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ORIGINAL ARTICLE

Drug Allergy, Insect Sting Allergy, and Anaphylaxis

Synthetic antigenic determinants of clavulanic acid induce dendritic cell maturation and specific T cell proliferation in patients with immediate hypersensitivity reactions

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Abstract

Background: Immediate drug hypersensitivity reactions (IDHRs) to clavulanic acid (CLV) have increased in the last decades due to a higher consumption alongside amoxicillin (AX). Due to its chemical instability, diagnostic procedures to evaluate IDHRs to CLV are difficult, and current in vitro assays do not have an optimal sensitivity. The inclusion of the specific metabolites after CLV degradation, which are efficiently recognised by the immune system, could help to improve sensitivity of in vitro tests.

Methods: Recognition by dendritic cells (DCs) of CLV and the synthetic analogues of two of its hypothesised antigenic determinants (ADs) was evaluated by flow cytometry in 27 allergic patients (AP) and healthy controls (HC). Their ability to trigger the proliferation of T cells was also analysed by flow cytometry.

Results: The inclusion of synthetic analogues of CLV ADs, significantly increased the expression of maturation markers on DCs from AP compared to HC. A different recognition pattern could be observed with each AD, and, therefore, the inclusion of both ADs achieves an improved sensitivity. The addition of synthetic ADs analogues increased the proliferative response of $CD4^{+}$Th2 compared to the addition of native CLV. The combination of results from both ADs increased the sensitivity of proliferative assays from 19% to 65% with a specificity higher than 90%.

Conclusions: Synthetic ADs from CLV are efficiently recognised by DCs with ability to activate $CD4^{+}$Th2 cells from AP. The combination of analogues from both ADs,

Abbreviations: ADf, antigenic determinant; AP, allergic patients; APC, antigen presenting cell; AX, amoxicillin; BAT, basophil activation test; BCR, B cell receptor; BL, betalactams; BPOL, benzylpenicilloyl octa-L-lysine; CCR7, C-C chemokine receptor type 7; CFSE, carboxyfluorescein succinimidyl ester; CLV, clavulanic acid; DC, dendritic cell; DPT, drug provocation test; EAACI, European Academy of Allergy and Clinical Immunology; GM-CSF, granulocyte-macrophage colony stimulating factor; HC, healthy control; HRT, histamine release test; HRUM, Hospital Regional Universitario de Malaga; HSA, human serum albumin; ICON, Internal Consensus on drug allergy; IDHR, immediate drug hypersensitivity reaction; LPS, lipopolysaccharide; LTT, lymphocyte transformation test; MD, minor determinant; MHC, major histocompatibility complex; MI, maturation Index; moDCs, monocyte-derived dendritic cells; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PI, proliferation index; PV, penicillin V; sIgE, specific immunoglobulin E; SPT, skin prick test; ST, skin test; TCR, T cell receptor.

RFS, GB and MIM; TDF, CM and MJT contibuted equally to this work.

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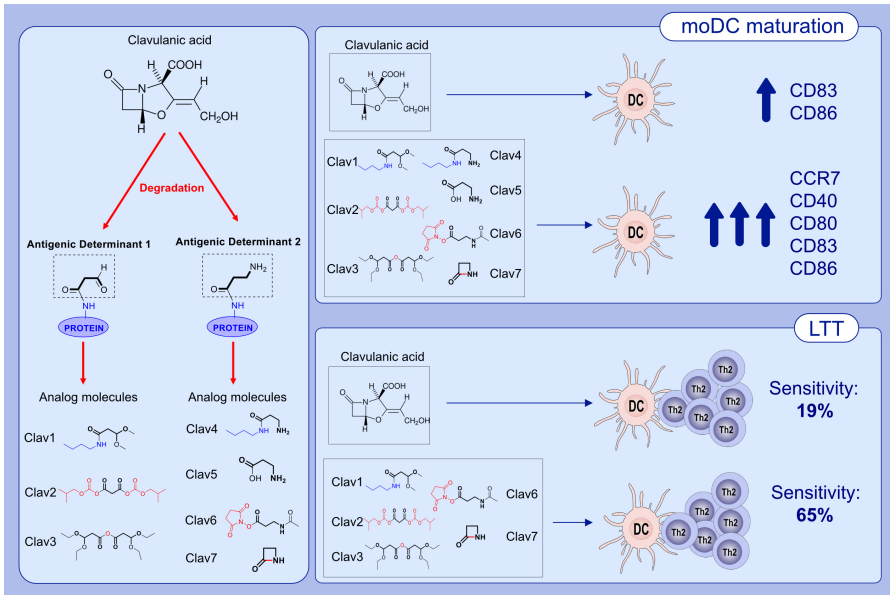
significantly increased the sensitivity of DC maturation and T-cell proliferation compared to native CLV.

KEYWORDS

antigenic determinants, clavulanic acid, immediate drug hypersensitivity reactions, maturation, proliferation



GRAPHICAL ABSTRACT

CLV synthetic antigenic determinants (AD-I and AD-II) are efficiently recognised by moDCs from CLV allergic patients. Some CLV-ADs induce the expression of activation, maturation, and migration markers on moDCs from allergic patients. Pre-stimulated moDCs with some CLV-ADs induces CD4^{+}Th2 cell proliferation. The combination of the results from different CLV-AD analogues increases LTT sensitivity to 65%. Abbreviations: ADs, antigenic determinants; CCR7, C-C chemokine receptor type 7; CLV, clavulanic acid; LTT, lymphocyte transformation test; moDCs, monocyte-derived dendritic cells

1 | INTRODUCTION

Betalactam antibiotics (BL) are the drugs most frequently used to treat bacterial infections[1] as well as the most oftenly involved in the induction of immediate drug hypersensitivity reactions (IDHR).[2] Moreover, amoxicillin (AX), whether combined or not with clavulanic acid (CLV), is the BL most commonly involved in IDHRs, as demonstrated in children[3] and adults.[4] From these BLs, the immunological response to AX has been widely studied by both in vivo and in vitro approaches[5,6]; however, for CLV, there is a lack of studies.[7]

Because of its high betalactamase inhibitor activity, CLV is frequently prescribed alongside AX.[8,9] Although it was initially thought that CLV was not immunogenic,[10] IDHRs to CLV may occur.[9,11–13] Actually, they have been increasing in the last decades, accounting nowadays for 30% of the reactions to AX-CLV, probably because of a higher consumption pattern.[1,2,9,12,14]

