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A Cell-Free Protein Synthesis Platform to Produce a Clinically Relevant Allergen Panel

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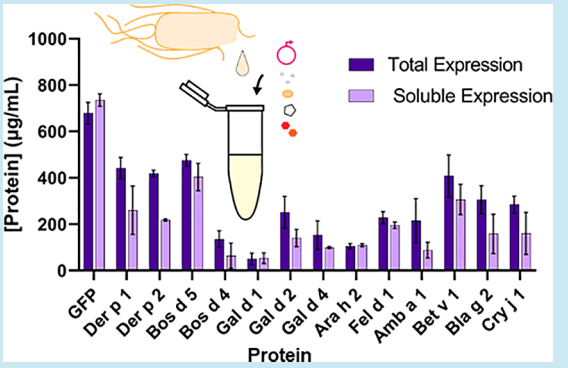
ABSTRACT: Allergens are used in the clinical diagnosis (e.g., skin tests) and treatment (e.g., immunotherapy) of allergic diseases. With growing interest in molecular allergy diagnostics and precision therapies, new tools are needed for producing allergen-based reagents. As a step to address this need, we demonstrate a cell-free protein synthesis approach for allergen production of a clinically relevant allergen panel composed of common allergens spanning a wide range of phylogenetic kingdoms. We show that allergens produced with this approach can be recognized by allergen-specific immunoglobulin E (IgE), either monoclonals or in patient sera. We also show that a cell-free expressed allergen can activate human cells such as peripheral blood basophils and CD34+ progenitor-derived mast cells in an IgE-dependent manner. We anticipate that this cell-free platform for allergen production will enable diagnostic and therapeutic technologies, providing useful tools and treatments for both the allergist and allergic patient.



KEYWORDS: *cell-free protein synthesis, allergen, IgE recognition, mast cells, basophils*

INTRODUCTION

The allergic response requires the presence of three key players: production by plasma cells of allergen-specific immunoglobulin E (IgE), allergen-responsive cells by virtue of their surface expression of IgE receptors, and an allergen. The immediate allergic response is mediated by mast cells and basophils. Mast cells are tissue-resident cells, while basophils circulate in the blood. One of the distinguishing features of both mast cells and basophils is the presence of metachromatic-staining granules, which contain several of the preformed mediators that drive the allergic response. Upon activation, these cells release granule-stored and newly generated mediators such as histamine, prostaglandins, leukotrienes, proteases, cytokines, and other substances that cause inflammation, vasodilation, bronchoconstriction, diarrhea, rhinorrhea, sneezing, hives, and itch observed during an allergic response. Both mast cells and basophils express high-affinity IgE receptors on their surface (FcεRI) whose α-chain engages with unique sequences in the Fc region of IgE antibodies. An allergic patient will have allergen-specific IgE antibodies occupying these surface FcεRI. Allergen exposure to these cell surface IgE antibodies will induce receptor cross-linking, initiating a calcium- and kinase-dependent activating cascade through the FcεRI's immunoreceptor tyrosine-based activating motifs (ITAMs). This activating cascade leads to the fusion of preformed granules with the outer membrane of mast cells and basophils and the release of the mediators they contain in a process called degranulation.

In the clinic, immediate hypersensitivity allergy is diagnosed by the combination of clinical history with the detection of allergen-specific IgE using a variety of allergen-based reagents.[1] By performing skin testing, a positive test result can be determined from mast cell activation that results in the generation of a histamine-dependent wheal and flare response. Extracts from allergen-producing organisms, plants, foods, and drugs are introduced into the skin to screen for the development of localized allergic reactions. Purified and recombinant allergens are used in lab tests of patient sera to quantify allergen-specific IgE in a manner that also aids diagnosis and prognosis. Therapeutically, allergen extracts are used to desensitize the patient to the allergen with repeated exposures over time given orally or in "allergy shots". More recently, allergen extracts have been formulated as supplements[2] to aid in early oral introduction of food allergens, which have been shown to greatly reduce the risk of the development of food allergy.[3,4]

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New tools are needed to produce allergen-based reagents. The current state-of-the-art relies on allergen extract preparations. Allergen extracts are made from raw material (e.g., dust mite feces and cat dander), isolating various allergen proteins and suspending the mixture in solution. As a result, allergen extracts are relatively crude protein mixtures. Only two allergens are standardized by a specific protein (Fel d 1 for cat and Amb a 1 for short ragweed), and most others are standardized based on crude protein nitrogen content (PNU) or by arbitrary biological activity units (BAUs). Thus, allergen extracts often contain variable amounts of allergen from batch-to-batch and in ratios that are highly dependent upon the organism's expression.[5]

There has been a recent push in the field of allergy toward molecular diagnosis and treatment, as well as precision allergology, where patients are diagnosed based on the precise allergens to which they are sensitized and treated with personalized immunotherapies containing only those allergens.[6−8] Such an approach is not possible with existing allergen extract preparation techniques. A better approach would enable production of individual allergens with the opportunity to mix allergens in a personalized cocktail based on the patient's sensitivity to provide a diagnostic or treatment reagent that is tailored to each patient. An even more attractive approach would enable this type of preparation at the point-of-care. Though not the state-of-the-art, recombinant approaches for producing allergens could conceivably achieve production of individual allergens and could do so with more rigorous quality control from batch-to-batch than for traditional allergen extract preparations (e.g., based on precise allergen quantity rather than arbitrary PNUs and BAUs). However, some allergens can be cytotoxic and not amenable to production in traditional cell-based platforms. This has contributed to the slowed rate at which allergen and allergen derivatives can be produced and characterized using cell-based recombinant methods.[6] Also, cell-based recombinant methods are difficult to transfer to point-of-care manufacturing.

