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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Development and validation of an Allergen Challenge Theater for grass and ragweed

Allergen challenge facilities are useful to test new allergy medications because pollen levels can be controlled, studies can be conducted outside the natural pollen season, and statistical significance can be obtained with fewer subjects. Fundamentally, the facility must maintain consistent allergen concentrations within tests (exposures ≥ 3 hours) as well as between tests.[1]

The Ottawa Allergen Challenge Theater (ACT) (Figure S1) consists of four quadrants of theater seating, each having a dedicated allergen supply. This allows flexibility to run quadrants independently or simultaneously. In the present configuration, quadrants are run independently using different allergens. The objective of this work was to perform independent validation studies for Timothy-grass (*Phleum pratense*) and ragweed (*Ambrosia artemisiifolia*) (Greer) in the largest quadrant having a 40-person capacity (8 seats across, 5 rows deep). Future validations will be necessary for operating other zones, other allergens, and for running multiple zones simultaneously.

Pollen concentrations were measured by laser particle counters (LPC) (Model R5102, Lighthouse Worldwide Solutions, Inc). The output from the driving LPC was fed back into a programmable logic controller which adjusted the amount of pollen injected into the airflow in order to maintain the selected level in the ACT. LPC provide rapid assessments of particle counts but do not discriminate between dust and pollen. Therefore, pollen counts were verified using impact samplers (IS) (GRIPS-99M, Aerobiology Research Laboratories), which capture pollen on the surface of two rotating rods. The rods were stained, and the pollen identified and counted using light microscopy.

Technical validation studies evaluated (a) the relationship between LPC counts and IS pollen counts, (b) stability of pollen levels, and (c) variability of pollen counts in the seating area.

The LPC-IS relationship for both pollens was linear over a wide range of concentrations: grass r^2: 0.85 ragweed r^2: 0.93, (Figure S3). Ragweed and grass concentrations were found to be stable over several hours, responsive to changes in the pollen setpoint (Figure 1B), and sufficient to induce symptoms. Multiple IS or LPC were placed within the seating area and demonstrated acceptable uniformity of pollen exposure (See Appendix S1 for details). These studies demonstrated that the ACT is able to generate reproducible pollen concentrations comparable to those seen in other facilities[2-4] which are associated with the ability to induce ocular and nasal symptoms of appropriate intensity.

Clinical validation studies were performed to test reproducibility of the allergic response to grass and ragweed in participants meeting

