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Dendrimeric Antigens for Passive Mast Cell Activation in the Evaluation of Amoxicillin Allergy

Amene Tesfaye Ayane[1,2] | Maria Salas[1,3,4] | Sara Benede[5] | Maria J. Rodriguez-Sanchez[6,7] | Maria J. Rodriguez-Sojo[6,7] | Isabel M. Jiménez-Sánchez[1] | Gador Bogas[1,3] | Ezequiel Perez-Inestrosa[2,8] | Cristobalina Mayorga[1,3] | Juan L. Paris[1] | Alba Rodríguez-Nogales[5,6] | María J. Torres[1,3,4] | María I. Montañez[1,2]

[1]Allergy Research Group, Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina—IBIMA Plataforma BIONAND, Málaga, Spain | [2]Departamento de Química Orgánica, Universidad de Málaga, Málaga, Spain | [3]Allergy Unit, Hospital Regional Universitario de Málaga-HRUM, Málaga, Spain | [4]Departamento de Medicina y Dermatología, Universidad de Málaga, Málaga, Spain | [5]Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, Madrid, Spain | [6]Department of Pharmacology, Center for Biomedical Research (CIBM), University of Granada, Granada, Spain | [7]Instituto de Investigación Biosanitaria de Granada (ibs. GRANADA), Granada, Spain | [8]Instituto de Investigación Biomédica de Málaga y Plataforma en Nanomedicina—IBIMA Plataforma BIONAND, Málaga, Spain

Correspondence: Alba Rodríguez-Nogales (albarn@ugr.es) | María I. Montañez (maribelmv@uma.es)

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ABSTRACT

Background: Amoxicillin (AX) is frequently implicated in immediate IgE-mediated allergic reactions. Diagnosis is challenging, highlighting the need for new approaches enhancing in vitro sensitivity and specificity. Engineered nanostructures can mimic immunological recognition of hapten-carrier conjugates, offering a strategy to improve diagnostic accuracy.

Methods: Dendrimeric Antigens (DeAns), with controlled size (1st–5th generation) and multivalent AX determinants (8–128 units, respectively), were synthesized for in vitro immunological evaluation. In vitro IgE recognition was studied by competitive radio-immunoassay. Allergenic activity was evaluated in mouse bone marrow-derived mast cells (MC) sensitized with mouse anti-AX IgE monoclonal antibody and humanized RBL-2H3 (huRBL-2H3) and LUVA cells sensitized with sera from β-lactam-allergic subjects and tolerant controls, measuring degranulation in response to DeAns stimulation.

Results: Five different DeAns were obtained as pure compounds. All DeAns were recognized by AX-sIgE. A clear size-dependent activation pattern was observed in the three cell models: lower-generation (1st–2nd) DeAns failed to induce degranulation, whereas DeAns of bigger size (3rd–5th generation) triggered significant, dose-dependent activation. Notably, no activation was observed in tolerants and unsensitized cells or with blank dendrimers. In patient-sera assays, the passive MC activation test (pMAT) with DeAns provided complete diagnostic discrimination, with activation restricted to AX-allergic patients.

Conclusions: DeAns are effective platforms for investigating effector cell activation in AX allergy. By fine-tuning structural attributes—size and multivalence—we reveal the promising utility of DeAns in pMAT that leverage commercial cell lines and patient sera. This approach could address key limitations of β-lactam allergy diagnostics, enabling more reliable and standardized in vitro testing.

Abbreviations: AX, amoxicillin; AXO, amoxicilloyl; AXO-Bu, amoxicilloyl-butylamine; BAT, basophil activation tests; BiAn, bidendron antigens; DeAn, dendrimeric antigen; DPTs, drug provocation tests; G, generation; GPC, gel permeation chromatography; HSA, human serum albumin; Hu, humanized; MCs, mast cells; MoAb, monoclonal antibodies; NMR, Nuclear Magnetic Resonance; PAMAM, polyamidoamine; PEG, polyethylenglycol; PLL, poly-L-lysine; pMAT, passive mast cell activation test; RAST, radioallergosorbent test; RBL, rat basophilic leukemia cell; STs, skin tests.

Amene Tesfaye Ayane and Maria Salas contributed equally and are considered joint first authors.

Maria Jose Torres and Maria Isabel Montañez contributed equally and are considered joint senior authors.

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1 | Introduction

Amoxicillin (AX) is a widely prescribed semi-synthetic β-lactam antibiotic, alone or with clavulanic acid, and frequently implicated in allergic reactions, which are mainly induced by IgE-mediated mechanisms [1]. The allergic reaction occurs when the drug, acting as a hapten, forms multivalent protein conjugates of sufficient size for crosslinking specific IgE (sIgE) on basophils or mast cells (MCs) surface, leading to their activation, degranulation, and the release of inflammatory mediators that manifest as clinical allergic symptoms. Chemically, after AX administration, the drug undergoes a nucleophilic attack by primary amino groups on endogenous proteins, forming amoxicilloyl (AXO) determinants with an opened β-lactam ring covalently attached to proteins [2]. The immune recognition of these hapten-protein conjugates and further effector response critically depends on their size and multivalency [3, 4], which are necessary to effectively crosslink IgE bound to FcεRI receptors on effector cell surface. This highlights the importance of chemical structural factors in both immune activation and diagnostic testing of drug allergy [5].

The diagnosis of drug allergy, including β-lactam, remains a significant clinical challenge due to the complexity of immune mechanisms involved and the limitations of available diagnostic tools. Standard diagnostic methods rely on both in vivo and/or in vitro tests [6]. Confirmation of β-lactam allergy is achieved using in vivo assays, skin tests (STs) and drug provocation tests (DPTs), but with notable drawbacks [7, 8]. DPTs, although regarded as the gold standard, are time-consuming, expensive, require expert personnel and facilities and are not recommended in patients with life-threatening reactions [9–11]. In vitro alternatives like sIgE determination (ImmunoCAP) [12] and basophil activation tests (BAT) offer safer options but with limited sensitivity, particularly for β-lactam-induced allergies [13, 14]. BAT, in particular, is limited by its dependence on fresh blood samples, a narrow testing window (typically within 24 h), and a significant proportion of non-releasers, with sensitivity for β-lactam antibiotics reported as 55% [7, 15–16]. The low sensitivity of in vitro assays is attributed in part to the incomplete understanding of the in vivo formation of drug-protein conjugates and their structural requirement for effector cells' activation [14, 17–18].