Here, we describe a cell-free platform for producing recombinant allergens that overcomes the shortcomings of traditional allergen extract preparations and endows several favorable features beyond the capabilities of cell-based recombinant methods. Cell-free protein synthesis (CFPS)[9−11] is a technique that has been applied to protein biologic discovery,[12,13] glycoprotein synthesis,[14−19] point-of-care manufacturing,[20−25] distribution of medicines to resource-limited settings,[26,27] small molecules,[28−30] diagnostics,[31−33] and education.[34−36]

CFPS involves the isolation of transcription and translation machinery from lysed cells, such as those from *Escherichia coli*, and collection in a test tube, where supplementation of necessary building blocks, buffers, and cofactors in addition to plasmid encoding a protein of interest enables expression of desired proteins, obviating the need for live cells in culture.[37,38] This particular feature grants access to clinically relevant allergens that would otherwise be cytotoxic to common expression hosts. CFPS offers several other unique features including (i) an open reaction environment, which grants a level of control over reaction conditions difficult to achieve in a cell cytoplasm (e.g., to facilitate disulfide bonding), (ii) production of one protein per reaction, which enables diagnosis and treatment at the level of the allergen rather than the organism—a key tenet of precision allergology, and (iii) the opportunity to multiplex individual production of many components, which will better facilitate point-of-care formulation of personalized cocktails based on patient sensitization profiles. These features can also be leveraged in the laboratory to rapidly produce several allergens or allergen derivatives for high-throughput characterization of IgE-mediated responses that would enhance our understanding of allergy at a molecular level. Applying this CFPS platform toward the production of allergens makes these features available for the field and has the potential to accelerate innovation of diagnostic and therapeutic technologies in the allergy space.

RESULTS

Cell-Free Expression of a Clinically Relevant Allergen Panel. First, we wanted to test whether a bacterial, cell-free system would be amenable to expression of protein allergens from a wide range of taxonomic kingdoms, including insects, plants, and mammals (Table 1, Figure 1). We curated a list of common allergens implicated in food allergy and environmental allergy, and we aimed to select allergens with high rates of IgE positivity in sensitized patients. Our list comprises a combination of common indoor and outdoor aeroallergens and food allergens, including foods associated with a higher risk of anaphylaxis (Tables 1 and S1). Addition of ^{14}C-leucine into the CFPS reaction enabled quantification of the protein yield by detection of radioactive leucine incorporated into the final protein product (Figure 1A). Based on this quantification, all allergens expressed in our system, with an average total and soluble yield of 267 ± 132 μg/mL and 174 ± 98 μg/mL, respectively. Even those allergens expressed in the lowest yields would be amenable to production for downstream applications with simple scale-up (Figure 1B). A key feature of a cell-free approach is the opportunity to optimize reaction conditions for expression. We tested this potential using dust mite allergen Der p 2, which has three disulfide bonds, and observed that by altering the reaction environment to generate oxidizing

Table 1. Panel of Allergens Used in This Study: Selected Based on Clinical Relevance and Prevalence of IgE Reactivity and Includes Those Implicated in Food Allergy and Environmental Allergy

Allergen Panel	
Allergen	Source
Common Indoor Allergens	
Der p 1	Dust mite
Der p 2	Dust mite
Major Milk and Egg Allergens	
Bos d 5	Milk
Bos d 4	Milk
Gal d 1	Egg (ovomucoid)
Gal d 2	Egg (ovalbumin)
Gal d 4	Egg (lysozyme)
Major Peanut Allergen	
Ara h 2	Peanut
Other Common Aeroallergens	
Fel d 1	Cat (dander)
Amb a 1	Ragweed (pollen)
Bet v 1	Birch (pollen)
Bla g 2	Cockroach
Cry j 1	Japanese cedar (pollen)