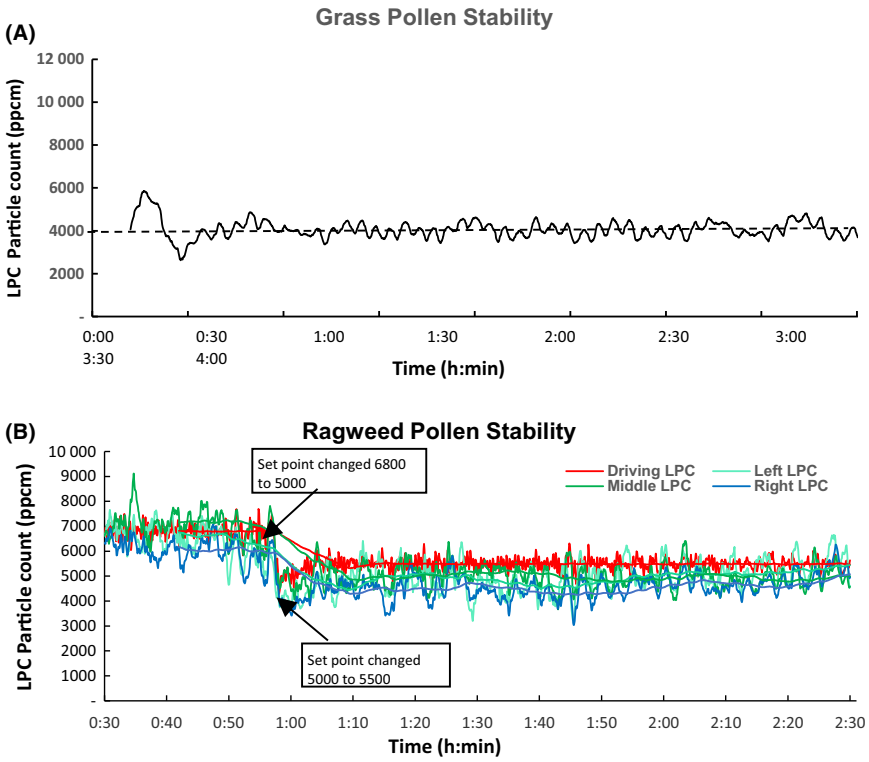

FIGURE 1 Panel A, Grass pollen concentration over 4 h, the dashed line represents the pollen setpoint. Panel B, Ragweed pollen concentrations over a 2-h period. One LPC was designated as the driving LPC (red line), and the others were placed at the right (blue), middle (green), and left (aqua) in the center of one zone. Solid lines are 12-minute running averages overlaying fainter lines which are raw data transmitted every 3 s. The driving LPC was the one that controlled the pollen levels in the ACT. Its particle count was always higher than the others as it was closer to the vents where the pollen enters the room but all LPC were stable over the time of measurement and responsive to changes in setpoint concentration. (IS = impact sampler; LPC = laser particle counter; ppcm = particles per cubic meter)

the inclusion/exclusion criteria (See Appendix S1 for details). The studies were approved by an Ontario Provincial Research Ethics Board (IRB Services).

For the grass study, reproducibility was assessed by comparing Total Nasal Symptom Score change from baseline to end of challenge (ΔTNSS) on two challenge days separated by a day. Mean pollen concentrations for Challenge 1 (3359 ± 1344 grains/m^3) and Challenge 2 (3468 ± 1258 grains/m^3) were comparable. ΔTNSS reached a plateau at 120 minutes and was numerically higher during the second challenge from this point onwards (Figure 2A). In 13/30 (43%) subjects, Challenge 1 TNSS was 1.5 ± 1.4 at 180 minutes and Challenge 2 TNSS at 180 minutes was 2.73 ± 1.95, P = 0.005. Repeated exposure to allergen promotes epithelial accumulation of inflammatory cells. As a consequence, the response to a second exposure could be increased, a process which has been called "priming." Other centers have used one or more "priming challenges" to stimulate the allergic response out of season and reduce the variability of outcome measures. Ellis et al[5] demonstrated the wide range of symptoms induced by an initial priming challenge and noted that skin prick test reactivity did not correlate with clinical symptoms. As we did not prime subjects, we evaluated subjects who achieved TNSS ≥ 5 at any time during Challenge 1. In the seventeen subjects with TNSS ≥ 5 (Figure 2B), the two challenges overlapped; ΔTNSS at 180 minutes was 4.30 ± 2.21 for Challenge 1 and 4.89 ± 2.28 for Challenge 2, P = 0.33.

ΔTOSS in these subjects was numerically higher at all time points during Challenge 2 but only significantly different at 60 and 180 minutes (Figure 2C). At 180 minutes, ΔTOSS was 1.24 ± 1.30 vs 2.46 ± 2.27, P = 0.01 for Challenges 1 and 2, respectively.

In the ragweed study, the primary outcome was difference in ΔTNSS over the last hour (180-240 minutes) on the two challenge days. Mean pollen concentration was 3929 ± 526 grains/m^3 for Challenge 1 and 4100 ± 678 grains/m^3 for Challenge 2. ΔTNSS for Challenge 1 was slightly higher than for Challenge 2 (Figure 2D), and the mean ΔTNSS plateau was 4.89 ± 2.19 vs 4.28 ± 2.69 for Challenges 1 and 2, respectively, P = 0.04. The difference was due to the higher baseline for Challenge 2 (2.04 ± 1.49) compared with Challenge 1 (1.6 ± 1.22), P = 0.24, but, apart from the first two time points, TNSS scores were virtually superimposed (Figure 2E). ΔTOSS (Figure 2F) was the same for both challenges with the exception of the 180- and 210-minute measurements. At 240 minutes, ΔTOSS was 3.01 ± 1.33 vs 3.0 ± 1.94, P = 0.95 for Challenges 1 and 2, respectively.

Average TNSS at the end of the ragweed challenges (6.76 ± 2.18, Challenge 1 and 6.52 ± 2.74, Challenge 2) was lower than TNSS in response to ragweed challenges reported by other facilities.[6,7] This was not due to ACT pollen concentrations as our reported levels are slightly higher than other centers. Selection criteria may have played a role. Other centers selected subjects based on TNSS scores ≥ 6.[7,8] In a report by Ellis et al[9] where subjects were selected on the basis of achieving a TNSS of 4 in a priming challenge, peak TNSS was comparable to our results. Ocular symptoms in both studies were in the range reported in the literature.[10]

We have demonstrated that the ACT is able to generate reproducible pollen concentrations comparable to those seen in other facilities. Allergy symptoms in both grass and ragweed allergic patients were also induced with good reproducibility, but clearly illustrating the importance of subject selection and the time interval between the challenges.