The diagnosis of IDHRs after the administration of AX-CLV is complex, mainly based on clinical history, followed by skin testing (ST), and drug provocation test (DPT).[15,16] As clinical history is often unreliable, ST to CLV has not optimal sensitivity and is not available in all countries,[9,12,17] DPT is sometimes the uniquely available in vivo alternative. Nevertheless, it is not recommended for severe reactions,[18] and it cannot be performed with CLV itself, so DPT has to be based on the good tolerance to AX after a positive reaction with AX-CLV.[19–21] For this reason, risk-free in vitro tests can be considered as useful diagnostic tools to evaluate IDHRs to AX-CLV,[7,22] avoiding or reducing the need of DPT, as well as allowing the correct identification of the specific drug responsible for the IDHR.[22–24]

Despite AX and CLV being BLs, cross reactivity between them is uncommon because of differences between their chemical structures,[8,17,25] as well as the specific metabolites derived after drug metabolism.[7,8,26] Only two studies reported that a patient can be simultaneously sensitised to both drugs.[14,27] Thus, the correct identification of the culprit drug has important implications because selective patients to CLV can tolerate other BL, including AX[7,17,28]; this avoids the need for alternative drugs, often with more adverse effects and higher costs for health systems.[29] For this reason, in vitro tests are crucial complementary diagnostic tools to evaluate IDHRs.[7]

Differently from other BLs, the detection of serum-specific IgE (sIgE) to CLV is nowadays not possible by immunoassays.[11,30] Therefore, basophil activation test (BAT) and histamine release test (HRT) are the unique in vitro tests available for the diagnosis of

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IDHRs to CLV.[2,24,31] Nevertheless, their sensitivity is not optimal, ranging from 52% to 60%.[1,2,7,24] A possible explanation for this low sensitivity could be that CLV is highly metabolised into different products,[11] with heterogeneous immunogenicities,[12,19] being only some of them optimally recognised by the immune system. In a previous study, we demonstrated that the inclusion of metabolites from pyrazolones increased BAT sensitivity to identify anaphylaxis to metamizole.[32] In the case of CLV, the specific metabolites generated after its degradation are not well known. This is due to its complex chemical structure and unpredictable reactivity, forming unstable adducts with further degradation, being only some of them specifically recognised by the immunological system.[8,20,26,30]

Similarly to the rest of BL, CLV has a low molecular weight, and accordingly to the hapten hypothesis, it needs to bind covalently to proteins to be efficiently recognised by the immune system.[6,8,33] A recent study described that the detection of human serum albumin modified sites after culturing with CLV,[20] although other candidate protein carriers such as haptoglobin and immunoglobulin chains have also been proposed.[30] The hapten-carrier conjugates are recognised by antigen presenting cells (APCs), and, after processing, peptides are presented to B and T cells, triggering their activation.[34–36]

Based on all of this, our group has recently proposed two potential antigenic determinants (ADs) from CLV, following two different degradation pathways after protein conjugation: AD-I (N -protein, 3-oxopropanamide) and AD-II (N -protein, 3-aminopropanamide). Moreover, synthetic analogues from each AD with different capacities to modify proteins were synthesised and evaluated by BAT, obtaining promising results, mainly with those from AD-I.[20]

To deepen the understanding on the immunological recognition of AD-I and AD-II from patients with IDHRs to CLV, we evaluated their recognition by dendritic cells (DCs) and their capacity to trigger proliferation of different T cell populations. This knowledge will be important to better understand the underlying mechanism involved in the immunological response of IDHRs to CLV.

2 | METHODS

2.1 | Patients and healthy control selection

Patients who attended to the Allergy Unit of Hospital Regional Universitario de Malaga (HRUM) from 2018 to 2020 with a compatible history of an IDHR after the intake of AX-CLV were evaluated according to the Internal Consensus on drug allergy (ICON).[37] Those confirmed as selective reactors to CLV with good tolerance to AX were finally included. The diagnostic procedure was carried out following the European Academy of Allergy and Clinical Immunology (EAACI) guidelines.[38,39] A group of 13 sex–age matched subjects with no reported hypersensitivity reactions to any drug was included in the study as healthy controls (HC). All participants were correctly informed and signed an informed consent to participate in the study. The research was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Committee of Malaga.

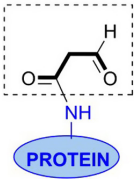
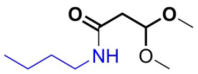
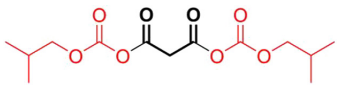
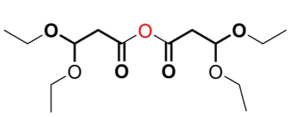
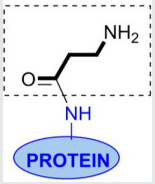
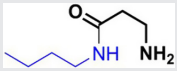
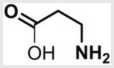
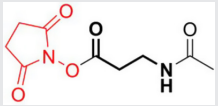
2.2 | Skin test

Skin prick tests (SPT) and, if negative, intradermal tests (IDT) were performed as described.[38,39] CLV and AX were used at 20 mg/ml; benzylpenicilloyl octa-L-lysine (BP-OL) was used at 0.04 mg/ml and penicillin minor determinants (MD) at 0.5 mg/ml (all from Diater). Readings were done after 20 min and a wheal larger than the 3 mm surrounded by erythema with negative result to control saline was considered positive.

2.3 | Drug provocation test

In patients with positive ST to CLV and negative to AX, BPOL, and MD, the good tolerance to AX was assessed by single-blind placebo-controlled oral DPT, as described.[14,38] Briefly, AX was administered with increasing doses (50, 100, 150, 200 mg), until reaching the cumulative dose of 500 mg to AX followed by a 2-day therapeutic course every 8 h at home.

TABLE 1 Antigenic determinants of clavulanic acid and synthetic analogues

Proposed antigenic determinants	Analogue molecules	Name
AD-I 		Clav1
		Clav2
		Clav3
AD-II 		Clav4
		Clav5
		Clav6
		Clav7

Note: Chemical structures of proposed antigenic determinants of clavulanic acid, I and II, and their corresponding synthetic analogues (Clav1-Clav7). Black bold structure corresponds to the fragment of the antigenic determinant; blue structures to the small fragments (butylamine) that mimic conjugated protein in a "reduced version"; red labels indicate the functional groups or part of the molecule that activate binding to the protein, which would be cleavaged upon conjugation or hydrolysis.

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2.4 | Generation of CLV antigenic determinants and synthetic analogues

A series of analogues from two different ADs from CLV (AD-I and AD-II) were synthesised following synthetic methodologies previously described by our group (Table 1).[20] Three different synthetic analogues for AD-I (clav1-3) and four for AD-II (clav4-7) were synthesised with different chemical functional groups (Table 1), except clav7, which is commercially available (Merck), providing different stability and ability to modify proteins.