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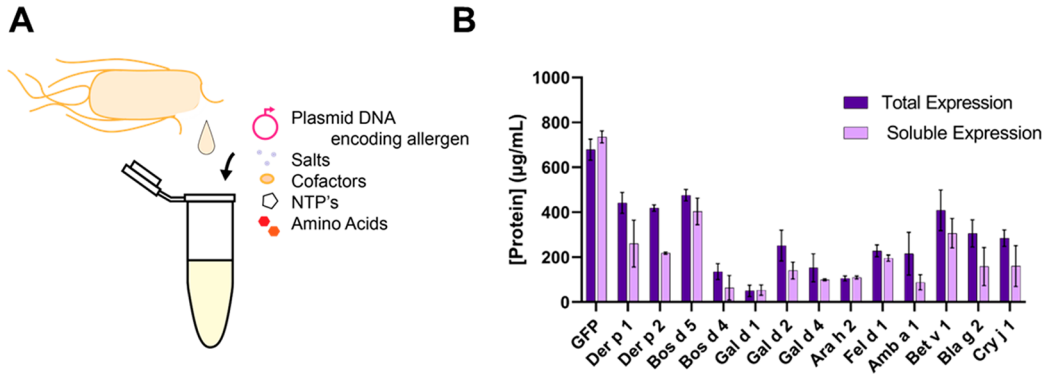

Figure 1. Cell-free expression of a clinically relevant allergen panel. (A) Cell-free protein synthesis (CFPS) involves isolating the transcription and translation machinery of *E. coli* cells post lysis in a test tube. Building blocks, cofactors, and plasmid DNA encoding the desired proteins for expression are supplemented. After protein expression occurs, the protein of interest can be isolated and used for subsequent purposes. (B) Expression of each allergen was quantified by incorporation of radioactive ^{14}C-leucine and prepared to measure both the total and soluble expression. Green fluorescent protein (GFP) is included as a reference point for high expression. All allergens were amenable to cell-free expression with relatively good solubility. Even those with lower yields (<100 μg/mL) are expressed at levels that could readily be used for clinically relevant applications after scale-up of the CFPS reaction (n = 3, error bars = standard deviation).

