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

Subtitle: A Proof-of-Concept Demonstration with Der p 2 Dust Mite Allergen

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Abstract

Allergen is used in the clinical diagnosis (e.g., skin tests) and treatment (e.g., immunotherapy) of allergic disease. 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 comprised of common allergens spanning a wide range of phylogenetic kingdoms. We show that allergen produced with this approach can be recognized by allergen-specific immunoglobulin E (IgE), either monoclonals or in patient sera. We also show that 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 the allergic patient.

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 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. Upon activation, these cells release several preformed and newly generated mediators such as histamine, prostaglandins, leukotrienes, proteases, cytokines, and other substances that cause inflammation, vasodilation, bronchoconstriction, diarrhea, rhinorrhea, sneeze, hives, and itch observed during an allergic response. Both mast cells and basophils express high-affinity IgE receptors on their surface (FcεRI) whose alpha 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 release of the mediators they contain in a process called degranulation.

In the clinic, allergy is diagnosed by detecting allergen-specific IgE with 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. Crude 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 enzyme-linked immunoassay (ELISA) lab tests of patient sera to quantify allergen-specific IgE in a manner that also aids-in diagnosis and prognosis. Therapeutically, allergen extracts are used to desensitize the patient to the allergen with repeated small 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]

New tools are needed to produce allergen-based reagents. The current state-of-the-art relies on allergen extract preparations. Allergen extracts are made by crushing raw material (i.e., dust mite colonies, cat dander), isolating the allergen proteins with solvents, and suspending the mixture in a liquid suspension. As a result, allergen extracts are crude protein mixtures. Only two allergens are standardized by a specific protein (Can f 1 for dog 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 (BAU). 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 of allergy 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, 7] Such an approach is not possible with existing allergen extract preparation approaches. A better approach would enable production of individual allergen with the opportunity to mix allergens in a personalized cocktail based on the patient's sensitivity to provide a diagnostic and/or treatment reagent that is tailored to the 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 allergen could conceivably achieve production of individual allergen, and it could do so with more rigorous quality control from batch-to-batch than for traditional allergen extract preparations (i.e., based on precise allergen quantity rather than arbitrary PNU and BAU units). 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 for point-of-care manufacturing.

Here, we describe a cell-free platform for producing recombinant allergen 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)[8-10] is a technique that has been applied to protein biologic discovery,[11, 12] glycoprotein synthesis,[13-18] point-of-care manufacturing,[19-24] distribution of medicines to resource-limited settings,[25, 26] small molecules,[27-29] diagnostics,[30-32] and education.[33-35]

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.[36, 37] 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 (i.e., 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's sensitization profiles. These features can also be leveraged in the laboratory to rapidly produce several allergen 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 allergen 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 includes the most common allergens, the predominant allergens in several common food allergies as well as the food allergen to which sensitivity is associated with the highest risk of anaphylaxis, and 5 of the top aeroallergens (**Table 1, Figure S1**). Addition of ^{14}C-leucine into the CFPS reaction enabled quantification of 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 $\pm$ 132 μg/mL and 174 $\pm$ 98 μg/mL, respectively. Even those allergens expressed at 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 conditions, we gained higher levels of soluble expression (**Figure S1A-B**).

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)

Table 1. A panel of allergens was selected based on clinical relevance and prevalence of IgE reactivity that includes those implicated in food allergy and environmental allergy.