Passive MC activation test (pMAT) has emerged as a promising new approach to address the limitations of current in vitro diagnostic tests [19, 20]. pMAT involves sensitizing healthy donor-derived or immortalized MC lines with patient sera, followed by stimulation with the suspected allergen/drug to assess effector cell degranulation. This approach avoids the need for fresh blood, reduces the issue of non-releasers, and has shown high analytical sensitivity [16, 21–22]. Recent studies have demonstrated the utility of pMAT [20] in diagnosing IgE-mediated allergies to drugs, including reactions to chlorhexidine [22, 23], rocuronium [24] and platins [25] with promising results. In contrast, the only study reporting the use of pMAT for detecting AX allergy demonstrated limited diagnostic value, comparable to that of the conventional BAT [26].

To develop more reliable diagnostic tools for β-lactam allergy, we have focused on elucidating the structural and conformational requirements for optimal immunological recognition. To this end, we use drug-protein conjugates [2, 27] as well as drug-dendrimer based synthetic nanostructures [5, 28–31] as model systems, which also demonstrated efficient immunologic recognition (Figure 1). Dendritic structures are particularly attractive for this purpose because they mimic protein-like features and allow precise control over key nanoscale parameters, including size, shape, surface chemistry, flexibility/rigidity, and overall architecture [32–34]. Among them, dendrimers are complete, highly branched macromolecules with a central core, whereas dendrons represent the individual branched segments. Both exhibit monodispersity and allow progressive increases in size and in the number of peripheral groups with each increasing generation, to which derived BLs, such as AXO moieties, can be efficiently coupled [28–31]. Our previous work with Bidendron Antigens (BiAns), consisting of two dendrons exhibiting eight AXO units each and linked at both ends of flexible polyethylene glycol (PEG) spacers of several lengths, demonstrated that IgE receptor crosslinking required for effector cell degranulation

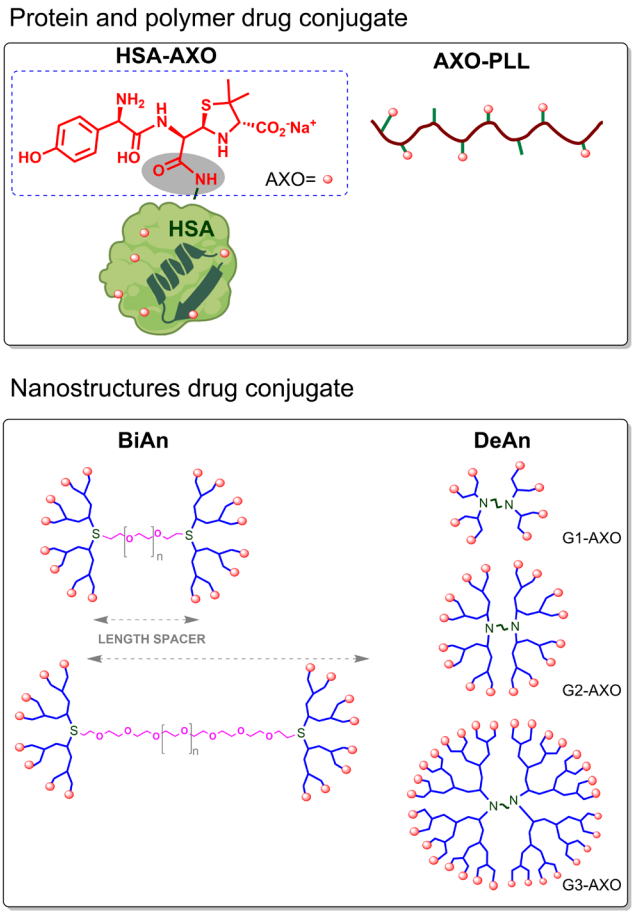

FIGURE 1 | Architectural diversity of hapten carriers. Representative structures of hapten carriers used in conjugate formation. HSA offers biological relevance but low hapten density and limited chemical control. PLL provides multivalency but suffers from polydispersity. Nanostructures such as BiAns and DeAns are well-defined structures exhibiting a precise number of hapten presentations. DeAn size increases with generation, becoming globular, while BiAn size depends on spacer linker (PEG) length, which may fold and shield epitopes in solution, complicating in vitro immunological interpretation.

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occurs only when the nanostructure achieves an optimal hapten (AX) spacing/distance (with a minimum PEG length of 6000 Da) [5]. Although BiAns highlight the importance of adduct size in effector cell activation for AX allergy, their utility in studying activation mechanisms and enhancing the sensitivity of BAT or pMAT could be constrained by several factors like the strong tendency of PEG spacers to fold in aqueous solution, and the possible shielding of antigenic sites due to longer PEG chains that may hinder interaction with cells.

As part of this study, these limitations prompted us to develop a simpler and more defined model, the Dendrimeric Antigens (DeAns), which are drug-functionalized dendritic nanostructures lacking PEG linkers. DeAns allow precise control over key structural parameters such as size, shape, and valency of AXO determinants. To investigate the structural requirements of DeAns for FcεRI-mediated effector cell activation, we employed three different cellular degranulation models. As a first and simpler approach for setting up experiments, we used mouse bone marrow-derived MCs passively sensitized with mouse monoclonal anti-AX IgE antibodies. Then, the other two cell models, LUVA and humanized RBL-2H3 (huRBL-2H3), were selected because of their ability to respond to human IgE sensitization using patient sera, thus allowing evaluation of their suitability for in vitro drug allergy diagnosis. LUVA is a human MC line derived from CD34+ progenitors, showing a mature MC phenotype with FcεRI, FcγRI, and FcγRII expression and FcεRI-mediated degranulation, although with modest β-hexosaminidase release [35]. RBL-2H3, a rat basophilic leukemia line with a MC–basophil intermediate phenotype, grows rapidly, is easy to culture, and expresses functional FcεRI, although with suboptimal degranulation capacity and fluctuating FcεRI expression [16]. LUVA better mimics human MC physiology, whereas RBL-2H3 is a robust, cost-effective model but less representative of human primary cells [35]. Previous successful use of huRBL-2H3 for analogous nanostructure evaluation [5] supports its inclusion in this study, while LUVA provides a more physiologically relevant representation of human MCs.

Therefore, this study aimed to synthesize well-defined DeAns to elucidate the structural features that govern FcεRI-mediated effector cell activation across two cellular models (huRBL-2H3 and LUVA cells), to further evaluate their potential application in pMAT as a reliable tool for in vitro diagnosis of AX allergy.

2 | Methods

2.1 | Patients and Controls

Patients with a confirmed immediate allergic reaction to β-lactams, as well as tolerant subjects, were selected according to guidelines of the European Academy of Allergy and Clinical Immunology (EAACI) [12, 36]. The study protocol adhered strictly to the principles outlined in the Declaration of Helsinki and received approval from the Provincial Ethics Committee of Malaga. Prior to participation, all subjects were informed about the study and completed a written consent form.