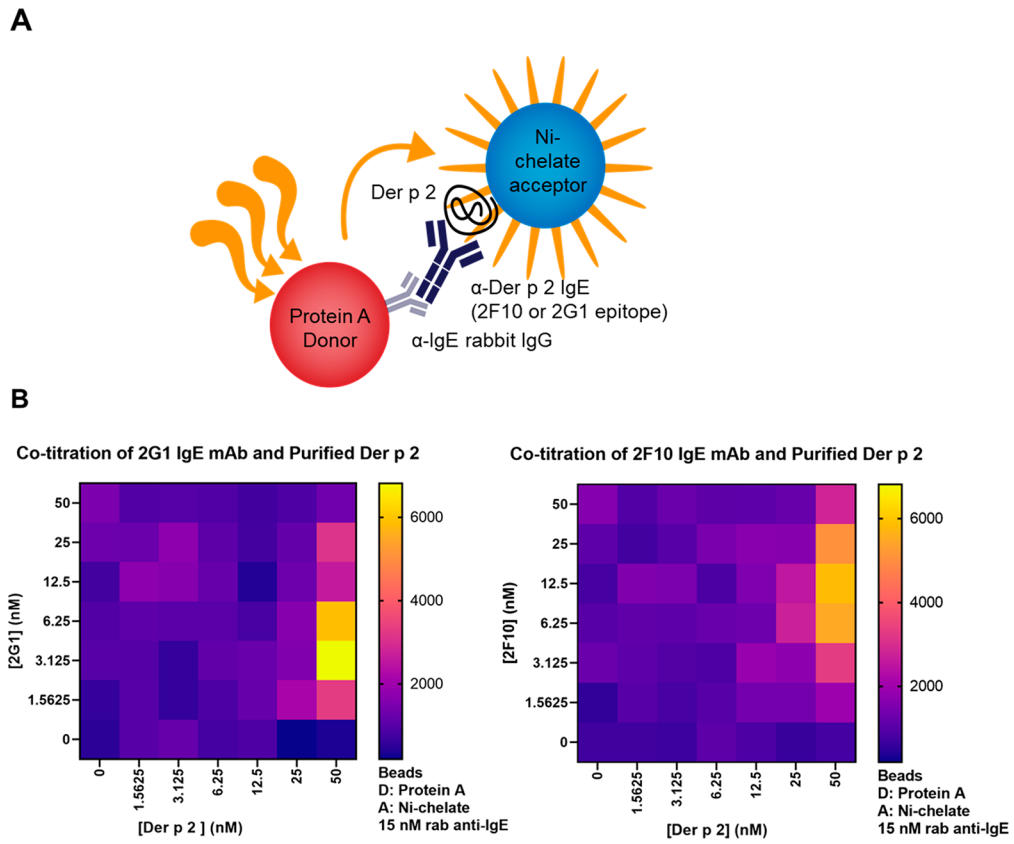

Figure 2. Purified CFPS-expressed Der p 2 is recognized by monoclonal IgE. (A) An AlphaLISA assay detects IgE binding of a purified dust mite allergen, Der p 2. The Protein A donor bead (D:) associates with α-IgE rabbit IgG, and a Ni-chelate acceptor bead (A:) associates with dust mite allergen Der p 2. If monoclonal 2G1 or 2F10 IgE recognizes and binds CFPS-expressed Der p 2, then it will bridge the two proteins and their associated beads, leading to an energy transfer between the beads once in close proximity, yielding an emission of light that can be read on a plate reader. (B) Titrating CFPS-expressed Der p 2 and each monoclonal IgE reveals a typical binding pattern, where yellow indicates a signal and purple indicates no signal (e.g., no binding). In the zero "0" condition (left most column and bottom most row), when either species is absent, there is no binding detected as expected. At an optimal concentration of 50 nM Der p 2 and 3−12 nM IgE, optimal bead interactions lead to the highest level of the signal, and the signal gradually decreases as the concentration moves away from those optimums. At higher concentrations, an excess free binding partner occupies binding sites and disrupts bead interaction attributable to the Hook effect. At lower concentrations, insufficient amounts of either binding partner prevent enough bead interactions to occur to elicit a visible signal. Data are representative of three independent experiments.

conditions, we gained higher levels of soluble expression (Figure S1A-B).

Recognition of CFPS-Expressed Der p 2 by Monoclonal Allergen-Specific IgE. After demonstrating soluble

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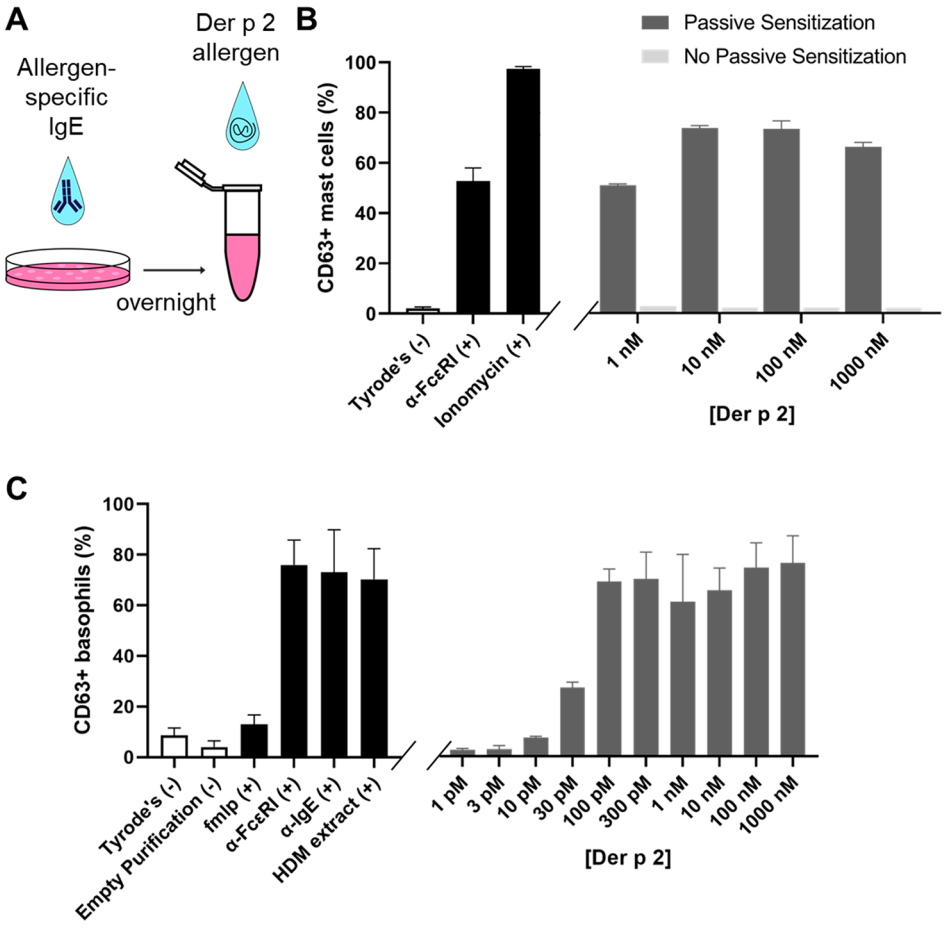

Figure 3. Der p 2 activation of human basophils and mast cells. (A) CD34+ progenitors are isolated from human peripheral blood and differentiated into primary human CD34+ progenitor-derived mast cells in culture with cytokines necessary for their selective differentiation. CD34+ culture-derived mast cells are then passively sensitized with serum from a Der p-sensitized donor (Table S2) and incubated with the Der p 2 allergen. (B) These mast cells respond to negative (−) and positive (+) controls as expected (n = 2, error bars = standard error) and respond to CFPS-expressed Der p 2 in a dose-dependent manner (n = 3, error bars = standard deviation), where 10 nM appears to be optimal for FcεRI cross-linking. Without passive sensitization, stimulation with the Der p 2 allergen results in background levels of activation, indicating that CFPS-expressed Der p 2 effects are IgE-mediated. (C) Peripheral blood human basophils from whole blood are tested for allergen reactivity. Patients sensitized to Der p 2 have Der p 2-specific IgE already occupying the FcεRI receptors on the surface of their basophils and will degranulate upon incubation with Der p 2. Degranulation can be identified and assessed with proper gating strategies without purification for enhanced levels of surface CD63 detected by flow cytometry. Peripheral blood human basophils respond to negative (−) and positive (+) controls as expected and degranulate upon Der p 2 exposure in a dose-dependent manner with >100 pM being the optimal concentration for cross-linking (n = 2 samples, 1 HDM-sensitized patient donor, error bars = standard error).