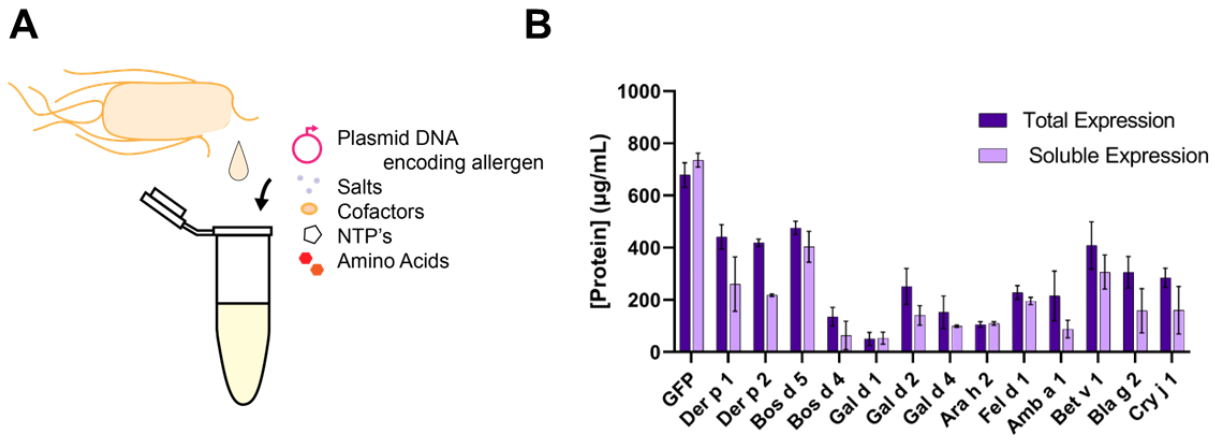

Figure 1. Cell-free expression of clinically relevant allergen panel. (A) Cell-free protein synthesis (CFPS) involves isolating the transcription and translation machinery of *E. coli* cells post-lysis in a test tube. Building blocks, cofactors, and plasmid DNA encoding the desired protein for expression are supplemented. After time for protein expression to occur, 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 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).

Recognition of CFPS-expressed Der p 2 by monoclonal allergen-specific IgE

After demonstrating soluble synthesis of numerous allergens, we next sought to determine whether the CFPS-expressed allergen retains its function insofar as being able to bind IgE. As a model prototype, 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 the 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 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 co-titration 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.[38] On the final readout of an AlphaLISA, 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. Background signal is determined by the zero conditions (“0”) where either IgE (bottom row) or Der p 2 (left column) is absent. The pattern of signal in both co-titrations, 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**). This

demonstrates that CFPS-expressed Der p 2 retains its ability to be recognized by IgE, a key interaction underpinning allergic responses and diagnostics. Of note, altering reaction conditions for expression of Der p 2 did not significantly alter IgE binding (**Figure S1C**).