2.2 | Production of Conjugates

AX was conjugated to different carriers: dendrimer nanostructures to yield DeAns ($G_{1\text{-}5}$-AXO), human serum albumin (HSA) to yield HSA-AXO, and butylamine monomer to obtain AXO-Bu. Conjugation consisted of nucleophilic reactions involving free amine groups on polyamidoamine (PAMAM) dendrimers, HSA, and butylamine, which selectively reacted with the β-lactam ring of AX, forming stable AXO-derived conjugates, following previous protocols [5]. The detailed experimental procedures for the synthesis, purification, and characterization of DeAns by Nuclear Magnetic Resonance (NMR) techniques and Gel Permeation Chromatography (GPC) are provided in Supporting Information: Methods section.

2.3 | Immunoassays

FEIA ImmunoCAP (Thermo-Fisher) was used for quantifying serum benzylpenicillin-sIgE (with allergen c1, penicilloyl G), AX-sIgE (with allergen c6, AXO), and total IgE levels in patients following manufacturer's instructions. Results of drug-sIgE were considered positive according to the cutoff ≥0.1 kU A/L.

Radioimmunoassays were performed in individual sera from 14 AX allergic patients (Table S1) using the radioallergosorbent test (RAST) as previously described [2] employing as solid phase poly-L-lysine (PLL) bound to cellulose discs and conjugated to the β-lactam (β-lactam-PLL cellulose discs) and ^{125}I-labeled anti-IgE antibodies as secondary antibodies [37]. Positive results were defined as those with label uptake exceeding 2.5%, a threshold derived from the mean ±2 SD of the negative control group, ensuring stringent criteria for IgE reactivity.

Competitive inhibition immunoassays were performed using one pool of sera from 14 patients with RAST values ≥7% (Table S1). The sera were pre-incubated with the inhibitors AXO-Bu and the series of DeAns at three concentrations (60, 30, and 6 mM of AXO equivalents) as fluid phase for 18 h at room temperature. Then, AXO-PLL cellulose discs were added to the test assay and incubated for 3 h, and the RAST protocol was executed as previously described [2]. Results were quantified as inhibition percentage over non-inhibited samples.

2.4 | Basophil Activation Test

An in-house BAT was performed following a standardized consensus protocol [38] on whole-blood samples from patients, using freshly prepared AX solutions at 2.5, 1.25, and 0.25 mg/mL. For further details, see the Supporting Information: Methods section.

2.5 | Cell Degranulation and Viability Assays

Three different cell models have been used to evaluate degranulation:

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1. Mouse bone marrow cells were collected and differentiated into MCs and cultured prior to assay following established protocols [5, 39]. Then, mouse MCs were sensitized for 4 h with 1 μg/mL of mouse anti-AX IgE MoAb [40]. After washing, MCs were resuspended in Tyrode's buffer and incubated with DeAns at concentrations of 1, 5, 10, 50, and 100 μM (AXO equivalents), as well as with drug-free PAMAM dendrimers (at concentration equivalent to DeAns) for 1 h. The HSA-AXO conjugate, displaying an average of 14 AXO units (10 μM of AXO moieties), served as a positive control, while phosphate-buffered saline (PBS) and HSA were employed as negative controls. Parallel tests were carried out utilizing unsensitized cells. All experiments were performed in triplicate. Animal protocols were approved by the Comunidad de Madrid (Ref PROEX 286.8 20) in accordance with European regulations (Directive 2010/63/EU).
2. HuRBL-2H3 cells were cultured as previously reported [5].
3. The human MCs line (LUVA) was maintained in StemPro-34 SFM (ThermoFisher), supplemented with L-glutamine (2 mM), 100 μg/mL penicillin, 100 U/mL streptomycin and 50 mg/mL primocin, in a humidified 5% CO_2 atmosphere at 37°C. Cells were seeded in 96-well plates at a density of 2×10^5 cells/mL.

Both confluent huRBL-2H3 and LUVA cells were then sensitized with (50% v/v) individual sera from 16 patients allergic to AX or cefuroxime (Tables S2 and S3) and 10 tolerant controls for 48 h at 37°C. Unsensitized cells were used as negative controls. Following sensitization, cells were stimulated with DeAns at different concentrations of AXO units (10, 20, 50, and 100 μM) for 2 h. In parallel experiments, cells were treated with the corresponding blank dendrimers (PAMAM lacking AXO functionalization) as negative controls. The HSA-AXO conjugate (10 μM AXO) served as the positive control, while ionomycin (10 μM) and C48/80 (50 μg/mL) were used as positive controls of non-IgE mediated degranulation.

In the three cell models, cell degranulation was assessed by measuring the release of β-hexosaminidase in the supernatants [41], after incubation with the substrate solution and addition of stop solution, through measuring absorption at 405 nm in an ELISA reader [41]. Data were expressed as a percentage of the total cellular β-hexosaminidase content, which was determined after cell lysis.

The effects of DeAns on cell viability were determined by a non-radioactive cell toxicity test, as described in Supporting Information: Methods section.

3 | Results

3.1 | Dendrimeric Antigen Nanostructures

Multivalent nanostructures with precisely controlled chemical architecture, termed DeAns, were developed as platforms for evaluating effector cell activation in the context of AX allergy (Figure 2). The DeAns design incorporates two main features that are systematically modified as each generation (G) grows: (i) a controlled, linear increase in the size of the conjugates, and (ii) an exponential increment in the number of antigenic determinants (AXO) in their periphery.

Five generations (G1–G5) of PAMAM dendrimers, built upon an ethylenediamine core, were strategically employed as starting multivalent scaffolds, selected for their abundant peripheral primary amino groups, which at basic pH facilitate covalent conjugation of AX, resulting in dendrimers decorated with AXO moieties. The structures of the resulting conjugates were elucidated by various NMR techniques, and were in agreement with previous descriptions [28, 31]. ^{1}H-NMR spectra displayed a single set of signals corresponding to the AXO determinants, with integrations consistent with the expected number of AXO moieties per DeAn generation. In the dendrimer scaffold, the shift of the methylene protons adjacent to the terminal amino groups to 3.22 ppm in ^{1}H-NMR, together with the disappearance of the 41.7 and 39.9 ppm signals in ^{13}C-NMR corresponding to the peripheral ethylene carbons of native PAMAM, confirmed the complete functionalization with AXO (Figures S3–S8). The increase in molecular weight of the DeAn conjugates relative to the starting dendrimers scaffold was confirmed using GPC, and corroborated by reduced diffusion coefficients with larger generation structures (Figure 2). The resulting DeAns conjugates (Gn-AXO, $n = 1$–5) showed a controlled and linear expansion in size. Simultaneously, the terminal groups proliferate exponentially, from 8 (G1) to 128 (G5) [42], thereby augmenting both the multivalency and antigenic density of the constructs.