2.5 | Biological sample collection

Forty ml of peripheral blood were obtained by venipuncture from each participant. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient (Rafer SL), frozen, and stored by Biobank of IBIMA-HRUM.

2.6 | moDCs isolation

Monocytes were isolated from PBMCs by positive selection (Miltenyi Biotec). Monocytes were transformed into monocyte-derived DCs (moDCs) by culturing with 100 ng/ml of IL-4 and 200 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) (both from R&D Systems Inc) for 5 days as previously described.[40,41]

2.7 | DC maturation after stimulation with CLV ADs synthetic analogues

Immature moDCs were cultured with two different concentrations (1 and 0.5 mM) of CLV and AD-I/II synthetic analogues for 3 days. These optimal concentrations were selected after dose–response curves to rule out the cytotoxic concentrations (data not shown). Immature moDCs were cultured with CLV and AD-I/II synthetic analogues for 3 days. Non-stimulated moDCs and lipopolysaccharide (LPS) (InvivoGen) were used as negative and positive controls, respectively. The expression of C-C chemokine receptor type 7 (CCR7), CD40, CD80, CD83, and CD86 was analysed to assess the maturation status of moDCs in a FACS CANTO II flow cytometer (BD). moDCs viability after culturing with CLV and its synthetic analogues was always determined by using Live-Dead™ fixable near IR dead cell staining (ThermoFisher Scientific). Representative examples of moDCs viability after culturing with the different stimuli are represented in Figure S1 (allergic patient) and Figure S2 (healthy control). Results were presented as maturation index (MI), calculated as the ratio between stimulated and unstimulated moDCs. Positive results were considered when MI ≥2.[41] moDCs gating strategy is represented in Figure S3A, and representative moDC maturation examples from a CLV allergic patient and a healthy control after culturing with LPS, CLV/synthetic CLV AD or without stimulus are represented as dot plots in Figure S3B.

2.8 | Proliferative response after stimulation with CLV ADs synthetic analogues

After positive selection of monocytes ($CD14^+$ cells), the negative fraction was labelled with carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific). Then, 1.5×10^5 of CFSE-$CD14^-$ cells were cultured for 7 days with 1.5×10^4 moDCs pre-stimulated with 1 and 0.5 mM of CLV or AD-I/II synthetic analogues for 3 days, after washing and removing the drug from the culture medium. Non-stimulated moDCs were included as negative control and 20 μg/ml of phytohemaglutinin (PHA) (Sigma) was used as positive control. Proliferation was assessed by analysing the expression of $CFSE_{Dim}$ in a FACS CANTO II flow cytometer of different subpopulations: $CD3^+$T, $CD8^+$T, $CD4^+$T, $CD4^+$Th1 ($CD3^+CD4^+CCR5^+$),[42] and $CD4^+$Th2 ($CD3^+CD4^+CRTH2^+$)[43] cells. Results were expressed as proliferation index (PI) calculated as follows[40]:

$$PI = \frac{(\%\text{CFSEdim stimulated lymphocytes} + \text{mo} - \text{DCs}) - (\%\text{CFSEdim unstimulates lymphocytes} + \text{mo} - \text{DCs})}{\%\text{CFSEdim unstimulated lymphocytes}}$$

Positive results were considered when PI ≥2.[15,44] LTT gating strategy is shown in Figure S4A and representative dot-plot proliferation examples from different cell populations and stimuli are represented in Figure S4B.

2.9 | Statistical analysis

Kolmogorov–Smirnov test was used to assess the normality of the data. Mann–Whitney test was used for quantitative comparisons without a normal distribution. Chi-square (X^2) test was used for qualitative comparisons. *p*-value ≤.05 was considered statistically significant. Statistical analysis was performed by using GraphPad PRISM v7.

3 | RESULTS

Twenty-seven allergic patients with confirmed selective IgE-mediated IDHRs to CLV were included in the study (Table 2). Sixteen were female (59.3%) and 11 were male (40.7%). The average age was of 52.07 ± 14.60 years. The most frequent clinical entity was anaphylaxis in 22 cases (81.48%), followed by urticaria with or without angioedema in 3 (11.11%) and anaphylactic shock in 2 (7.41%). The mean time interval between drug administration and the appearance of symptoms was 33.96 ± 27.85 min and the mean time interval between

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TABLE 2 Demographic and clinical data from the allergological workup of patients with immediate hypersensitivity reactions to clavulanic acid

Patients	Sex	Age	Reaction	Severity grade	Time interval (days) reaction/study	Time interval (minutes) drug administration/onset of symptoms	Drug	Episodes	ST			DPT
									CLV	AX	BP-OL/MD	AX
P-1	F	42	Anaphylaxis	II	60	60	AX-CLV	1	+	–	–	–
P-2	F	33	Anaphylaxis	III	2920	30	AX-CLV	2	+	–	–	–
P-3	F	66	Anaphylaxis	I	3650	30	AX-CLV	1	+	–	–	–
P-4	M	66	Urticaria/ Angioedema	I	30	60	AX-CLV	1	+	–	–	–
P-5	M	69	Anaphylaxis	I	3650	60	AX-CLV	1	+	–	–	–
P-6	F	47	Anaphylaxis	II	60	15	AX-CLV	2	+	–	–	–
P-7	F	34	Anaphylaxis	III	185	60	AX-CLV	1	+	–	–	–
P-8	F	42	Urticaria	I	730	60	AX-CLV	1	+	–	–	–
P-9	F	66	Anaphylaxis	I	167	5	AX-CLV	1	+	–	–	–
P-10	F	37	Anaphylaxis	I	210	10	AX-CLV	2	+	–	–	–
P-11	M	66	Anaphylaxis	II	30	60	AX-CLV	2	+	–	–	–
P-12	M	44	Anaphylaxis	III	130	30	AX-CLV	1	+	–	–	–
P-13	M	40	Anaphylaxis	III	31	30	AX-CLV	1	+	–	–	–
P-14	M	33	Anaphylaxis	I	240	120	AX-CLV	1	+	–	–	–
P-15	F	28	Anaphylaxis	III	570	15	AX-CLV	1	+	–	–	–
P-16	M	31	Urticaria/ Angioedema	I	1825	10	AX-CLV	1	+	–	–	–
P-17	F	33	Anaphylactic Shock	IV	365	40	AX-CLV	2	+	–	–	–
P-18	M	62	Anaphylaxis	II	1765	45	AX-CLV	1	+	–	–	–
P-19	F	48	Anaphylaxis	I	153	15	AX-CLV	1	+	–	–	–
P-20	M	56	Anaphylaxis	II	730	10	AX-CLV	1	+	–	–	–
P-21	F	39	Anaphylaxis	III	134	5	AX-CLV	1	+	–	–	–
P-22	M	58	Angioedema	I	35	72	AX-CLV	1	+	–	–	–
P-23	F	66	Anaphylaxis	II	55	5	AX-CLV	2	+	–	–	–
P-24	M	71	Anaphylactic Shock	IV	30	5	AX-CLV	1	+	–	–	–
P-25	F	71	Anaphylaxis	I	30	30	AX-CLV	1	+	–	–	–
P-26	F	41	Anaphylaxis	I	212	30	AX-CLV	1	+	–	–	–
P-27	F	60	Anaphylaxis	II	1460	5	AX-CLV	2	+	–	–	–

Abbreviations: AX, amoxicillin; BP-OL, benzylpenicilloyl octa-L-lysine; CLV, clavulanic acid; DPT, drug provocation test; F, female; M, Male; MD, minor determinant; ND, not done; SPT, skin prick test.

