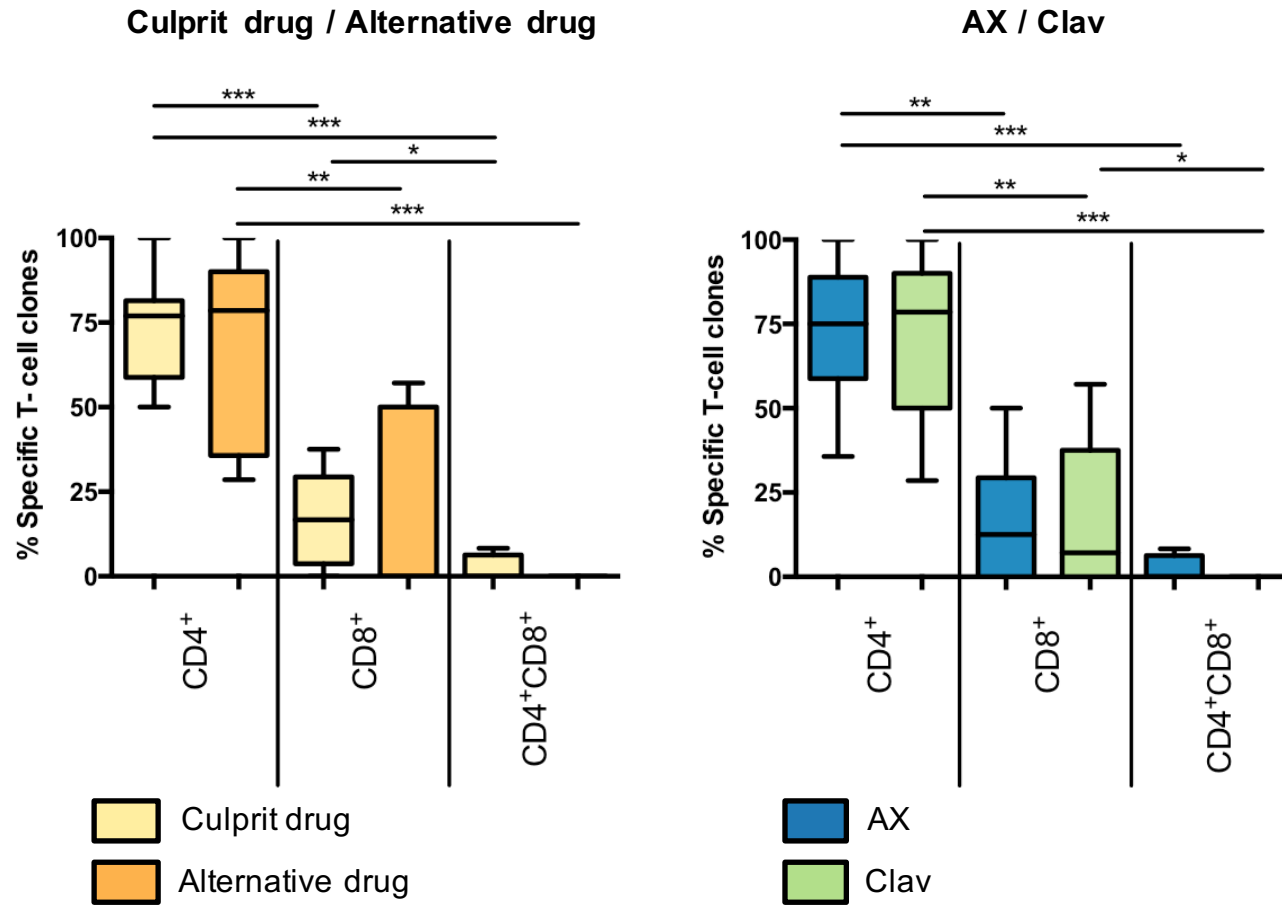


Figure S3