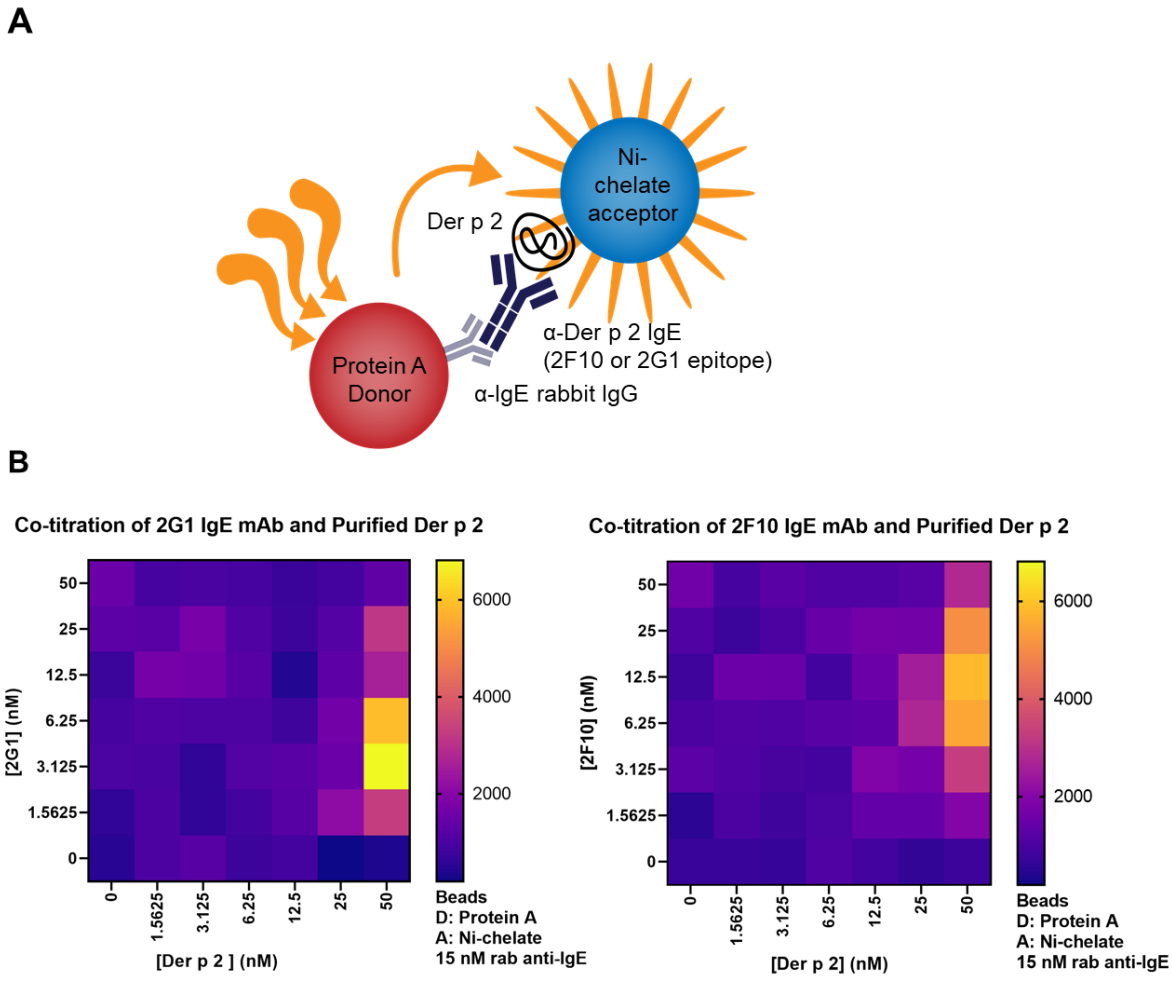

Figure 2. Purified CFPS-expressed Der p 2 is recognized by monoclonal IgE. (A) An AlphaLISA assay detects IgE binding of purified dust mite allergen, Der p 2. The Protein A donor bead associates with α-IgE rabbit IgG and a Ni-chelate acceptor bead associates with dust mite allergen Der p 2. If monoclonal 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 signal and purple indicates no signal (i.e., 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 of IgE, optimal bead interactions lead to the highest level of signal, and the signal gradually decreases as the concentration moves away from those optimums. At higher concentrations, 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 visible signal. Data representative of three independent experiments.

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 crosslinking 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.[39]

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. At initial inoculation, cells are small and round and are differentiated into mast cells by culture 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.[40] Over time, cells become larger with the appearance of intracellular granules and irregular borders characteristic of mast cell morphology (**Figure S2A**). They now display surface expression of typical mast cell markers CD117/KIT, CD33, FcεRIα, Siglec-6, and Siglec-8 (**Figure S2B**), and are capable of degranulation, as assessed by the appearance of CD107a/LAMP1 and CD63

when stimulated with α-FcεRI antibody or ionomycin, a calcium ionophore that triggers degranulation by raising intracellular [Ca^{2+}] in a non-IgE mediated manner (**Figure S3**). At this stage in culture, mast cells can be passively sensitized by incubation with allergen-specific IgE as monoclonals or serum from a sensitized donor. 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 scale from 1 nM to 1 μM resulted in a positive response at all treatment concentrations with 10 nM being the optimal concentration for crosslinking (**Figure 3B**). A positive response is observed at Der p 2 concentrations as low as 3 pM (**Figure S4**). One possible drawback of bacterial systems is the carryover of components, such as endotoxin, that may lead to non-IgE mediated activation. To account for this possibility, we quantified endotoxin in the purified allergen sample finding levels to be below the FDA limit (**Figure S5**). We also included a non-passively sensitized control sample at each Der p 2 concentration 1nM-1μM. 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, levels of CD63 are determined. 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 little to no non-IgE-mediated activation by carry over components from *E. coli* lysates. Exposure to a non-IgE-mediated, 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 crosslinking. 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 CF-expressed Der p 2 doses are comparable to previously published studies using traditional methods for allergen-based reagent preparation.[41, 42] These data indicate that CF-expressed Der p 2 can activate human mast cells and basophils in a dose-dependent manner, which is the key feature of clinically useful allergen-based reagents.

