**Characterization of amoxicillin and clavulanic acid specific T-cell clones from amoxicillin-clavulanic acid hypersensitivity patients**

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**Abstract:**

Background: Betalactam (BL) antibiotics are most common cause of drug hypersensitivity reactions. Amoxicillin (AX), which is often prescribed alongside the β-lactamase inhibitor clavulanic acid (Clav), is the most common elicitor. Objective: 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 pathways of T-cell activation.

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+ and chemokine receptor expression were analyzed by flow cytometry.

Results: 110 AX-specific and 96 Clav-specific T-cell clones were generated from 7 patients with positive skin test to either AX or Clav. Proliferation of AX- and Clav-specific T-cell 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-cell by MHC class II and I, respectively. A higher secretion of IL-13 and IL-5 was detected when clones were stimulated with the culprit drug compared with the alternative drug. Clones expressed high levels of CD69, CCR4, CXCR3 and CCR10.

Conclusion: 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 drug, although differences in cytokine secretion were observed.

**Key Messages**

What is already known about this topic?

* Amoxicillin (AX), prescribed alone or in combination with clavulanic acid (Clav), is the most common elicitor of drug hypersensitivity reactions (DHRs). No cross-reactivity between AX and Clav has been described.
* It is unclear why patients with hypersensitivity to the combination AX-Clav develop a selective reaction to AX while tolerating Clav and *vice versa*.

What does this study add?

* Our study details the antigen specificity and phenotype of AX- and Clav-specific T-cell clones generated from patients with AX-Clav-induced immediate hypersensitivity diagnosed by positive skin test.
* Both AX and Clav-specific T-cell clones could be generated from the same patient, irrespective of whether AX or Clav was the culprit drug. 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 that currently are not included in diagnostic tests, to go further in the improvement of the sensitivity of the in vitro diagnostic tests.

**Capsule summary**

This study characterized AX- and Clav-specific T-cell clones generated from patients with AX-Clav-induced immediate hypersensitivity and showed differences in cytokine secretion between specific clones to the drug patients selectively responded or to the alternative drug.

**Key words**

Amoxicillin; clavulanic acid; skin test; T-cell; immediate hypersensitivity; specificity; phenotype

**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 IsoTioCyanate; 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

**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 non-immediate, 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). Non-immediate reactions occur at least 1 h 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 and consumption (2). Nowadays, amoxicillin (AX) is the most common elicitor, in both children (6) 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 component, 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, with more cases of immediate than non-immediate reactions (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 in the same patient (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/naive 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 (i) determine whether AX and Clav-specific T-cells are detectable, (ii) assess whether T-cells display the same specificity as that detected in skin testing and (iii) explore pathways of T-cell activation.

**Methods**

**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) (. 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.

**Skin testing (ST):** 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: benzylpenicilloyl-octa-L-lysine (BP-OL) 0.04 mg/mL (8.64x10−5 M of the benzylpenicilloyl (BPO) moiety); minor determinant (MD) 0.5 mg/mL (1.5x10−3 M of the sodium benzylpenilloate); AX 20 mg/mL (5x10−2 M), and Clav 20 mg/mL (8.4x10-2 M) all from Diater-Ferrer (Madrid, Spain). 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 min after testing. An increase in the wheal diameter greater than 3 mm was considered positive.

**Drug provocation test (DPT):** 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 min) to achieve the therapeutic dose, followed by the administration at home for two days. First, oral penicillin V (ERN, Barcelona, Spain) 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, Madrid, Spain) was administered at the clinical setting (500 mg) followed by two days of oral AX at home (500 mg/12 h).

**Biological samples/cell isolation:** Biological samples were processed following standard procedures by the Malaga Hospital-IBIMA Biobank. Venous blood (70 ml) was collected from all the subjects and PBMCs were isolated by density gradient separation.

**Generation of Epstein-Barr virus (EBV)-transformed B-cell lines:** 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, Paisley, UK), L-glutamine (100 mM), penicillin (100 µg/mL) and streptomycin (100 IU/mL).

**Medium for T-cell culture and cloning:** Culture medium consisted of 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.

**Generation of T-cell clones:** PBMCs from all the subjects (1x106 cells/well; 0.5 ml) were cultured with AX (0.5 and 1 mM) or Clav (0.05 and 0.1 mM) 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).