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the reaction and the study was of 696 ± 10,638 days. Severity of each reaction was graded from I-IV, following Ring and Messmer classification for anaphylactoid reactions.[45] All patients reported an allergic episode after the intake of AX-CLV, 20 of them suffered a unique episode (74.07%), whereas 7 reported two (25.93%). The mean age of the healthy control group was of 37.26 ± 11.97 years, being 8 females (61.54%) and 5 males (38.46%).

3.1 | DC maturation after stimulation with CLV or its AD synthetic analogues

The inclusion of CLV only significantly increased the expression of CD83 and CD86 in moDCs from allergic patients (AP) compared to HC (Figure 1D,E). The inclusion of different synthetic analogues from both ADs increased significantly the expression of CD40, CD80, and CD83 compared to HC (Figure 1B,D). It was only observed a significantly higher expression of CD86 after the inclusion of clav1, 2, 6, and 7 in AP compared to HC (Figure 1E). Similarly, only higher expression of CCR7 was observed after the inclusion of clav1, 2, and 6 in AP (Figure 1A).

The inclusion of CLV reported the lowest % of positivity independently of the marker analysed (Figure 1F–J). The inclusion of clav2 induced the expression of CCR7 and CD40 (29% and 43% respectively), followed by clav3 and clav6 for CCR7 (14% for both), and clav5 for CD40 (33%) (Figure 1F,G). Higher % of positivity was obtained for CD80 and CD83 after the inclusion of analogues from AD-II, except for clav4, compared to those from AD-I (Figure 1H,I). The % of positivity for CD86 was higher after the inclusion of clav2 and clav6 (29% both), followed by clav3 and clav5 (24% both) (Figure 1J). The % of positivity in HC was lower than 10% in all cases, except for CCR7 with clav2 and CD83 with clav7 (15% both).

The analysis of the results from moDCs maturation, combining synthetic analogues from each AD, increased the % of positivity, independently of the marker analysed, except for CCR7 and CD40, compared to each synthetic analogue alone, with a specificity higher than 75% in all cases (Figure 2A–E). Combining the results obtained from synthetic analogues from both ADs, the % of positivity increased for all markers without a reduction in terms of specificity (Figure 2A–E). The inclusion of CLV did not increase the % of positive cases with any marker, with the exception of CD40, although there was a reduction in terms of specificity, from 77% to 69% (Figure 2B).

Focusing only on AP positive cases, we observed a different pattern of maturation depending on the AD included. Most positive cases for CCR7 (70%) and CD40 (52%) were induced exclusively after the inclusion of synthetic analogues from AD-I (Figure 2F,G). On the contrary, higher percentage of positive cases for CD80, CD83, and CD86 (36%, 25%, and 23%, respectively) was selectively detected after the inclusion of synthetic analogues from AD-II (Figure 2H–J).

3.2 | Proliferative response after stimulation with CLV or its AD synthetic analogues

The inclusion of CLV did not significantly increase the proliferation of $CD3^+$T, $CD8^+$T, $CD4^+$T, $CD4^+$Th1, and $CD4^+$Th2 cells (Figures 3A–C and S5A,B). Higher proliferation of $CD3^+$T cells was observed after the inclusion of clav4 and clav5 (Figure 3A) and of $CD4^+$T cells after the inclusion of clav1, 2, 4, and 5 (Figure 3B). No significantly increase in proliferation was observed in $CD8^+$T cells, independently of the synthetic analogue included (Figure S5A). Moreover, significantly higher proliferation was observed in $CD4^+$Th2 cells after the inclusion of clav2 and clav4-7 (Figure 3C) but not in $CD4^+$Th1 cells for any of the synthetic analogues (Figure S5B).

The % of positive cases with CLV was 12% for both, $CD3^+$ (Figure 3D) and $CD4^+$T cells (Figure 3E), and 19% for $CD4^+$Th2 cells (Figure 3F). Although the inclusion of synthetic analogues in most cases increased the % of positivity, the sensitivity did not significantly improve for any of the synthetic analogues included independently and the T cell population analysed (Figure 3D–F). The combination of synthetic analogues from either AD-I or II increased the % of positivity in all cases in AP, without differences between ADs, except for $CD4^+$Th2 cells, with a positivity of 42% for AD-I and 54% for AD-II (Figure 4A–C). The combination of results of synthetic analogues from both ADs increased the % of positivity to 46%, 50%, and 65% for $CD3^+$T, $CD4^+$T, and $CD4^+$Th2 cells, respectively (Figure 4A–C). The inclusion of proliferation results from CLV did not add any effect in terms of positivity; however, it increased the number of positive cases in HC (Figure 4A–C). Half of AP with positive $CD3^+$ T cell proliferation results recognised analogues from both ADs, whereas 25% only selectively recognised one AD or another (Figure 4D). Similar recognition was observed for $CD4^+$T cells (Figure 4E). Of note, 47% of $CD4^+$Th2 positive cases recognised both ADs, although a higher proportion specifically recognised analogues from AD-II (35%) compared to the ones from AD-I (18%) (Figure 4F). Higher % of $CD4^+$Th2 cells was observed in allergic patients compared to healthy controls, whereas higher % of $CD4^+$Th1 cells was detected in healthy controls compared to allergic patients (Figure S5C,D). $CD4^+$Th2/$CD4^+$Th1 ratio revealed a clear Th2 profile in allergic patients compared to healthy controls, although no differences were observed between conditions (Figure S5E).