3.2 | IgE Recognition of DeAns

A competitive RAST inhibition assay was performed to evaluate the recognition of the nanostructures by AX-sIgE present in a pool of sera from allergic patients to AX (Table S1). In this immunoassay, DeAns were used as inhibitors in the fluid phase at three different concentrations, calculated in terms of AXO moieties, against AXO-PLL cellulose solid phase. AXO-Bu monomeric conjugate was included as a control inhibitor. The results demonstrated concentration-dependent recognition across all conjugates, both DeAns and AXO-Bu (Figure 3). All conjugates were effectively recognized by AX-sIgE at the two higher concentrations (60 and 30 mM of AXO moieties), while only the AXO-Bu monomer and the smaller DeAn, G1-AXO, exhibited significant inhibition (> 50%) at the lower concentration (6 mM). This suggests that larger molecules, despite bearing more AXO moieties, experience greater steric hindrance, which could impair efficient IgE recognition.

3.3 | Effects of DeAns on Degranulation of Mouse Bone Marrow-Derived MCs via IgE Activation

The ability of nanostructures to induce IgE-dependent degranulation in mouse bone marrow-derived MCs was evaluated. Cells were first sensitized with mouse anti-AX IgE MoAb (AO6.2 specific to the AX side chain), then incubated with the series of DeAns at different concentrations of AXO units, and the resulting β-hexosaminidase release was measured. The HSA-AXO control (10 μM AXO moieties) elicited up to 23% β-hexosaminidase

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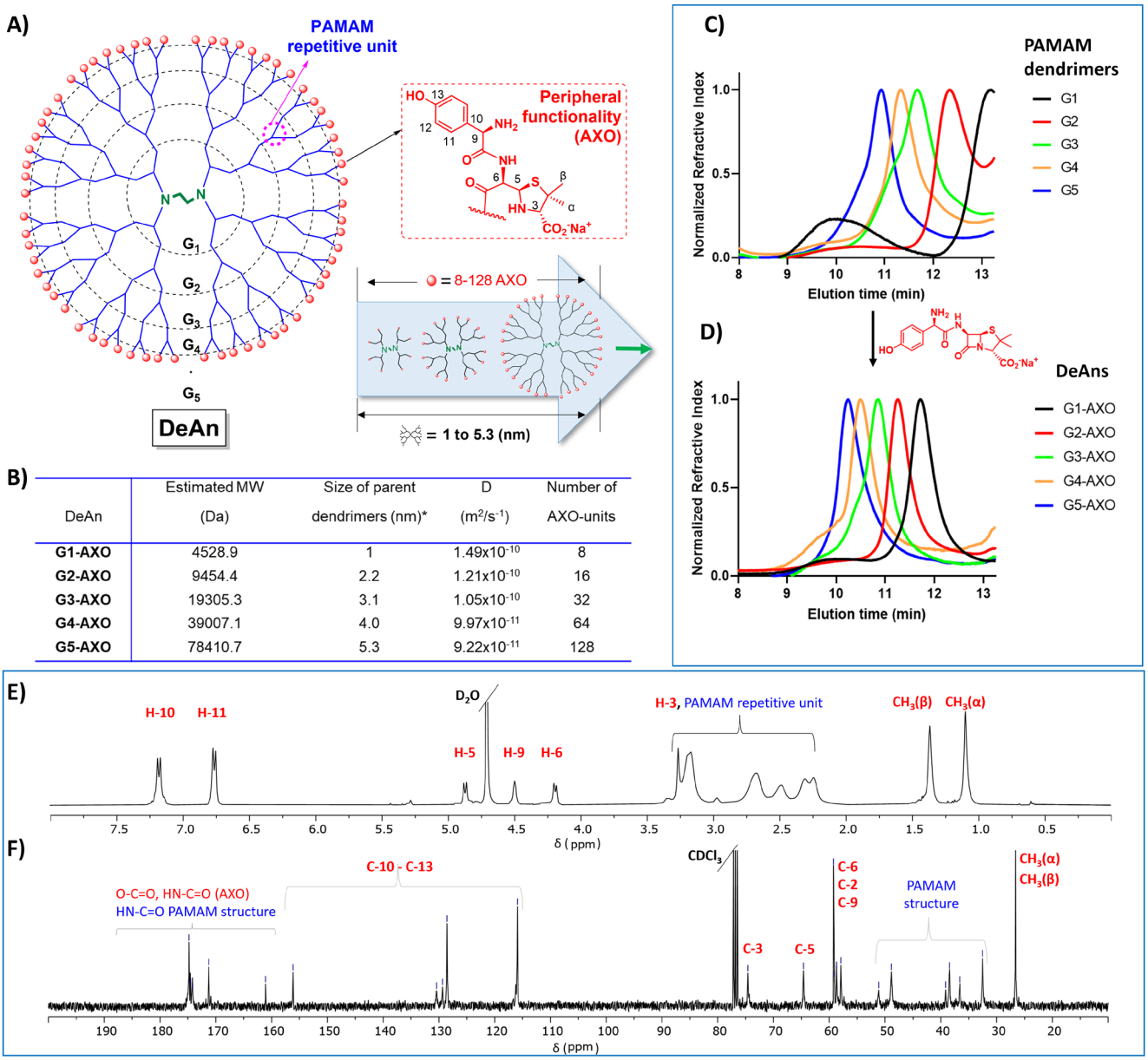

B)

DeAn	Estimated MW (Da)	Size of parent dendrimers (nm)*	D (m^2/s^{-1})	Number of AXO-units
G1-AXO	4528.9	1	$1.49x10^{-10}$	8
G2-AXO	9454.4	2.2	$1.21x10^{-10}$	16
G3-AXO	19305.3	3.1	$1.05x10^{-10}$	32
G4-AXO	39007.1	4.0	$9.97x10^{-11}$	64
G5-AXO	78410.7	5.3	$9.22x10^{-11}$	128

FIGURE 2 | Structural information of Dendrimeric Antigens (DeAn, Gn-AXO) and GPC analysis. (A) Schematic representation of structures with size variation depending on the dendrimer generation (G1 to G5) in DeAn (with valency ranging from 8 to 128 AXO); (B) the descriptive data of all synthetic DeAns are indicated in table inset, molecular weight (MW), estimated size of parent dendrimer structure, diffusion coefficient (*D*) and the number of AXO units on the periphery of DeAns; (C) the GPC traces of the parent unfunctionalized dendrimers, without AXO; (D) the GPC traces of DeAns after AX conjugation (E) ^{1}H NMR in D_2O of G5-AXO; (F) ^{13}C NMR in D_2O ($CDCl_3$ internal reference) of G5-AXO.

release. Lower-generation conjugates (G1-AXO with 8 AXO and G2-AXO with 16 AXO) did not induce degranulation, while higher-generation DeAns G3-AXO (from 5 to 100 μM) and G4-AXO and G5-AXO (across all tested concentrations from 1 to 100 μM) resulted in significant β-hexosaminidase release when compared with PBS and HSA negative controls ($p < 0.0001$), with a β-hexosaminidase release percentage comparable (no statistical differences) to the HSA-AXO treatment group (Figure 4). The degranulation observed with higher-generation DeAns is likely due to increased size, which facilitates effective crosslinking of IgE receptors, along with enhanced multivalent antigen presentation. No degranulation was observed in sensitized cells treated with native PAMAM dendrimers (without the drug), evaluated as blank structures. Importantly, no degranulation occurred in unsensitized cells treated with DeAns, confirming that activation was exclusively mediated by a specific interaction of the DeAns with anti-AX IgE.