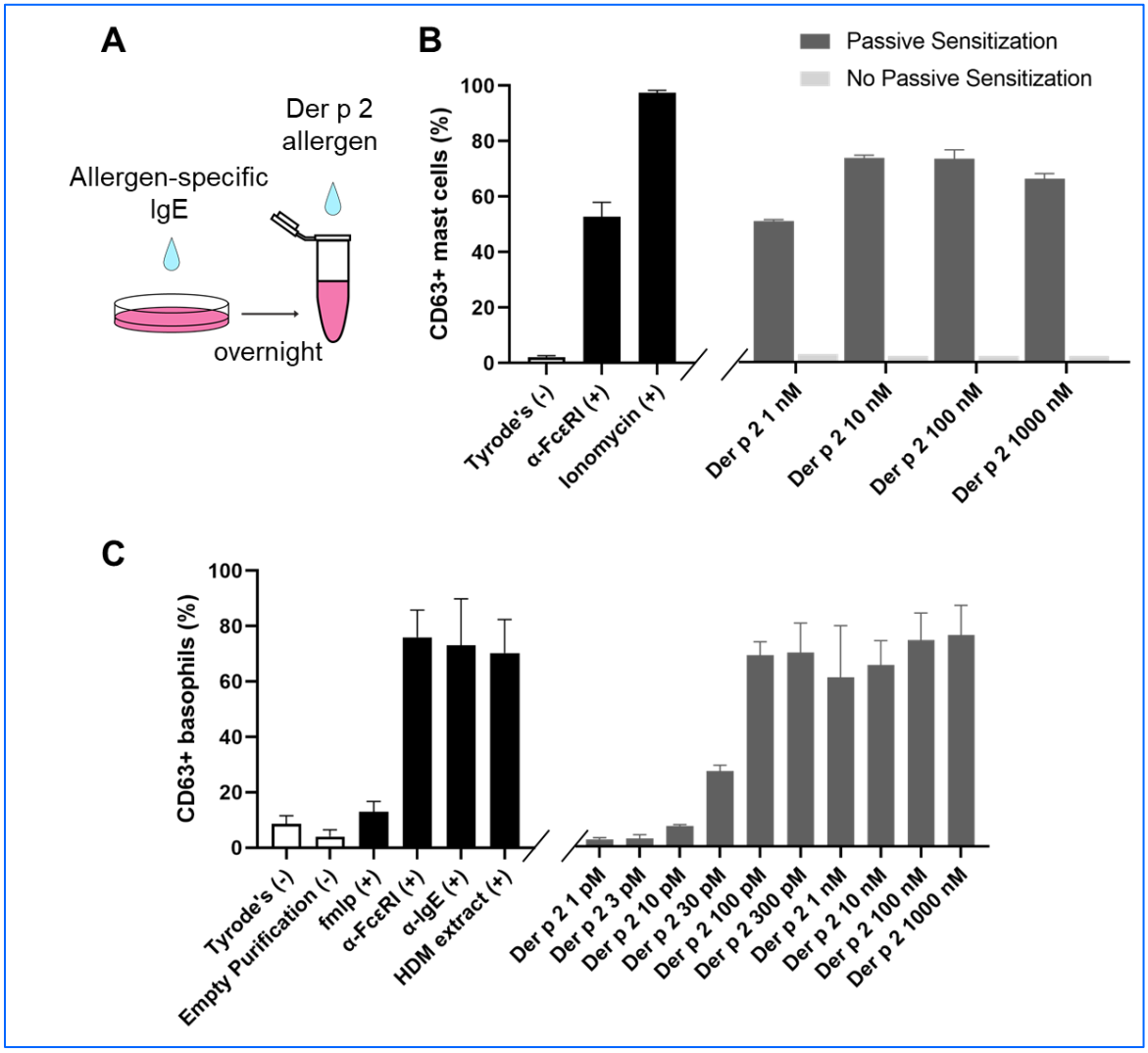

Figure 3. Der p 2 activation of human basophils and mast cells. (A) CD34+ progenitors are isolated from human peripheral blood and differentiated into primary human CD34+ progenitor-derived mast cells in culture with cytokines necessary for their selective differentiation. CD34+ culture-derived mast cells are then passively sensitized with serum from a Der p-sensitized donor (**Table S2**) and incubated with 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 crosslinking. Without passive sensitization, stimulation with 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 crosslinking. (n=2 samples, 1 HDM-sensitized patient donor, error bars = standard error).

Discussion

Recombinant allergen is a useful tool in the clinical diagnosis and treatment of allergic disease.[5-7, 43] 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 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 CFPS-expressed allergen retains its ability to be recognized by IgE and activate human basophils and mast cells *in vitro* at the picomolar range. More studies need to be done to confirm these important characteristics with other allergens.