The proliferation analysis of $CD4^+$Th2 cells after combining the results from synthetic analogues of both ADs reported the highest % of positivity (65%) (Figure 4C), without the need of including other cell populations into the analysis (Figure 4G). Next, we evaluated which concrete analogues have a positive influence in the proliferation results. As it is shown in Figure 4H, all synthetic analogues except clav4 and clav5 improved the sensitivity of the test, with a contribution in terms of positivity from the highest to the lowest: clav1 > clav6 > clav2 > > clav3 > clav7.

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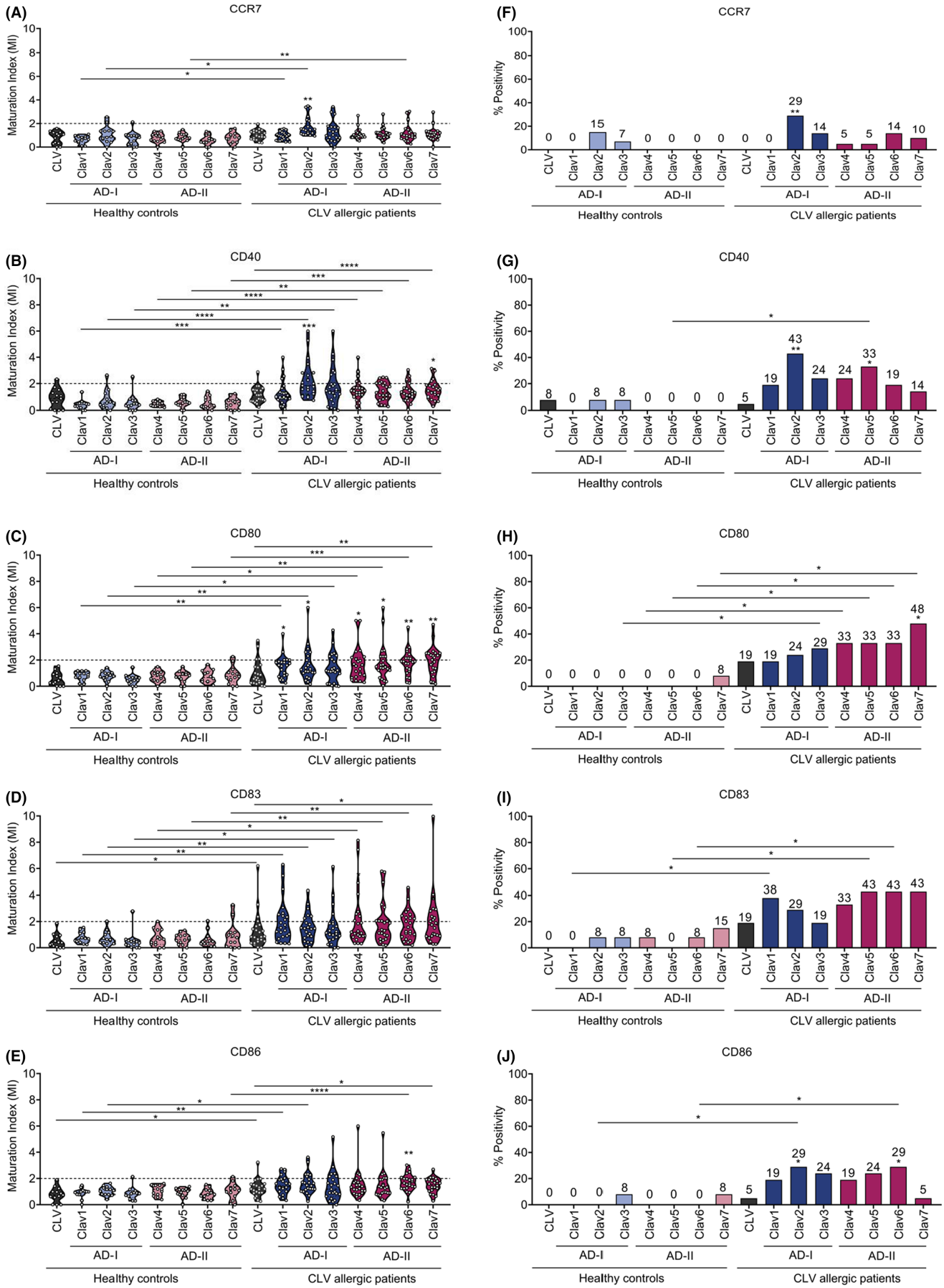

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FIGURE 1 Dendritic cell maturation induced by clavulanic acid and its antigenic determinants. (A–E) Violin plots represent maturation index (MI) of (A) CCR7, (B) CD40, (C) CD80, (D) CD83, and (E) CD86 from allergic patients and healthy controls. (F–J) Bars represent % of positivity based on MI ≥2 of (F) CCR7, (G) CD40, (H) CD80, (I) CD83, and (J) CD86 from allergic patients and healthy controls. Comparisons in terms of positivity by X^2 test and MI by Mann–Whitney test (*$p<.05$; **$p<.01$; ***$p<.001$; ****$p<.0001$)

4 | DISCUSSION

Selective IDHRs to CLV have increased in the last decades, mainly due to the higher consumption of AX-CLV.[1,2,9] The diagnostic procedure is complex,[15,16] being DPT a frequent in vivo method used to assess good tolerance to other BLs.[19,21] However, it is not recommended for most severe reactions.[18]

In vitro tests are good alternatives to safely help in the diagnosis of selective IDHRs to CLV.[22,23] Nevertheless, the detection of sIgE to CLV is not possible by immunoassays,[11,30] and it has been reported that other in vitro tests such as BAT and HRT, although useful, do not have an optimal sensitivity.[2,24] In an attempt to improve the sensitivity of in vitro testing, different drug metabolites which are efficiently recognised by the immune system have been included.[32,34,46] Nevertheless, the high degradative concentration and instability of its intermediates have made the characterisation of the CLV metabolites responsible for triggering an IDHRs very difficult.[20,26] Previous studies have reported that CLV degradation occurs between 5–10 times faster than penicillin G and AX,[47,48] detecting degradative CLV products within few minutes in neutral solution.[49] This degradation is also self-accelerated, because its decomposition products act as similarly strong general-base catalysts.[26,50] These molecules are formed after CLV hydrolysis due to the instability of the hydrolysed CLV molecule (with opened betalactam ring). Comparable low stability of the CLV-protein conjugate (covalently bound through betalactam ring) may lead to different degradation products.[48]

In a previous study, we found that one of the proposed AD of CLV (AD-I) was able to trigger basophil degranulation in AP with IDHRs.[20] Nevertheless, the complete immunological picture involved in this recognition is not completely understood. For this reason, we have stepped back to investigate the ability of CLV and different synthetic analogues of the ADs proposed to be recognised by DCs, as well as to promote the specific proliferative response of different T cell populations.