3.4 | Cytotoxicity Assay

The impact of DeAns on the viability of huRBL-2H3 and LUVA cells revealed that survival rates consistently exceeded 95% across all tested concentrations (Figures S1 and S2). These results suggest that DeAns do not significantly affect the cell viability of either cell type, suggesting that the tested nanostructures do not display overt cytotoxic effects under the evaluated conditions.

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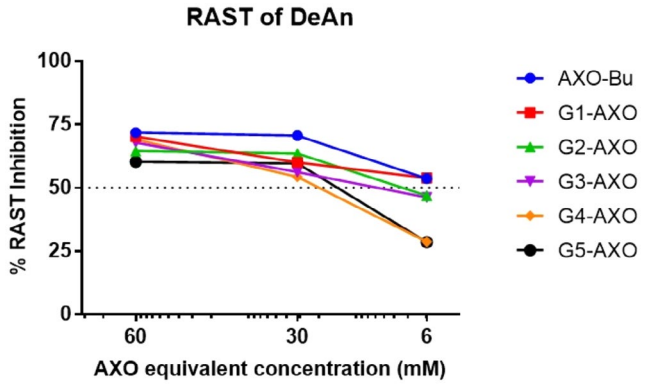

FIGURE 3 | RAST inhibition assays performed with a pool of sera from patients allergic to AX. Cellulose discs coated with AXO-PLL were used as the solid phase. The series of DeAns (Gn-AXO, $n = 1–5$) and AXO-Bu conjugate were used as inhibitors. Specific IgE recognition is considered significant when inhibition is ≥ 50%.

3.5 | Effects of DeAns on Degranulation in huRBL-2H3 and LUVA Cells Sensitized With Sera From β-Lactam Allergic Patients

HuRBL-2H3 cells, which share characteristics of both MCs and basophils [43], together with LUVA cells [44] (immortalized human MCs) expressing high levels of FcεRI receptors, were passively sensitized with sera from three groups of subjects: 14 allergic to AX (Table S2), 2 allergic to cefuroxime (Table S3), and 10 tolerant to β-lactams. These selected serum samples showed a wide range of total IgE levels, measured using the ImmunoCAP, from as low as 12 to ≥ 5000 kU/L.

In huRBL-2H3 cells, no degranulation was observed following stimulation with blank dendrimers or after sensitization with sera from tolerant subjects or cefuroxime-allergic patients (Figure 5A). In contrast, cells sensitized with sera from patients allergic to AX exhibited significant degranulation upon