synthesis of numerous allergens, we next sought to determine whether the CFPS-expressed allergen retains its function insofar as being able to bind to IgE. As a model, we selected dust mite allergen Der p 2, one of the higher expressing allergens, because of its clinical relevance as one of the most common allergens. To detect binding of IgE to the allergen, we utilized an in-solution, bead-based ELISA assay called AlphaLISA. Here, a protein A coated donor bead associates with α-IgE rabbit IgG, and a nickel coated acceptor bead associates with the His-tag-purified Der p 2 allergen. If IgE successfully binds to Der p 2, then it will bridge the two species, bringing their associated beads in proximity. Once in proximity, an energy transfer reaction will take place between the donor and acceptor bead that produces an emission of light that can be read on a plate reader (Figure 2A).

After purification of the allergen (Figure S1B, lane 4), we performed a cotitration of IgE and purified CFPS-expressed Der p 2 with the AlphaLISA assay using two monoclonal IgE antibodies, 2G1 and 2F10, that each recognize a distinct epitope on opposite poles of the Der p 2 allergen.[39−41] On the final readout of an AlphaLISA assay, successful binding is indicated by an enhanced level of signal over background at some optimal concentration, with lower signal gradually fanning out as the concentration moves away from the optimum. A background signal is determined by the zero condition ("0") where either IgE (bottom row) or Der p 2 (left column) is absent. The pattern of signal in both cotitrations, and optimal binding at nM concentrations, is indicative of successful binding by both monoclonal IgE antibodies recognizing either the 2G1 or 2F10 epitope (Figure 2B). Of note, altering reaction conditions for expression of Der p 2 did not significantly alter IgE binding (Figure S1C).

To ensure that the AlphaLISA binding signal was allergen-specific and not confounded by possible contaminants in the purified reaction, we performed a similar cotitration where Der p 2 was modified with a trisaccharide to block the epitope.[42] Installing the trisaccharide abrogated IgE binding, indicating that binding only occurs when the Der p 2 epitope is present

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and accessible (Figure S2). This demonstrates that unmodified CFPS-expressed Der p 2 retains its ability to be recognized by IgE, a key interaction underpinning allergic responses and diagnostics.

Activation of Human Allergic Effector Cells by CFPS-Expressed Der p 2. To further confirm bioactivity, it is important to demonstrate that IgE binding and cross-linking of FcεRI translates to a cellular response against our CFPS-expressed allergen. To do this, we utilized two human allergic effector cell models involving culture-derived mast cells and primary blood basophils to assess activation and degranulation upon exposure to relevant concentrations of CFPS-expressed Der p 2. Degranulation can be assessed by detecting proteins associated with intracellular granules that appear on the cell surface during granule membrane fusion and mediator release. These surface activation markers, typically detected by flow cytometry, include CD63 for basophils and mast cells and CD107a (LAMP1) for mast cells.[43]

The first cell model utilizes human CD34+ progenitor cells isolated from peripheral blood to generate mast cells in culture. One advantage of this approach is that the mast cells will not have any prior IgE in their receptors, unlike primary mast cells isolated from human tissues. After initial inoculation, CD34+ cells turn into mast cells by culturing with mediators of mast cell differentiation and survival—recombinant human interleukin-3 (IL-3), recombinant human interleukin-6 (IL-6), and recombinant human stem cell factor—as described.[44] Over time, cells become larger with the appearance of intracellular granules and irregular borders characteristic of the mast cell morphology (Figure S3A). They now display surface expression of typical mast cell markers CD117/KIT, CD33, FcεRIα, Siglec-6, and Siglec-8 (Figure S3B) and are capable of degranulation, as assessed by the appearance of CD107a/LAMP1 and CD63 when stimulated with the α-FcεRI antibody or ionomycin, a bacterial-derived ionophore that triggers degranulation by raising intracellular $[Ca^{2+}]$ in an IgE-independent manner (Figure S4). At this stage in the culture, mast cells can be passively sensitized by incubation with either allergen-specific IgE monoclonals or in serum from a sensitized donor (Figure 3A). CD34+ progenitor-derived human mast cells were sensitized with human serum from a Der p-sensitized donor at a dilution ratio of 1:10 (Table S2).