Previous studies have reported that drugs and their metabolites can induce the activation and maturation of DCs,[35,41] able to trigger a T cell response in NIDHRs.[34,46,51] Based on the hapten hypothesis, well established for BLs,[5,52,53] they spontaneously interact with proteins present in the culture medium. These drug-carrier compounds are recognised by moDCs, which uptake, process, and present them to T cells through major histocompatibility complexes (MHCs) by increasing the expression of activation and maturation markers on DCs,[54] such as CD40, CD80, CD83, and CD86.

In the present study, we observed that reactive CLV metabolites are more active than the native drug in interacting with DCs. In fact, after the inclusion of CLV, only differential upregulation of CD83 and CD86 was observed in AP compared to HC, whereas the inclusion of CLV synthetic analogues from both ADs increased the expression of CD80, CD83, and CD40, and, although in a less extent, also of CCR7 and CD86. Moreover, synthetic analogues differentially interacted with DCs. Synthetic analogues from AD-I preferentially promoted the expression of CCR7 and CD40, whereas analogues from AD-II mainly induced the expression of CD80, CD83, and CD86. These results could explain the need for including synthetic analogues from both ADs to improve the sensitivity of the in vitro test.

Different studies have investigated the role of lymphocyte transformation test (LTT) in the evaluation of NIDHRs with heterogeneous results, although they have shown promising results in NIDHRs to BL.[23,55–58] In a recent paediatric study, LTT reported a sensitivity of 33.3% for CLV in NIDHRs.[15] Nevertheless, due to the low number of studies focused on this topic in IDHRs, and because the methodology used in this study was significantly different from others, the comparison of results should be carefully taken into account. In the present study, we obtained a lower sensitivity, 12%–19% in IDHRs when native CLV was included in the test. This low sensitivity could be explained because: (i) although DCs have the best capacity to induce a proliferative response on T cell populations,[35,40] they would not achieve a complete maturation status; thus, not efficiently present the antigens to T cells[35,59,60]; and (ii) CLV is spontaneously degraded into different metabolites, being only some of them efficiently recognised by the immune system.[46] This heterogeneity and concentration of the specific metabolites generated could have an important influence in moDCs maturation and T cell proliferation.

In a recent study, we demonstrated that the determination of proliferative response in the effector cell subpopulations involved in the reaction increased the LTT sensitivity in NIDHRs.[40] In this study, the highest value of sensitivity was found when the proliferation of $CD4^+$Th2 cells was analysed, without the need for including other subpopulations, since these are the relevant cells to promote the necessary microenvironment for IgE isotype switching in IDHR.[7,35,36,61]

Besides, the inclusion of different drug metabolites to increase LTT sensitivity has also been broadly investigated to evaluate NIDHRs to sulphamethoxazole.[51,62,63] In spite of the low LTT sensitivity found in our study with CLV itself, the inclusion of synthetic analogues from both ADs, increased the sensitivity to 65%, with a specificity higher than 90%, similarly to previous studies with different drugs in both, NIDHRs,[46] and IDHRs,[61] although the latest with a different methodology. It is important to note that certain factors can have an influence in in vitro test sensitivity such as the severity of the reaction and the time interval between the reaction and in vitro test performance. This last factor has been described to be critical in IDHRs for BAT and immunoassays, due to the clearance of sIgE with time.[64] Nevertheless, this seems not to be so important for LTT,[56,61] because the proliferation of mostly memory T

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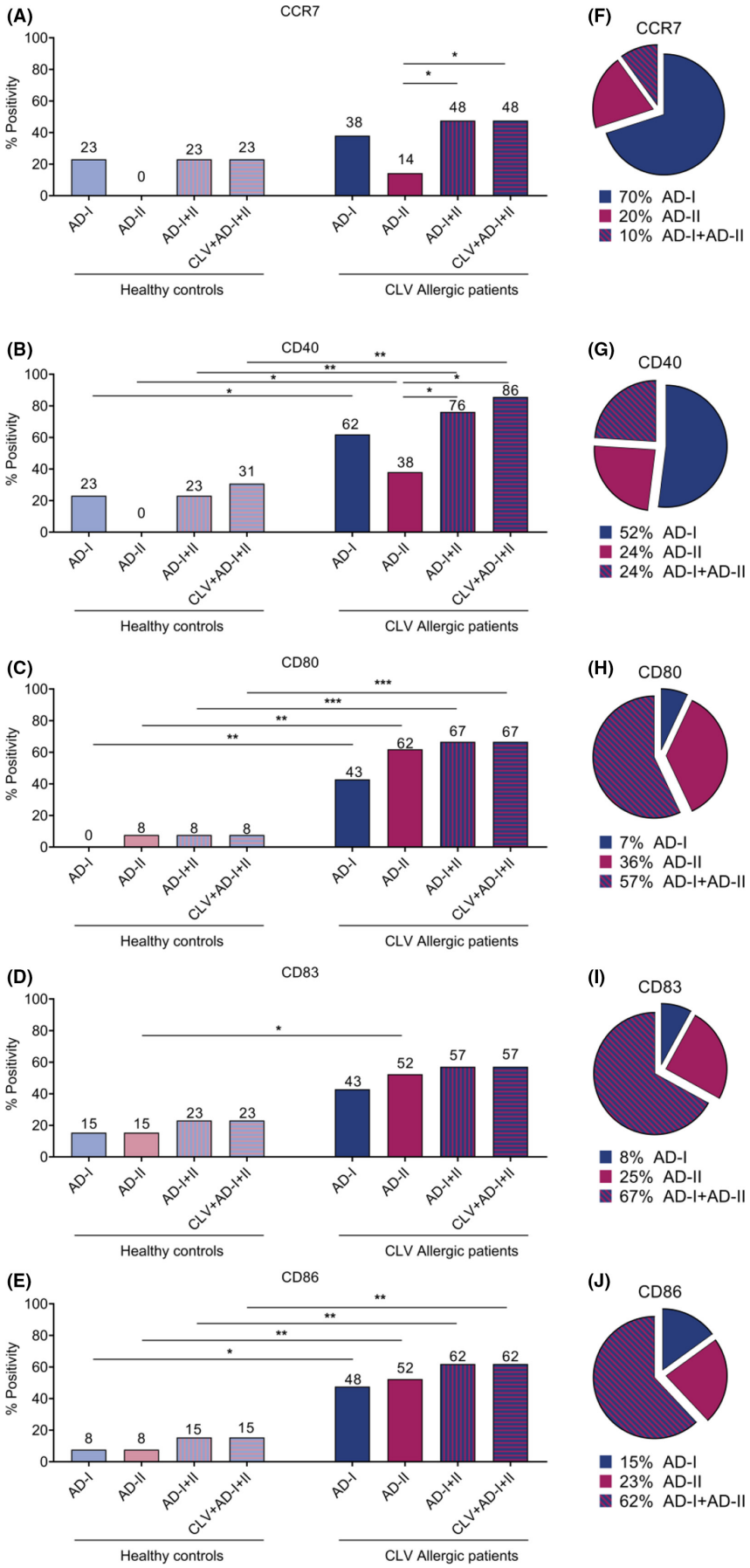