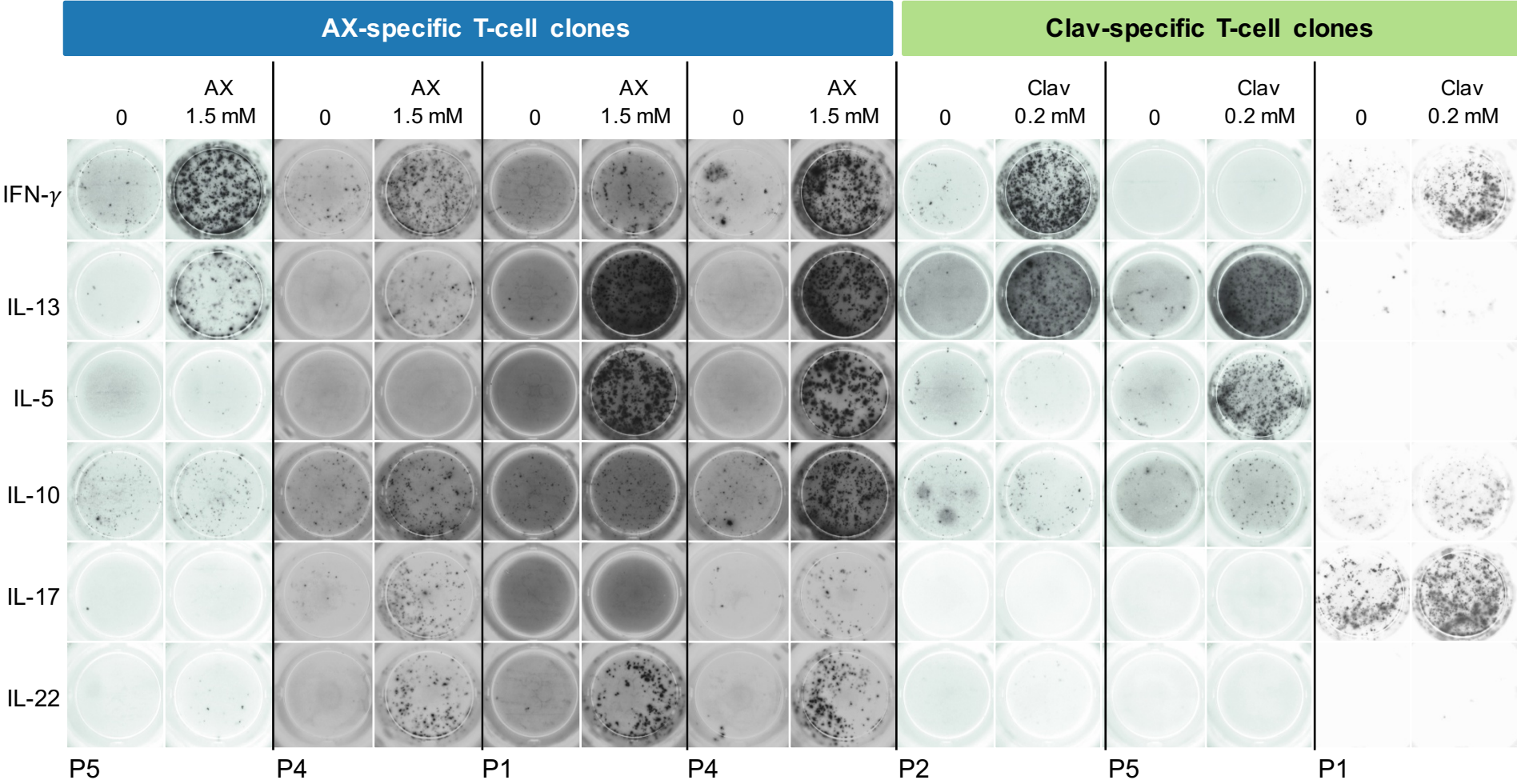


Figure S4