Incubation with CFPS-expressed Der p 2 over a logarithmic range from 1 nM to 1 μM resulted in a positive response at all treatment concentrations with 10 nM being the optimal concentration for activation (Figure 3B). A positive response is observed at Der p 2 concentrations as low as 3 pM (Figure S5). One possible drawback of bacterial systems is the carryover of components, such as endotoxin, that may lead to non-IgE mediated activation. To assess this possibility, we quantified endotoxin in the purified allergen sample finding levels to be below the FDA limit (Figure S6). We also included a nonpassively sensitized control sample at each Der p 2 concentration (1 nM-1 μM). At these concentrations in the absence of IgE sensitization, we observed 3 ± 0.5% CD63 positivity, similar to levels of background activation. This demonstrates that the activating effect of CFPS-expressed Der p 2 is IgE-dependent and not confounded by components, which may be carried over from *E. coli* lysates from which Der p 2 was purified after expression.

The second cell model utilizes basophils in peripheral blood from a house dust mite (HDM)-sensitized donor. Anticoagulated whole blood is used in the FlowCAST Basophil Activation Test where allergen is added to the sample and basophils are gated on by both light scatter and the basophil-selective marker, CCR3. To assess activation, we determined levels of CD63. Under negative control buffer-only conditions, only 9 ± 3% basophils are CD63 positive. Stimulation with a purified sample where no Der p 2 was expressed yielded CD63 positivity in only 2 ± 6% of basophils, indicating no activation by carry over components from *E. coli* lysates. Exposure to an IgE-independent, bacterial tripeptide activator (fMLP) leads to CD63 positivity in 13 ± 4% of basophils. Incubation with α-FcεRIα antibody as one positive control leads to CD63 positivity in 76 ± 10% of basophils, while incubation with α-IgE antibody as a second positive control leads to CD63 positivity in 73 ± 17% of basophils. Finally, as hoped, basophils incubated with either HDM extract or CFPS-expressed Der p 2, the latter over a logarithmic range from 1 pM to 1 μM, showed marked CD63 positivity at all concentrations with >100 pM being optimal for FcεRI cross-linking. The half maximal CFPS-expressed Der p 2 dose is >30 pM and <100 pM. At 3 pM, CD63 positivity remains near background levels (Figure 3C).

The levels of human mast cell and human basophil activation at corresponding CFPS-expressed Der p 2 doses are comparable to previously published studies using traditional methods for allergen-based reagent preparation.[45,46] These data indicate that CFPS-expressed Der p 2 can activate human mast cells and basophils in a dose-dependent manner, which is a key feature of clinically useful allergen-based reagents.

DISCUSSION

Recombinant allergen is a useful tool in the clinical diagnosis and treatment of allergic disease.[5−7,47] CFPS-based production possesses several features that can be particularly useful for allergen production and can aid in the implementation of molecular diagnosis and treatment of allergy as well as precision allergology. These features include an open reaction environment, portability that could enable point-of-care use, single allergen production with direct control over dosing, and the ability to rapidly multiplex multiple protein allergens. In our development of a CFPS-based platform for biomanufacturing allergen, we observed soluble yields >100 μg/mL for 9 out of 13 tested allergens included in our panel and >50 μg/mL for all allergens. This provides a proof-of-concept that protein allergens are amenable to cell-free production even across a wide range of phyla.

Using Der p 2 as a model system, we show that the CFPS-expressed allergen retains its ability to be recognized by IgE and activate human basophils and mast cells *in vitro* in the picomolar range. More studies need to be done to confirm these important characteristics with other allergens.

Additional work may also be needed to overcome the presence of endotoxin in *E. coli* lysates for clinical applications. Since endotoxin paired with an allergen is a common approach for sensitization in allergy mouse models,[48,49] its presence would not be suitable for human use in immunotherapy or skin testing. Here, purification alone was able to achieve endotoxin levels of 0.16 EU per a typical immunotherapy dose, but ideal preparations would achieve undetectable levels (Figure S6). Optimization of endotoxin removal while minimizing protein loss could overcome this challenge. Also, this would not be a constraint for laboratory-based quantification of IgE nor for *in vitro* studies of allergen and immune reactions.

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There is a push in the field toward molecular diagnosis and treatment of allergy and precision allergology, and a CFPS-based approach for allergen production makes this more accessible than cell-based recombinant allergen methods alone. Ultimately, the cell-free platform for allergen production established here has the potential to not only facilitate improved molecular understanding of allergy but also open new capabilities and technologies for the clinical allergist and allergic patient.

MATERIALS AND METHODS

Plasmid Design and Synthesis. Allergen protein sequences were retrieved from Uniprot, synthetically modified, and then codon optimized for *E. coli* K12 strains into a DNA sequence (IDT Codon Optimization Tool). Additional synthetic sequences were added to optimize expression and enable affinity purification (Table S1). Inserts were synthesized into a pJL1 backbone at the NdeI and SalI restriction sites (Twist Biosciences).

Plasmid DNA used in CFPS reactions was purified from glycerol stocks provided by Twist Biosciences using a ZymoPURE Midi Kit (Zymo Research D4200).