Additional work may also need to overcome the presence of endotoxin in *E. coli* lysates for clinical applications. Since endotoxin paired with allergen is a common approach for oral sensitization in allergy mouse models,[44, 45] 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 S5**). Optimization of endotoxin removal while minimizing protein loss could overcome this challenge. Also, this would not be a constraint for lab tests such as IgE ELISA's, nor for *in vitro* studies of allergen and immune reactions.

There is a push in the field towards 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 to 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 NdeI and SalI restriction sites (Twist Biosciences).

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

Harvest and processing of *E. coli* lysate for CFPS

E. coli lysate was prepared using previously published methods [46] using engineered *E. coli* strain C321.ΔA.759, a highly productive MG1655-derived strain.[47] 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 10L 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 x g at 4°C and distributed to twelve 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 x 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 per gram of wet cell mass S30 buffer and aliquotted in 1.4 mL increments. Cells were kept on ice and underwent sonication for 45 s on, 59 s off at 50% amplitude until 950 joules was reached. Supernatant (820 µL) was collected and underwent a 1-hour runoff reaction at 37°C with agitation (250 rpm). Lysate was then centrifuged at 12,000 x 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.[46] 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 of plasmid encoding allergen in pJL1 backbone; and 27% v/v of *E. coli* crude lysate (prepared above). Reagents were sourced from Sigma-Aldrich unless stated otherwise.

Radioactive quantification of allergen expressed by cell-free protein synthesis

Allergen expressed was quantified by ^{14}C-leucine incorporation according to previously published methods.[48] In brief, 10.67 µL ^{14}C-leucine was supplemented into assembled CFPS reactions described above. After 20 hours of overnight expression, protein was precipitated by addition of 0.5N KOH in a 1:1 ratio to samples containing total protein and samples containing soluble protein (supernatant remaining after centrifugation at 12,000 x g for 5 min). Samples were incubated at 37°C for 20 min. Then, 4 µL of sample was dispensed onto a Filtermat (Perkin Elmer, 1450-421) and 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, cooled, and then signal was read using a Microbeta2 Scintillation Counter. Protein expression was calculated using following formula:

(((Signal of sample on washed filtermat – signal of no DNA negative control sample) / signal of sample on unwashed filtermat) * [^{14}C-leucine (µM)] * molecular weight of protein (g/mol)) / (# leucine residues x 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 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 manufacturer's instructions and collected in nuclease free water (Ambion, AM9937).

Detection of IgE binding by 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 2x serial dilution was prepared by adding half volume of the previous dilution to the next dilution and mixing by pipette in a serial fashion. AlphaLISA reactions were assembled 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 hour. Bead components were then distributed according to manufacturer's instructions and allowed to incubate for at least 1 additional hour. Signal was read on a plate reader (Tecan Infinite M1000).

Activation test of primary human basophils in peripheral blood

Venous blood was obtained from an allergic 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

manufacturer's instructions (Bühlmann Labs, FK-CCR-U). As positive controls, basophils in whole blood were stimulated with 5 µL 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.[40]

Passive sensitization and activation of human CD34+ progenitor-derived mast cells

Cultured CD34+ progenitor-derived mast cells were incubated overnight with allergic 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 10x 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 for 20 minutes at 37°C at 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 S2**).

For flow cytometry, cells were washed in PBS and incubated at 4°C for 30 minutes with live/dead staining (Invitrogen L23105) suspended in PBS according to 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 manufacturer's instructions (10-minute 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), PE α-human CD63 (BD 557305) all suspended in FACS buffer according to manufacturer's instructions and incubated at 45 minutes at 4°C. Cell 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.

Supporting Information

- **Supplementary Figures**
  - Figure S1: Der p 2 CFPS expression in reducing vs oxidizing conditions
  - Figure S2: Phenotypic characterization of culture-derived human mast cells from CD34+ cells
  - Figure S3: Optimization of CD34+ progenitor-derived mast cell stimulation with positive controls
  - Figure S4. Der p 2 activation of human mast cells at low doses
  - Figure S5: Quantification of endotoxin levels in Der p 2 allergen purified from CFPS
- **Supplementary Tables**
  - Table S1: Allergen accession ID and sequences
  - Table S2: Characteristics of Der p 2-allergic donor serum used for passive sensitization of CD34+ culture-derived mast cells

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Conflict of Interest

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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