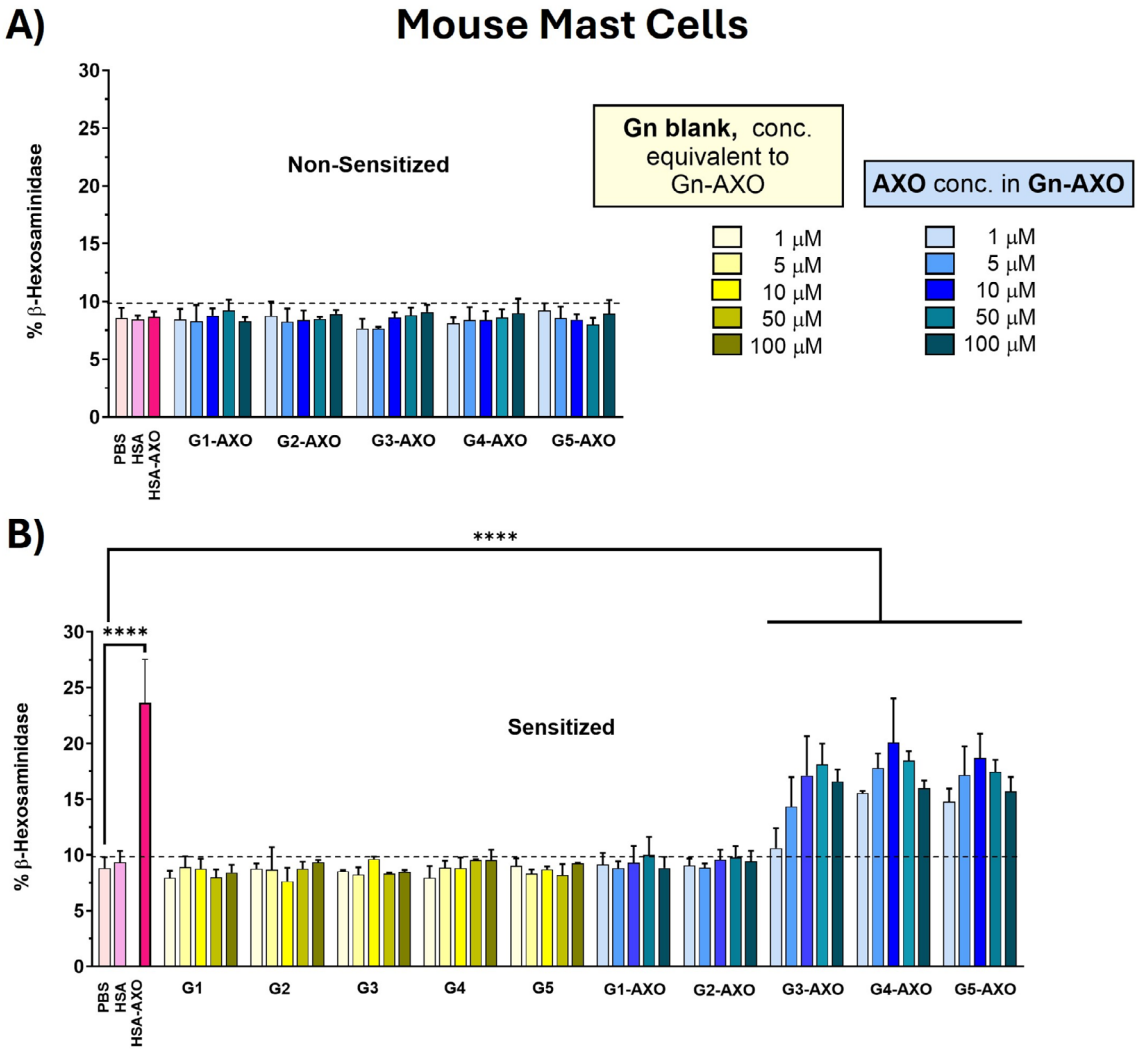

FIGURE 4 | Degranulation assays after incubating mouse MCs with a series of DeAns (blue) at concentrations of 1, 5, 10, 50, and 100 μM of AXO equivalents and the equivalent concentrations of native PAMAM dendrimers (yellow) as blank structures. PBS and HSA served as negative controls, while HSA-AXO at 10 μM served as positive controls. (A) Percentage of β-hexosaminidase release in unsensitized bone marrow-derived MCs. (B) Percentage of β-hexosaminidase release in sensitized bone marrow-derived MCs with AX-MoAb. The data are expressed as means ± SD ($n = 3$), with the baseline percentage of β-hexosaminidase release indicated by the dotted horizontal line. Statistical analysis by two-way ANOVA with Dunnett correction for multiple comparisons. ****$p < 0.0001$.

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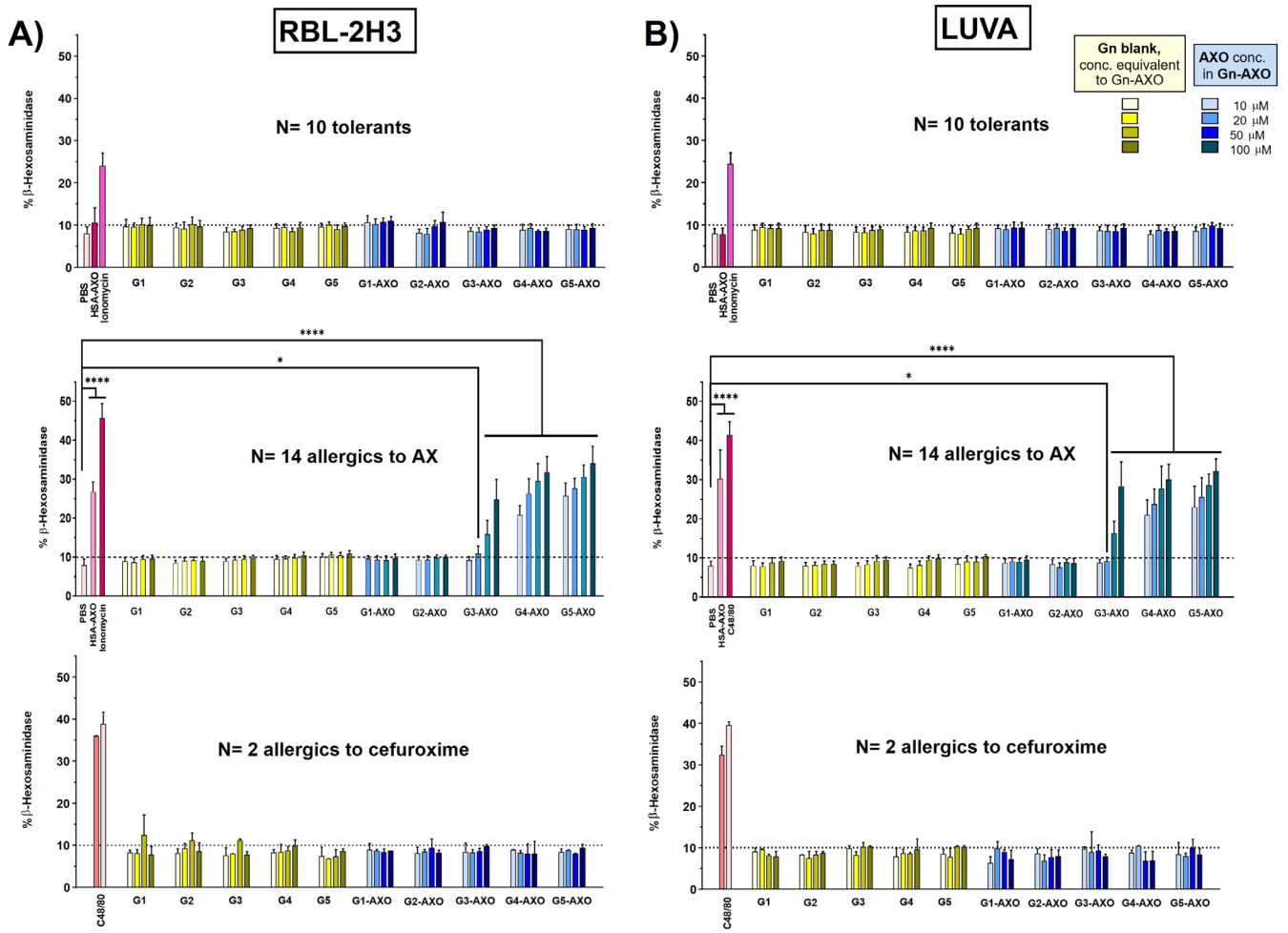

FIGURE 5 | Degranulation cell assays in response to DeAns at various concentrations (10, 20, 50, and 100 μM of AXO, shown in blue) compared to the equivalent concentrations of free dendrimers structures (blank, shown in yellow). HuRBL-2H3 and LUVA cells were primed with sera from patients allergic to AX, patients allergic to cefuroxime, and tolerant subjects as controls. (A) Degranulation study using huRBL-2H3 cells primed with patient sera and exposed to DeAns and PAMAM dendrimer at different concentrations. (B) Degranulation study using LUVA cells primed with patient sera and exposed to DeAns and PAMAM dendrimer at different concentrations. PBS was used as a negative control, while HSA-AXO served as a positive control. Additionally, ionomycin and C48/80 were to induce maximum degranulation. The data are expressed as means ± SD, with the baseline percentage of β-hexosaminidase release indicated by the dotted horizontal line. Statistical analysis by two-way ANOVA with Dunnett correction for multiple comparisons. *$p < 0.05$, ****$p < 0.0001$.

challenge with higher-generation DeAns. Specifically, G3-AXO induced detectable activity starting at 50 μM, whereas G4-AXO and G5-AXO triggered robust degranulation responses across all tested concentrations ($p < 0.0001$ vs. PBS negative control). Notably, the degree of degranulation induced by G4-AXO (containing 64 AXO units) and G5-AXO (with 128 AXO units), although lower than those observed with the HSA-AXO control, did not differ significantly (Figure 5A).