**Specificity of T-Cell clones:** T-cell clones were tested for AX and Clav specificity by measuring proliferation. T-cell clones (5x104 cells/well) were cultured with autologous irradiated EBV-transformed B-cells (1x104 cells/well) and AX (1 mM) or Clav (0.1 mM) for 48 h. Proliferation was measured by the addition of [3H]thymidine (0.5 µCi/well, 5 Ci/mmol; Morovek Biochemicals, Brea, CA, USA) for the last 16 h of the culture period. T-cell clones with a stimulation index (SI) greater than 1.5 were expanded by repetitive stimulation with irradiated allogenic PBMCs (5x104 cells/well; 200 µl), PHA (10 µg/mL) and IL-2 (700 IU/mL). T-cell clones with SI lower than 1.5 were expanded if they showed a positive release of IFN-γ or IL-13 in the presence of the drug by ELISpot assay.

**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 mM) and Clav (0.025-0.2 mM), and cross-reactivity between both drugs was also analyzed. Proliferation was measured by [3H]thymidine incorporation as described above.

**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 (5x104 cells/well, 100 µL) were cultured with autologous irradiated EBV-transformed B cells (1x104 cells/well, 50 µL) and AX (1.5 mM) or Clav (0.2 mM) on plates precoated with capture antibody for 48 h (Mabtech, Sweden). 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.

**Phenotype of T-Cell clones:** CD phenotype ofAX- and Clav-specific T-cell clones were characterized by flow cytometry. Antibodies used for flow cytometry staining were CD4-fluorescein (FITC) and CD8-phycoerythrin (PE) (BD, New Jersey, USA). Chemokine receptors were also analyzed by flow cytometry, and the antibodies used were CXCR3-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 for the whole population of each analyzed T-cell clone.

**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: i) APC fixation assay to determine the role of intracellular processing 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: incubation with 0.05% glutaraldehyde for 30 sec, followed by incubation with 1 mM glycine for 45 sec and extensive washing), ii) MHC restriction of APCs for AX- and Clav-specific T-cells clones was analyzed by the incubation of APC with MHC class I and II blocking antibodies for 30 min at 37ºC. Activation of T-cells was determined in both experiments by proliferation or IFN-γ and IL-13 secretion assays.

**Statistical Analysis:** Quantitative variables without a normal distribution were expressed as median and interquartile range, and comparisons were carried out using the Mann–Whitney and Wilcoxon tests for non-related and related samples, respectively. P values ≤ 0.05 were considered statistically significant.

**Results**

**Donor clinical characteristics:** Patients 1-7 (5 men and 2 women) were diagnosed with immediate hypersensitivity reaction after the administration of the combination AX-Clav (Table 1) ("see Table E1 in the Online Repository"). 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.

**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 3,100 T-cell cultures originating from single precursor cells. T-cells were then cultured with autologous EBV transformed B-cell lines as APCs and either AX (1 mM) or Clav (0.1 mM) and proliferation measured by incorporation of [3H]thymidine. A total of 110 and 97 T-cell clones displayed reactivity against AX and Clav, respectively, on initial testing T-cell clones were considered responders if SI > 1.5 (T-cell clones with SI < 1.5 were included if IFN-γ or IL-13 secretion was detected by ELISpot) (Table 2) ("see Figure E1 in the Online Repository").

We analyzed the percentage of responsive T-cell clones obtained and attempted to correlate the data obtained with time interval factors: i) time interval between drug administration and reaction (min), ii) 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 preseting AX- or Clav-diagnosed allergic reactions, with no significant difference in T-cell clone numbers when the patients were compared. T-cell clones selected on initial testing as responsive T-cell clones were expanded for further analysis.

**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-cellproliferation was obtained with both AX and Clav. No proliferation was detected when AX-responsive T-cell clones were cultured with Clav and *vise versa* (Figure 1).

**CD4+/CD8+ phenotype:** AX- and Clav-specific T-cell clones were characterized in terms of CD phenotype by flowcytometry. Clones displaying reactivity against AX or Clav were mainly CD4+ ("see Figure E2 and Table E2 in the Online Repository"). No significant difference in the percentage of CD4+ and CD8+ T-cell clones was observed between AX- or Clav-specific T-cell clones as well as between T-cell clones generated against the culprit or the alternative drug.