Harvest and Processing of *E. coli* Lysate for CFPS. *E. coli* lysate was prepared using previously published methods[50] using engineered *E. coli* strain C321.ΔA.759, a highly productive MG1655-derived strain.[51] Briefly, *E. coli* cells were inoculated at OD 0.08 into 2xYTPG media (yeast extract 10 g/L, tryptone 16 g/L, NaCl 5 g/L, K_2HPO_4 7 g/L, KH_2PO_4 3 g/L, and glucose 18 g/L, pH = 7.2) in a 10 L fermentor (Sartorius Biostat C+) and grown at 34 °C with agitation (250 rpm). Cultures were induced for T7 expression at OD 0.6−0.8 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich I6758) and harvested at OD 2.8−3.0. Cells were pelleted in a chilled JLA 8.1 rotor (Beckman Avanti J-25I Refrigerated Centrifuge) for 5 min at 8,000*g* at 4 °C and distributed to 12 50 mL Falcon tubes. Cells were then washed three times with chilled S30 buffer (10 mM Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate) with centrifugation for 2 min at 10,000*g* at 4 °C in a Thermo Heraeus Multifuge X3R Centrifuge in between. Pellets were then weighed and flash frozen in liquid nitrogen. For lysis, thawed cells were resuspended in 0.8 mL/g wet cell mass S30 buffer and aliquoted in 1.4 mL increments. Cells were kept on ice and underwent sonication for 45 s on and 59 s off at 50% amplitude until 950 J was reached. The supernatant (820 μL) was collected and underwent a 1-h runoff reaction at 37 °C with agitation (250 rpm). Lysate was then centrifuged at 12,000*g* for 10 min at 4 °C, and the supernatant was collected (500 μL), aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C for downstream use.

Cell-Free Protein Synthesis of Allergen. CFPS reactions were assembled based on previously published methods.[50] In brief, reactions were assembled with the following reagents and concentrations: 6 mM magnesium glutamate; 10 mM ammonium glutamate; 130 mM potassium glutamate; 2.646 mM ATP; 1.874 mM each of GTP, UTP, and CTP; 0.075 mg/mL folinic acid; 0.376 mg/mL *E. coli* tRNA mixture from strain MRE600 (Roche Applied Science); 0.33 mM nicotinamide adenine dinucleotide (NAD); 0.27 mM coenzyme-A (CoA); 4 mM oxalic acid; 1 mM putrescine; 1.5 mM spermidine; 57 mM HEPES at pH = 7.2; 2 mM of each of the 20 standard amino acids; 30 mM phosphoenolpyruvate (PEP) (Roche Applied Science); 13.3 μg/mL plasmid encoding allergen in pJL1 backbone; and 27% v/v of *E. coli* crude lysate (prepared above). For oxidizing conditions, 4 mM oxidixed L-glutathione (GSSG), 1 mM reduced L-glutathione (GSH), and 3 μM DsbC (Enzo ALX-201-268-C100) were also added to the reaction, and the *E. coli* crude lysate was incubated with 14.3 μM iodoacetamide for 30 min before addition. Reagents were sourced from Sigma-Aldrich unless stated otherwise.

Radioactive Quantification of Allergen Expressed by Cell-Free Protein Synthesis. An expressed allergen was quantified by ^{14}C-leucine incorporation according to previously published methods.[52] In brief, 10.67 μM of ^{14}C-leucine was supplemented into assembled CFPS reactions described above. After 20 h of overnight expression, protein was precipitated by addition of 0.5 N KOH in a 1:1 ratio to samples containing total protein and samples containing soluble protein (supernatant remaining after centrifugation at 12,000*g* for 5 min). Samples were incubated at 37 °C for 20 min. Then, 4 μL of the sample was dispensed onto a Filtermat (PerkinElmer, 1450-421), duplicated on a second Filtermat, and allowed to dry. One Filtermat was then washed in 5% trichloroacetic acid (Sigma-Aldrich T6399) three times for 15 min each at 4 °C and allowed to dry. Scintillation wax was then applied to the Filtermat over a hot plate and cooled, and then the signal was read using a Microbeta2 Scintillation Counter. Protein expression was calculated using the following formula:

$$(((\text{Signal of sample on washed filtermat} - \text{signal of no DNA negative control sample})/\text{signal of sample on unwashed filtermat}) \times [^{14}\text{C-leucine } (\mu\text{M})] \times \text{molecular weight of protein (g/mol)})/(\#\,\text{leucine residues} \times 1000)$$

Purification of CFPS-Expressed Allergens. Allergen was suspended in Buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, pH = 8.0) and isolated by affinity-tag purification with Ni-NTA magnetic beads (Invitrogen Dynabeads His-tag Isolation and Pulldown, 10104D) according to the manufacturer's instructions, where beads were washed with Buffer 1 and allergen was eluted with 500 mM imidazole (Sigma-Aldrich I5513) in Buffer 1.

The elution was desalted using Zeba spin columns (Thermo Scientific 89883) according to the manufacturer's instructions and collected in nuclease free water (Ambion, AM9937).