FIGURE 2 Maturation positivity induced by clavulanic acid and its antigenic determinants. (A–E) Bars represent % of positive cases (maturation index ≥2) of (A) CCR7, (B) CD40, (C) CD80, (D) CD83, and (E) CD86 in allergic patients and healthy controls after the combination of synthetic analogues from both antigenic determinants. (F–J) Parts of whole represent % of positive cases (maturation index ≥2) for (F) CCR7, (G) CD40, (H) CD80, (I) CD83, (J) CD86 in allergic patients with positive response. Comparisons in terms of positivity by X^2 test (*$p<.05$; **$p<.01$; ***$p<.001$)

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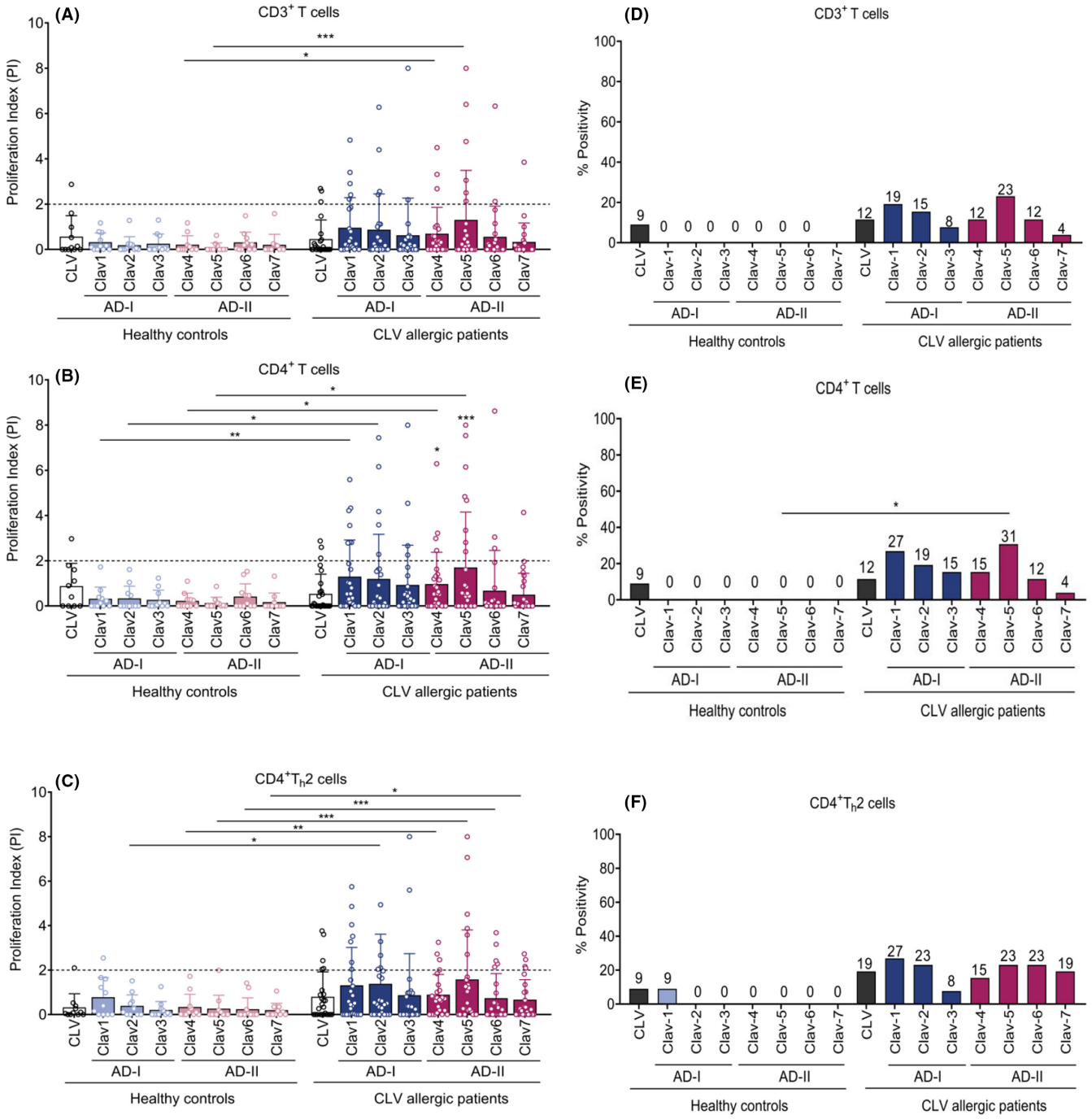

FIGURE 3 T cell proliferative response induced by clavulanic acid and its antigenic determinants. (A–C) Bars and standard deviation represent proliferation index (PI) of (A) CD3+T cells, (B) CD4+T cells, (C) CD4+Th2 cells. (D, E) Bars represent % of positivity based on PI ≥2 of (D) CD3+T cells, (E) CD4+T cells, (F) CD4+Th2 cells from allergic patients and healthy controls. Comparisons in terms of positivity by X^2 test and proliferation index by Mann–Whitney test (*$p<.05$; **$p<.01$; ***$p<.001$)

cells was detected in blood even after 20 years from the reaction.[56] In this study, we did not observe any correlation between the reaction severity or time elapsed between the reaction and the performance of the test and the sensitivity of DC maturation or LTT assays. Nevertheless, because of the reduced number of patients, this should not be completely discarded.

A different recognition pattern was observed depending on the AD included. 82% of patients with positive CD4+Th2 cell proliferative response recognised synthetic analogues from AD-II, whereas only 65% recognised those from AD-I. These observations may suggest that the upregulation of CD80, CD83, and CD86 on DCs induces a stronger positive response promoting the proliferation of CD4+Th2 cells than the expression of CCR7 and CD40. Nevertheless, as 18% and 35% of patients selectively recognised synthetic analogues from AD-I or AD-II, respectively, the inclusion of synthetic analogues from both ADs would be needed.