**Profile of cytokinescytolytic molecules secreted by specific T-cell clones:** Secretion of IFN-γ and IL-13 from AX- and Clav-specific T-cell clones were analyzed by ELISpot, by incubating cells with either AX (1.5 mM) or Clav (0.2 mM). 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. However, statistical analysis was not performed due to the low number of CD8+ clones obtained and tested (Figure 2A-B). Focusing on the analysis of CD4+ T-cell clones, the level of IFN-γ and IL-13 secreted was higher for the T-cell clones specific to the alternative drug than 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) (Figure 3A, “see Figure E3 in the Online Repository”). T-cell clones secreted higher levels of IL5 and IL13, and lower levels of IFN- γ 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).

**CD4+ and CD8+ T-cell clones are activated via processing-dependent pathway in a MHC-restricted manner:** To explore the pathways of drug antigen presentation for AX and Clav, T-cell clones were initially cultured with drug in presence or absence of APCs or in the presence of fixed APCs. 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). The importance of MHC class in the presentation of AX and Clav to specific T-cell clones was tested using MHC blocking antibodies (for MHC class I and II) in proliferation assays. 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).

**Chemokine receptor expression:** Expression of 12 chemokine receptors, CLA, CD69, and E-cadherin were compared on AX- and Clav-specific T-cell clones. Both specific T-cell clones were found to express high levels of CD69, followed by CCR4, CXCR3 and CCR10 when flow cytometry data were analyzed (“see Figure E4 in the Online Repository”). Higher expression of CCR10 was detected on T-cell clones displaying reactivity to the alternative drug (Figure 6).

**Discussion**

The combination of AX and Clav is widely used in Europe to control and prevent infections (22); however, nowadays it is also the most frequent cause of allergic drug reactions (2, 23). Most patients suffering a reaction after AX-Clav intake present selective sensitization to either AX or Clav (24-26). Although both drugs are BL antibiotics, no cross-reactivity has been described, mainly due to differences in their chemical structure and degradation patterns (8, 27-31) (24-26). An accurate diagnosis of these patients is crucial, because Clav-allergic subjects can tolerate other BLs, including AX (23). However, diagnosis is complex; 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 fluoroimmunossay (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 22-55% 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 with different optimal concentrations for each drug. The optimal concentration of AX and Clav required to active the T-cell clones (1-2 mM and 0.1-0.2 mM, respectively) was higher than circulating concentrations in plasma (about 0.1 mM for the oral administration of 250/125 mg of 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 al., (45) 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 (46).

 Until now, it is unclear why patients with hypersensitivity to the combination AX-Clav develop a selective reaction to AX while 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. However, when analyzing cytokines secreted from drug-responsive CD4+ T-cell clones (the most abundant clones), a higher secretion of IL-13 and IL-5 (Th2 cytokines) (47) 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) (48). These results suggest that the ratio of Th2/Th1 secreting T-cell clones is higher with T-cell clones displaying reactivity towards the culprit drug, that is associated with IgE-mediated allergic reactions. 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 fungi (49) and helps to maintain immune responses including allergic inflammation (50-52). IL-22 increases the innate immunity of tissue cells, protects tissues from damage and enhaces tissue regeneration, targeting specifically the skin, the digestive tract, the lungs, and kidney (53). In our study, IL-17 secretion was only observed in a small number of T-cell clones, all of them responsive towards 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) (54), CXCR3 (highly expressed on Th1 cells) (55), CCR10 and the activation marker CD69. CCR4 and CXCR3 are also expressed on AX- and Clav-specific T-cell clones from patients with AX-Clav-induced liver injury (46). 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 over expressed in inflammatory states (56).

Activation of T-cell clones with either AX or Clav was depedent 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. This supports the hapten hypothesis for the recognition and presentation of BL antibiotics (57). 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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**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 |

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

**Table 2.** 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.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **AX-specific T-cell clones** | | | **Clav-specific T-cell clones** | | |
|  | **ID** | **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

**Figure legends**

**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 [3H]-thymidine. Results are expressed as mean + SD of counts per minute (cpm).

**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. **2A**, 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). **2B,** Comparison of IFN-γ and IL-13 secreted by CD4+ and CD8+ T-cell clones derived from hypersensitive patients. **2C,** 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<0.05).

**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. **3A,** Profile of cytokines secreted by CD4+ AX- and Clav-specific T-cell clones. Results are expressed as mean+SD spot-forming units (sfu). **3B,** 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 median spot-forming units (sfu), with error bars showing 1st/99th percentiles.

**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).

**Figure 5.** Proliferation assay to analyze the antigen presentation to T-cell clones by MHC. APCs were incubated for 30 min at 37ºC 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).

**Figure 6.** 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. Mean fluorescence intensity detected for chemokine receptors on AX- and Clav-specific T-cell clones.