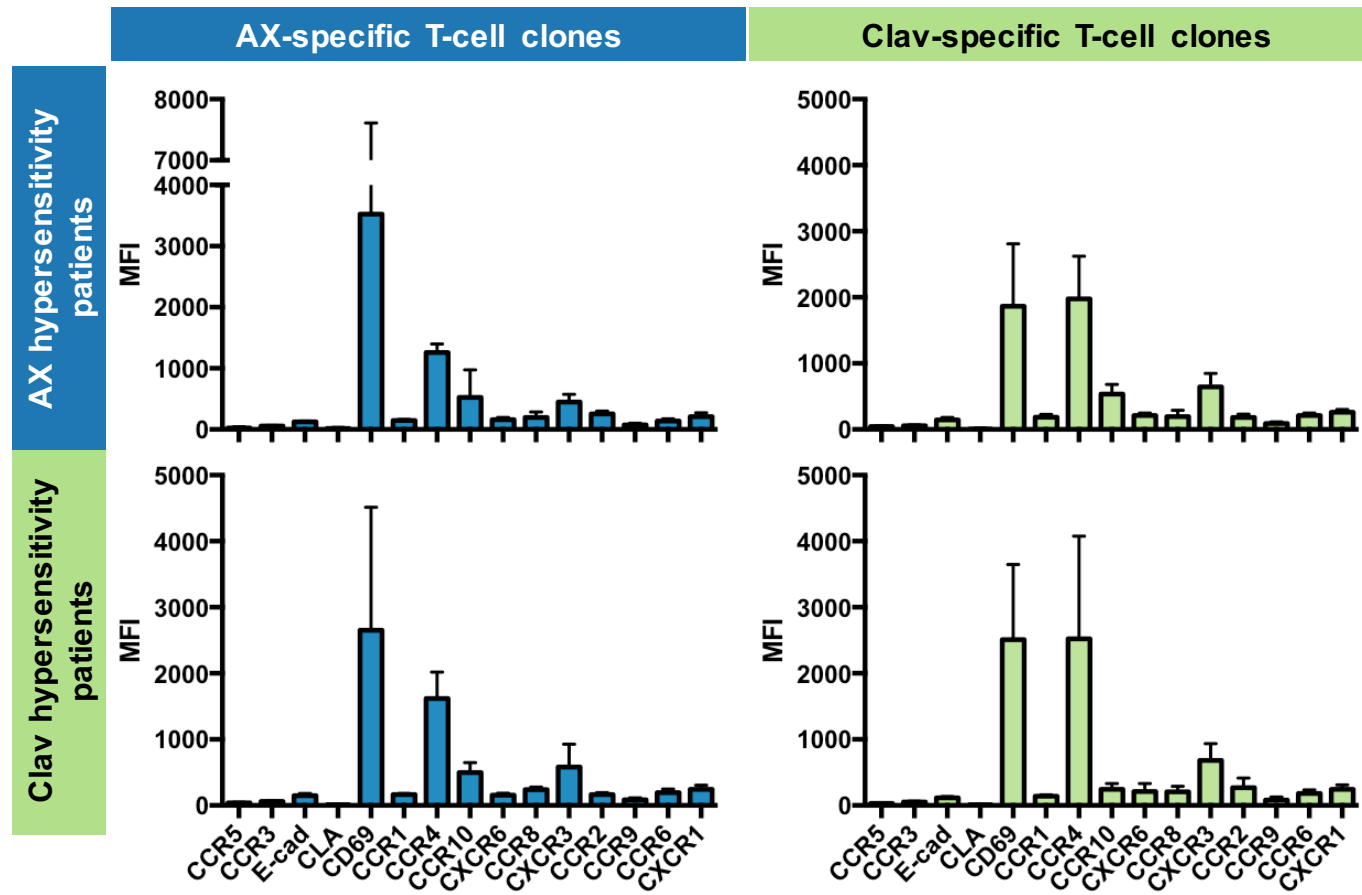
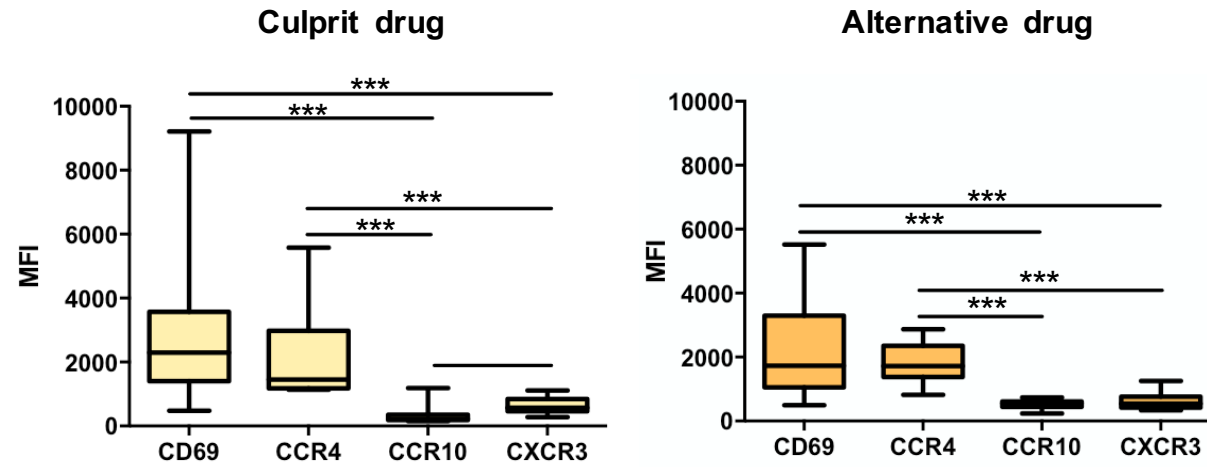