To further investigate the proof-of-concept application of DeAns in pMAT for AX allergy, complementary studies were conducted employing LUVA cells. Consistent with the huRBL-2H3 assay findings, no degranulation was observed in cells challenged with blank dendrimers or sensitized with sera from tolerant subjects or cefuroxime-allergic patients. Interestingly, LUVA cells primed with sera from AX-allergic patients showed marked degranulation in response to higher-generation DeAns. G3-AXO displayed activity from a 50 μM concentration, while both G4-AXO and G5-AXO consistently induced strong degranulation responses across all concentrations tested ($p < 0.0001$ compared to PBS negative control; Figure 5B). Importantly, the degranulation responses induced by the higher-generation DeAns (G4-AXO and G5-AXO) were comparable to those elicited by the HSA-AXO positive control (Figure 5B).

It is noteworthy that, in both cell systems, all 14 individual AX-allergic sera consistently tested positive for the same larger DeAn structures, whereas all sera from tolerant patients tested negative. Importantly, no discrepancies were detected between the cell models employed.

4 | Discussion

In this study, we evaluated a series of DeAns, monodisperse nanostructures with defined size that increase progressively with dendrimer generation, designed for multivalent and uniform presentation of AXO antigenic determinants, to assess

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in vitro IgE-mediated immune recognition in the context of AX allergy. Compared to conventional AXO-protein (HSA) [27] or AXO-polymer [45] (PLL) conjugates (Figure 1), DeAns offer precisely defined valency and nanoscale architecture, ensuring batch-to-batch consistency. This reliable platform enables robust effector cell assessment using patient sera and commercially available cell lines, providing a closer approximation to in vivo conditions and supporting more accurate clinical inferences in AX allergy studies.

The results of the RAST inhibition assays provide clear evidence that DeAns are recognized by AX-sIgE from allergic patients in a concentration-dependent manner (expressed in AXO units), consistent with earlier findings for similar DeAns containing different penicillin determinants [30, 31], further validating their function as synthetic allergen mimetics. At high concentrations of AXO equivalents (60–30 mM) present in DeAns conjugates and AXO-Bu, results in the inhibition assay were the same (Figure 3). Little differences can be observed only at low AXO concentration (6 mM) with higher generations, G4-AXO and G5-AXO showing less recognition.

The differences can be explained by steric hindrance rather than the affinity. In fact, the intrinsic affinity of individual AXO determinants is expected to be identical across generations. Differences in size and epitope spatial arrangement may affect steric hindrance, and therefore modulate IgE accessibility. Similar observations regarding the predominance of ligand spatial arrangement over valency have been reported for the biological activity of glycan-PAMAM dendrimer multivalent nanostructures [46]. Such behavior contrasts with the well-accepted paradigm that multivalent affinity generally rises with ligand valency, as shown in systems where valency critically influences biological efficacy [47, 48].

Degranulation assays across all cell models consistently revealed that higher-generation DeAns (G4-AXO and G5-AXO) induced robust, dose-dependent β-hexosaminidase release, highlighting the importance of antigen size and multivalency for effective FcεRI crosslinking and subsequent effector cell activation, as demonstrated in our previous study [5]. However, distinct differences were observed in how individual cell models responded at higher antigen doses. Specifically, a biphasic, non-linear response was uniquely noted in mouse MCs primed with mouse anti-AX IgE MoAb. In these cells, degranulation peaked at 10 μM and declined at higher concentrations (50 and 100 μM of AXO equivalents) with higher-generation DeAns, suggesting feedback inhibition. This saturation effect aligns with known immunological mechanisms where excessive allergen can reduce crosslinking efficiency via low-avidity monovalent interactions [49, 50]. In contrast, both cells containing human FcεRI, huRBL-2H3 and LUVA cells, demonstrated sustained, dose-dependent activation across all tested concentrations of G4-AXO and G5-AXO. Notably, only cells sensitized with sera from AX-allergic patients responded, while those from cefuroxime-allergic (β-lactam with no similarities at the R1 side chain with AX) or tolerant individuals showed no activation, confirming the specific recognition of DeAns by these IgE antibodies. HuRBL-2H3 cells, while widely used for IgE degranulation studies, may differ from human MCs in sensitivity and receptor expression. LUVA cells, therefore, provided a valuable complementary model due to their human origin, higher cell homogeneity and ease of culture [51]. In both systems, bigger DeAns bearing higher AXO valencies induced degranulation levels comparable to the HSA-AXO positive control, with approximately 30% β-hexosaminidase release, significantly exceeding those observed in all negative controls. Although G4-AXO and G5-AXO induced cell activation levels comparable to HSA-AXO, the latter consistently yielded slightly higher responses, likely due to the greater accessibility of AXO residues on HSA-AXO, which may facilitate crosslinking of more IgE molecules per conjugate. However, the inherent heterogeneity and undefined epitope distribution of HSA-AXO limit its reproducibility. In contrast, DeAns provide precise and reproducible control over size, valency, and epitope arrangement, making them more suitable for standardizing pMAT conditions.

This convergence of results across all models supports the hypothesis that high valency and optimal nanoscale geometry of DeAns enhance IgE crosslinking and downstream signaling [47]. Moreover, the absence of degranulation in cells treated with blank dendrimers underscores the strict IgE dependence of the observed activation.

Although the cellular assays may appear to contradict the RAST inhibition results regarding the fact that higher valency does not always lead to improved recognition, it is important to consider that these approaches assess fundamentally different outcomes. In cell-based assays, the readout depends not only on the intrinsic hapten–antibody interaction but also on the geometrical constraints required to achieve efficient IgE cross-linking on the cell surface.

Importantly, comparative structure–function analysis highlights that not only antigen valency but also spatial arrangement is critical. While in a previous work we reported that BiAns containing 16 AXO units triggered activation when appropriately spaced via flexible polymers, herein G2-AXO DeAn, displaying the same number of AXO determinants (Figure 1), failed to activate any effector cells [5]. Neither G1-AXO (8 units) nor G2-AXO (16 units) induced degranulation, likely due to insufficient distance to have a crosslinking capacity. This could stem from conformational constraints or intramolecular geometry that limits effective FcεRI clustering. While intramolecular IgE crosslinking has been postulated as a possible inhibitory factor, the ~13 nm distance between IgE Fab arms likely exceeds the epitope spacing required for such interactions [52]. These structural considerations reinforce that activation depends on precise molecular architecture, not merely hapten count.