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This fact could be explained because, due to the different chemical structure, each synthetic analogue could modify different carrier

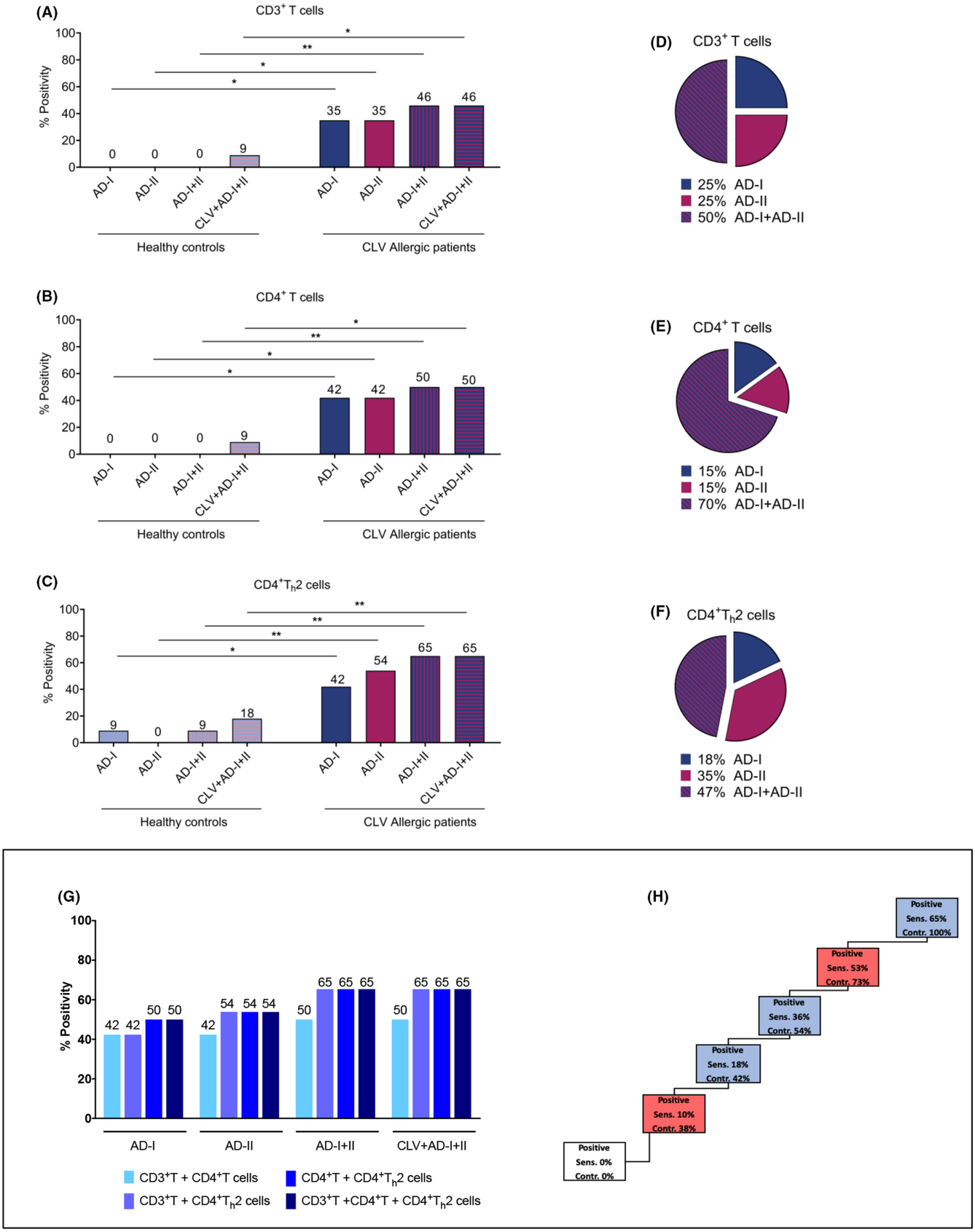

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FIGURE 4 T cell proliferation positivity induced by clavulanic acid and its antigenic determinants. (A–C) Bars represent % of positive cases (proliferation index ≥2) of (A) $CD3^+$T cells, (B) $CD4^+$T cells, (C) $CD4^+$Th2 cells in allergic patients and healthy controls after the combination of synthetic analogues from both antigenic determinants. (D–F) Parts of whole represent % of positive cases (proliferation index ≥2) of (D) $CD3^+$T cells, (E) $CD4^+$T cells, (F) $CD4^+$Th2 cells in allergic patients with positive response. (G) Bars represent the % of positivity from clavulanic acid and synthetic analogues from each antigenic determinant by combining the proliferative results of $CD3^+$T, $CD4^+$T, and $CD4^+$Th2 cells. (H) Diagram represent the % of sensitivity (sens.) and the % of contribution (contr.) regarding the total positive cases with the most relevant synthetic analogues in the proliferative response of $CD4^+$Th2 cells. Comparisons in terms of positivity by X^2 test (*$p<.05$; **$p<.01$; ***$p<.001$)

molecules, with differences regarding the density and distribution of the CLV on the carrier molecule, with important implications in their recognition.[8,65,66]

Although many degradation products can be proposed as a consequence of CLV instability upon protein conjugation, from the two ADs studied herein, only the identification of AD-I structure by mass spectroscopy had been previously confirmed.[20,26] The positive results for triggering T-cell response with all AD-I analogues are in agreement with these studies,[20,26] from which clav2 and clav3 were able to activate basophils from AP,[20] explained by the chemical structure of AD-I, but also by protein conjugation ability.[67] However, in the present study, the AD-I aldehyde functionality seems to be the most important factor, since clav1 is already conjugated to butylamine, acting as a short lysine residue. From analogues that do not trigger T cell response, clav4 and clav5 display the unaltered amine functionality of AD-II, but they are not activated to react with proteins. Regarding analogues of AD-II that showed ability of T cell activation, clav6 is much more reactive than clav7 for protein binding, and, in both, amine group is somehow protected.

In conclusion, we have demonstrated that synthetic analogues from CLV functionalised with both a carbonyl functional group (AD-I) and an amino group (AD-II) are efficiently recognised by DCs from AP. These cells can present the peptides to T cells, inducing proliferation mainly of those with a Th2 cytokine pattern in patients with IDHRs to CLV. The combination of synthetic analogues from AD-I and AD-II significantly increased not only the DC maturation but also LTT sensitivity compared to the results obtained with native CLV, with more than 90% of specificity. Further studies comparing BAT, DC maturation assays and LTT in the same patients after the inclusion of CLV synthetic analogues are required to better understand the specific mechanism involved in CLV recognition. The present findings, although in a very early stage, are promising to improve the diagnosis of IDHRs to CLV in future.

AUTHOR CONTRIBUTION

The authors approved the final version of the manuscript as submitted and agreed to be accountable for all aspects of the work.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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