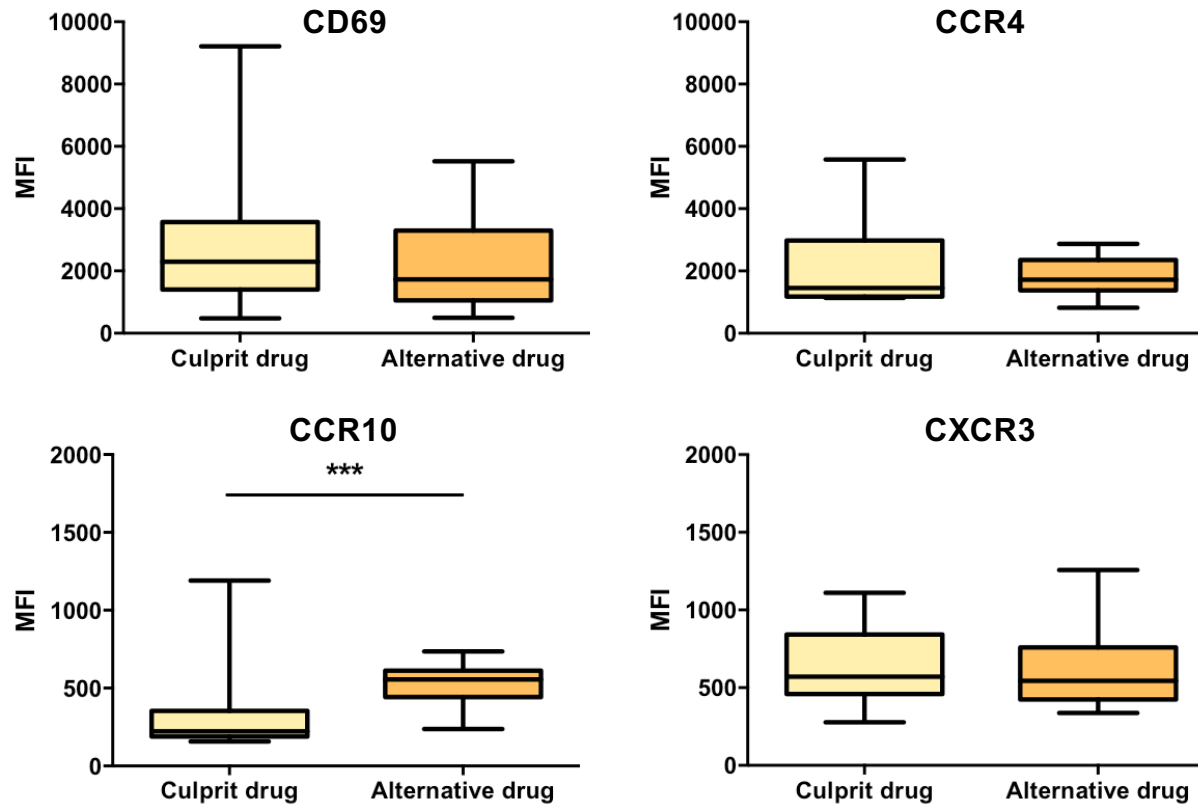