The marked differences in cellular activation observed between lower-generation (G1/G2) and higher-generation (G4/G5) DeAns demonstrate that scaffold size is a decisive determinant for FcεRI-mediated activation. Lower-generation DeAns, despite carrying AX residues, consistently failed to induce detectable degranulation, indicating that their limited size and surface area do not permit the spatial arrangement necessary to achieve productive IgE crosslinking. In contrast, higher-generation dendrimers provide a sufficiently large and multivalent platform to support effective receptor clustering. Although steric crowding in G4/G5 may restrict the

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simultaneous accessibility of all AX moieties, their overall dimensions and epitope density still allow efficient crosslinking, resulting in robust activation. Thus, scaffold size emerges as the primary factor governing DeAn-induced activation, with valency and steric accessibility contributing to secondary, fine-tuning effects.

Overall, these results demonstrate that DeAns can serve as highly controlled, bioactive constructs for dissecting the structural determinants of IgE-mediated effector cell activation, with significant implications for improving diagnostic precision in AX allergy. By using a combination of mouse MCs, RBL-2H3, and LUVA cells, we were able to assess the potential of DeAns to mimic allergenic AX-protein conjugates and induce effector cell degranulation. The comparative analysis of these cell models highlights important aspects of antigen structure–activity relationships and provides insights into the translational potential of DeAns for in vitro diagnostic purposes. Moreover, the multivalent AXO units and optimal nanostructure size of DeAns are tailored to fulfill the complex cellular requirements necessary for effector cell activation.

Importantly, the recognition of DeAns was evaluated using human sera spanning a range of clinical profiles, including diverse in vivo and in vitro diagnostics outcomes, ensuring relevance to real-world diagnostic challenges (Tables S2 and S3). The results obtained in the cell-based assays were consistent across the 2 sera from cefuroxime-allergic patients and the 10 sera from β-lactam-tolerant individuals, with no false-positive results observed, demonstrating the high specificity of the diagnostic approach. Additionally, the cell assays showed high sensitivity, as degranulation was consistently observed across the 14 sera from AX-allergic patients, showing degranulation in response to the larger DeAns, G4-AXO and G5-AXO, which may be considered as the optimal stimulators (triggers). Among these patients, 11 out of 14 were confirmed as allergic to AX with in vivo tests (10 by ST and 1 by DPT). Considering the in vitro tests, 12 out of 14 were positive to some kind of AX immunoassay (RAST and/or ImmunoCAP) and only 5 out of 11 performing BAT were positive to AX. This highlights the added value of pMAT with DeAn nanostructures for diagnosing AX allergy, as it can serve as a reliable diagnostic tool in cases with discordant sIgE and ST results. The described approach proved utility even in patients with AX-sIgE levels below the threshold of positivity, or with very low amounts of total IgE.

The present study enhances the diagnostic performance previously reported for pMAT in the diagnosis of AX allergy, which used primary human donor MCs and native AX, and showed results similar to those obtained with BAT [26]. In contrast, our study, employing humanized MC lines in combination with DeAn nanostructures, achieved positive pMAT results in all 14 allergic patients, thereby surpassing the diagnostic performance of BAT, which resulted positive in only 5 out of 11 cases. However, since the sample size is limited and BAT was not performed in all cases, these diagnostic conclusions are preliminary and require confirmation in larger patient cohorts. Similarly, previous proof-of-concept studies have shown that the incorporation of DeAn into BAT improves assay sensitivity compared to testing with the free drug (BP or AX), and that stimulation is more pronounced with higher DeAns generations [28], although further research including a higher number of patients are needed to elucidate the role of DeAn in BAT.

In relation to pMAT applied to other drugs, to date, only a few exceptions using native drugs (chlorhexidine [21–23], rocuronium [24]) or nanostructures (liposome-based synthetic allergen platform—nanoallergens—in the case of platins [25], BiAns [Figure 1]—spaced with a flexible linker in the case of AX [5]) have been successful in the diagnosis of IgE-mediated drug allergies. However, due to reported high sensitivity and specificity applied to food allergy [53], more studies focused on drugs are currently underway (such as in neuromuscular blocking agents) [54].

Taken together, these findings highlight key factors governing immune recognition of synthetic allergens and demonstrate the potential of DeAn platform to address current diagnostic challenges in drug allergy. The complex parameters influencing synthetic allergen binding, including hapten density (valency) [55], epitope heterogeneity [4], epitope spatial geometry [56], and structural flexibility [55] or rigidity [57–59] are all well-recognized drivers of immune recognition. DeAn platform fulfills most of these criteria, offering a tunable and reproducible model for studying AX allergy at the cellular level. Accordingly, DeAns may represent a transformative advance in AX allergy in vitro testing by enabling rigorous, high-precision antigen design that enhances diagnostic accuracy. This overcomes the limitations of existing hapten-carrier systems, such as polydispersity and inconsistent antigen presentation, which hinder clinical interpretation.

In conclusion, our results demonstrate that the optimized size, multivalency, and structural accuracy of DeAns effectively trigger cellular responses, confirming their potential as a powerful platform in pMAT for in vitro diagnosis of AX allergy. Extending this strategy to additional β-lactams constitutes an important next step to determine whether the diagnostic advantages observed for AX can be replicated in other β-lactam antibiotics, thus evaluating its broader applicability.

From a translational perspective, the use of DeAns in pMAT would offer potential advantages for routine diagnostic settings. Unlike BAT, pMAT does not require fresh blood samples and avoids the issue of non-releaser basophils, which could streamline clinical workflows and reduce repeat testing. Although the synthesis of DeAns is initially more complex than that of conventional hapten–protein conjugates, DeAns are monodisperse, highly stable, and batch-consistent, enabling their production as standardized reagents. This could ultimately lower per-test costs by minimizing variability and the need for repeated quality controls, thereby favoring the reproducibility of cell assays. Furthermore, the robust and specific activation observed with higher-generation DeAns suggests that pMAT based on these nanostructures could reduce the reliance on drug provocation tests, thereby decreasing healthcare costs and improving patient safety. Overall, the implementation of standardized DeAn-based pMAT has the potential to be both clinically applicable and cost-effective in routine allergy diagnostics to AX.

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Author Contributions

C.M., A.R.-N., M.J.T., and M.I.M. designed the study. M.S., G.B., and M.J.T. were responsible for subjects' data collection. A.T.A. performed chemical synthesis with input from E.P.-I., J.L.P., and M.I.M. S.B., M.J.R.-S., M.J.R.-S., and A.R.-N. performed cell assays. J.L.P. and A.R.-N. contributed to the statistical analysis. I.M.J.-S. and A.T.A. performed RAST assays and monoclonal antibody purification. A.T.A., M.S., S.B., M.J.R.-S., I.M.J.-S., G.B., E.P.-I., C.M., J.L.P., A.R.-N., M.J.T., and M.I.M. contributed to the interpretation of the data. A.T.A. and M.I.M. wrote the manuscript, with critical input of C.M., J.L.P., and A.R.-N. All authors critically revised the manuscript and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that supports the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** all70250-sup-0001-Supinfo.pdf.

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