Detection of IgE Binding by the AlphaLISA Assay. CFPS-expressed allergen concentration was measured on a nanodrop with Protein A280 based on extinction coefficients and molecular weights calculated using Expasy software. Allergen and IgE were diluted in AlphaLISA buffer (50 mM HEPES pH 7.4 with 150 mM NaCl, 0.015% v/v TritonX-100, and 1 g/L BSA), and a 2× serial dilution was prepared by adding half volume of the previous dilution to the next dilution and mixing by pipet in a serial fashion. AlphaLISA reactions were assembled by using an acoustic liquid handler (Echo 525). Protein components were distributed to a 384-well plate (PerkinElmer, 6008280) and allowed to incubate for at least 1 h. Bead components were then distributed according to the manufacturer's instructions and allowed to incubate for at least

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1 additional hour. A signal was read on a plate reader (Tecan Infinite M1000).

Glycosylation of the Der p 2 Allergen. Glycosylation was performed as previously described[42] to control for nonspecific IgE interactions.

Activation Test of Primary Human Basophils in Peripheral Blood. Venous blood was obtained from a sensitized donor after obtaining written informed consent for blood donation using an institutional review board-approved protocol at the Northwestern University Feinberg School of Medicine. Primary human basophils from whole blood were activated with the FlowCast Basophil Activation Test according to the manufacturer's instructions (Bühlmann Laboratories, FK-CCR-U). As positive controls, basophils in whole blood were stimulated with 5 μL of anti-IgE of a 0.5 mg/mL stock (BD 555894) and clinical-grade house dust mite extract at a 1:100 dilution.

Culture and Differentiation of Human Peripheral Blood CD34+ Progenitor Cells into Mast Cells. CD34+ progenitor cells from human peripheral blood were purchased from Stemcell Technologies and cultured as previously described.[44]

Passive Sensitization and Activation of Human CD34+ Progenitor-Derived Mast Cells. Cultured CD34+ progenitor-derived mast cells were incubated overnight with sensitized patient serum (Table S2) at a 1:10 dilution.

Mast cells were then collected and suspended in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 0.5 mg/mL bovine serum albumin (BSA), pH = 7.3). Allergen and control stimulants (10 μL of 10× stock) were distributed to 1.5 mL Eppendorf tubes, and 200k-250k cells (suspended in 90 μL Tyrode's buffer) were distributed to each stimulant sample. Cells were incubated with stimulant/allergen for 20 min at 37 °C and 170 rpm. Optimal positive control concentrations were determined to be 2 μM ionomycin (Thermo Scientific, J60628LB0) and 2 μL of a 50 mg/mL stock anti-FcεRIα (Biolegend, 334602) (Figure S4).

For flow cytometry, cells were washed in PBS and incubated at 4 °C for 30 min with live/dead staining (Invitrogen L23105) suspended in PBS according to the manufacturer's instructions. After washing with FACS buffer, cells were then blocked with α-human CD32 (Biolegend 334602) in FACS buffer (PBS, 2.5% BSA) according to the manufacturer's instructions (10 min incubation), then labeled with fluorescent antibodies FITC α-human FcεRIα (Biolegend 334608), APC α-human CD107a/LAMP1 (BD 641581), Bv421 α-human CD117/KIT (BD 562434), and PE α-human CD63 (BD 557305) all suspended in FACS buffer according to the manufacturer's instructions, and incubated at 45 min at 4 °C. Cells were washed in FACS buffer and run on a BD FACSymphony A3 5-laser cell analyzer using FACS Diva acquisition software and analyzed using FlowJo analysis software.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00269.

Figure S1: Der p 2 CFPS expression in reducing vs oxidizing conditions; Figure S2: IgE binding is specific to a Der p 2 epitope; Figure S3: Phenotypic characterization of culture-derived human mast cells from CD34+ cells; Figure S4: Optimization of CD34+ progenitor-derived mast cell stimulation with positive controls; Figure S5: Der p 2 activation of human mast cells at low doses; Figure S6: Quantification of endotoxin levels in Der p 2 allergen purified from CFPS; Table S1: Allergen accession ID and sequences; Table S2: Characteristics of Der p 2-sensitized donor serum used for passive sensitization of CD34+ culture-derived mast cells (PDF)

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Notes

The authors declare the following competing financial interest(s): B.S.B. receives remuneration for serving on the scientific advisory board of Allakos, Inc. and owns stock in Allakos. He receives consulting fees from Third Harmonic Bio, Lupagen, Sanofi, and Acelyrin. He receives publication-related royalty payments from Elsevier and UpToDate. He is a co-inventor on existing Siglec-8-related patents and thus may be entitled to a share of royalties received by Johns Hopkins University during development and potential sales of such products. B.S.B. is also a co-founder of Allakos, Inc. which makes him subject to certain restrictions under university policy. The terms of this arrangement are being managed by Johns Hopkins University and Northwestern University in accordance with their conflict-of-interest policies. M.C.J. has a financial interest in SwiftScale Biologics, Gauntlet Bio, Pearl Bio, Inc., Design Pharmaceutics, and Stemloop Inc. M.C.J.s interests are reviewed and managed by Northwestern University and Stanford University in accordance with their competing interest policies. All other authors declare no competing interests. The authors have filed an invention disclosure based on the work presented.

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