Figure S5

A



B



Supporting information

Title: Characterization of amoxicillin and clavulanic acid specific T-cell clones from patients with immediate drug hypersensitivity

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24 **Table S1.** Diagnostic evaluation of hypersensitivity patients included in the study.

ID	Clinical diagnosis	Skin test				Drug provocation test		
		PPL	MD	AX	Clav	PV	AX	AX-Clav
P1	BL hypers	-	-	+ (IDT)	-	+	ND	ND
P2	AX hypers	-	-	+ (IDT)	-	-	ND	ND
P3	AX hypers	-	-	+ (IDT)	-	-	ND	ND
P4	AX hypers	-	-	+ (SPT)	-	-	ND	ND
P5	Clav hypers	-	-	-	+ (IDT)	ND	-	ND
P6	Clav hypers	-	-	-	+ (IDT)	ND	-	ND
P7	Clav hypers	-	-	-	+ (IDT)	ND	-	ND

25

26 AX: amoxicillin; BL: betalactam; BP: benzylpenicillin; Clav: clavulanic acid; F:
 27 female; hyper: hypersensitivity; IDT: intradermal test; M: male; MD: minor
 28 determinant; ND: not done; P: patient; PPL: penicilloyl-polylysine; PV: penicillin V;
 29 SPT: skin prick test

30

Table S2. T-cell clones generated from blood of hypersensitivity patients to AX or Clav. Table shows the total number of T-cell clones generated and tested, the number of specific T-cell clones to AX and Clav and the stimulation index (SI) of the specific T-cell clones (expressed as median and minimum and maximum values). T-cell clones with SI < 1.5 were included if they were identified as specific T-cell clones by IFN- γ or IL-13 ELISpot.

	ID	AX-specific T-cell clones			Clav-specific T-cell clones		
		T- cell clones tested	Specific T-cell clones	Stim Index (median, min-max)	T-cell clones tested	Specific T-cell clones	Stim Index (median, min-max)
AX hypers patients	P1	350	24 (6.9%)	1.60 (1.50-6.40)	280	15 (5.4%)	1.50 (1.40-2.30)
	P2	186	14 (7.5%)	1.80 (1.40-5.10)	159	10 (6.5%)	1.60 (1.40-3.80)
	P3	281	35 (12.5%)	1.60 (1.30-5.70)	192	10 (5.2%)	1.70 (1.50-3.60)
	P4	216	9 (4.2%)	1.70 (1.40-2.70)	240	3 (1.3%)	1.40 (1.40-1.70)
Clav hypers patients	P5	112	10 (8.9%)	4.85 (1.70-16.60)	202	13 (6.4%)	1.80 (1.50-6.10)
	P6	216	14 (6.5%)	1.80 (1.50-4.80)	256	16 (6.3%)	1.60 (1.40-4.40)
	P7	190	4 (2.10%)	1.65 (1.50-2.20)	289	30 (10.4%)	1.60 (1.40-5.70)

AX: amoxicillin; Clav: clavulanic acid; Hyper: hypersensitivity; Stim: stimulation

Table S3. CD4⁺/CD8⁺ phenotype of drug-specific T-cell clones derived from blood of hypersensitivity patients to AX or Clav. Results analyzed by flow cytometry.

	ID	AX-specific T-cell clones				Clav-specific T-cell clones			
		n	CD4 ⁺	CD8 ⁺	CD4 ⁺ CD8 ⁺	n	CD4 ⁺	CD8 ⁺	CD4 ⁺ CD8 ⁺
AX hypersensitivity patients	P1	17	10 (58.8%)	5 (29.4%)	0 (0.0%)	14	11 (78.6%)	1 (7.1%)	0 (0.0%)
	P2	12	9 (75.0%)	2 (16.7%)	1 (8.3%)	10	9 (90.0%)	0 (0.0%)	0 (0.0%)
	P3	16	13 (81.3%)	2 (12.5%)	1 (6.2%)	7	2 (28.6%)	4 (57.1%)	0 (0.0%)
	P4	8	8 (100.0%)	0 (0.0%)	0 (0.0%)	3	3 (100.0%)	0 (0.0%)	0 (0.0%)
Clav hypersensitivity patients	P5	9	8 (88.9%)	0 (0.0%)	0 (0.0%)	13	10 (76.9%)	3 (23.1%)	0 (0.0%)
	P6	14	5 (35.7%)	7 (50.0%)	0 (0.0%)	16	8 (50.0%)	6 (37.5%)	0 (0.0%)
	P7	3	2 (66.7%)	0 (0.0%)	0 (0.0%)	27	22 (81.5%)	1 (3.7%)	0 (0.0%)

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42 AX: amoxicillin; Clav: clavulanic acid; Hyper: hypersensitivity

Figure S1. Generation of AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients to AX (**E1A**) and Clav (**E1B**). Approximately 3,100 individual T-cell clones were generated cultured with autologous APCs and either AX or Clav for 2 days. Proliferation was measured by the addition of [³H]-thymidine. Results are expressed as stimulation index (SI), calculated by dividing the mean counts per minute (cpm) of drug-stimulated cells by the mean cpm of non-stimulated cells. Results were considered positive if $SI \geq 1.5$.

Figure S2. CD4⁺/CD8⁺ phenotype of drug-responsive T-cell clones derived from blood of hypersensitivity patients to AX or Clav. CD4⁺/CD8⁺ phenotype was analyzed by using flow cytometry. Box plots show medians of percentage of T-cells clones, with error bars showing 1st/99th percentiles. Statistical analysis was performed using Mann-Whitney test (significant differences if $P \leq 0.05$). * $P \leq 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure S3. Profile of cytokines secreted by CD4⁺ AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients. T-cell clones were cultured with irradiated APCs and AX or Clav, and secreted cytokines were quantified and visualized by ELISpot. Images from seven representative AX- and Clav-specific T-cell clones.

Figure S4. Chemokine receptor expression on AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients. Results were analyzed by flow cytometry and expressed as mean+SD of mean fluorescence intensity (MFI). Data were normalized using unstained cells.

Figure S5. Chemokine receptor expression on AX- and Clav-specific T-cell clones derived from blood of hypersensitivity patients. Chemokine receptor expression was analyzed by flow cytometry. Results are expressed as median of the fluorescence intensity detected for chemokine receptors on AX- and Clav-specific T-cell clones, with error bars showing 1st/99th percentiles, statistical analysis was performed using

68 Wilcoxon test for paired samples (A) and Mann-Whitney test for unpaired samples (B)
69 (significant differences if $P < 0.05$). Data were normalized using unstained cells.
70 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; MFI: mean fluorescence intensity.