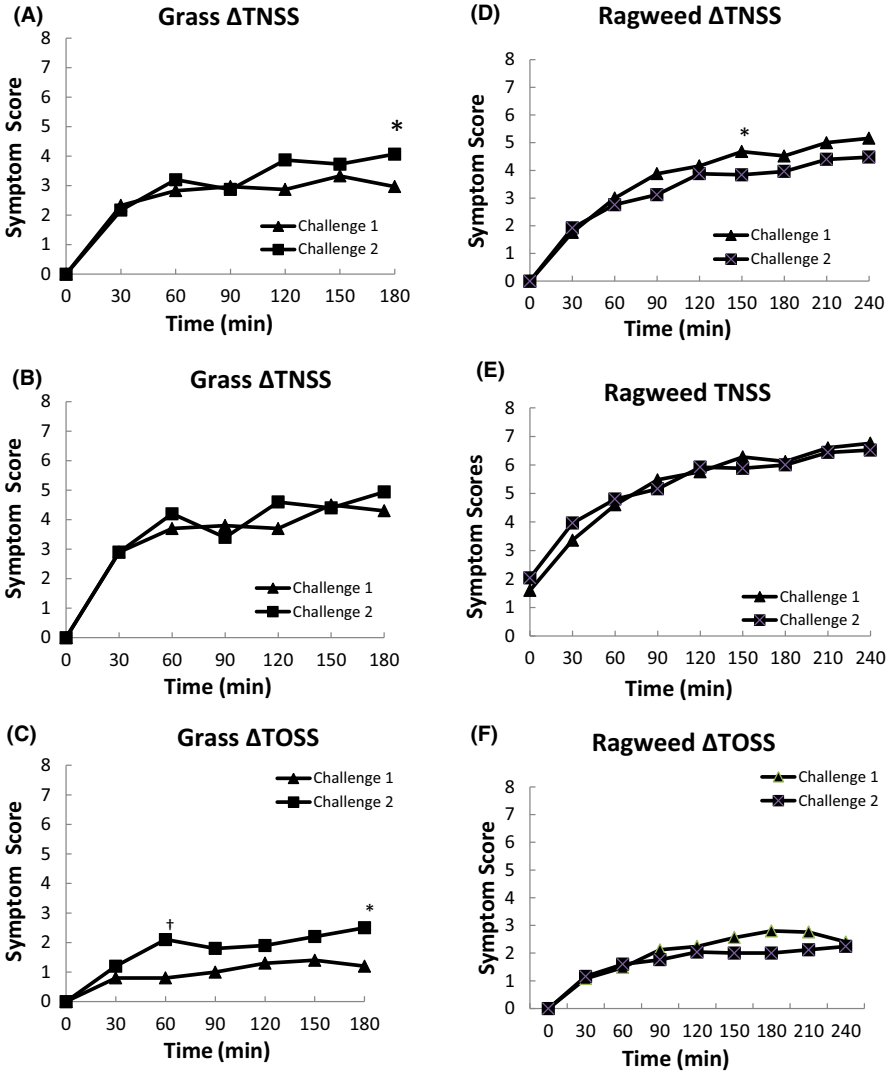

FIGURE 2 Nasal and ocular symptom scores during grass (A, B, and C) and ragweed challenges (D, E, and F). Panel A: nasal symptoms expressed as change from baseline in response to grass challenge in all subjects. Panel B: Nasal symptoms in a subset of 17 subjects who achieved a TNSS score ≥ 5 in Challenge 1. Panel C: Ocular symptoms expressed as change from baseline in the same subjects. Panel D: Nasal symptoms expressed as change from baseline in response to ragweed challenge. Panel E: Ragweed-induced nasal symptoms as absolute values. Panel F: Ragweed–induced ocular symptoms expressed as change from baseline. Paired *t* tests were used to compare the two challenges. No corrections were made for multiple comparisons. *, $P < 0.05$; †, $P < 0.001$. TNSS, Total Nasal Symptom Score (sum of congestion, rhinorrhea, sneezing, and itch scores; maximum score 12). TOSS, Total Ocular Symptom Score (sum of watery and itchy eyes scores; maximum score 6)

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CONFLICTS OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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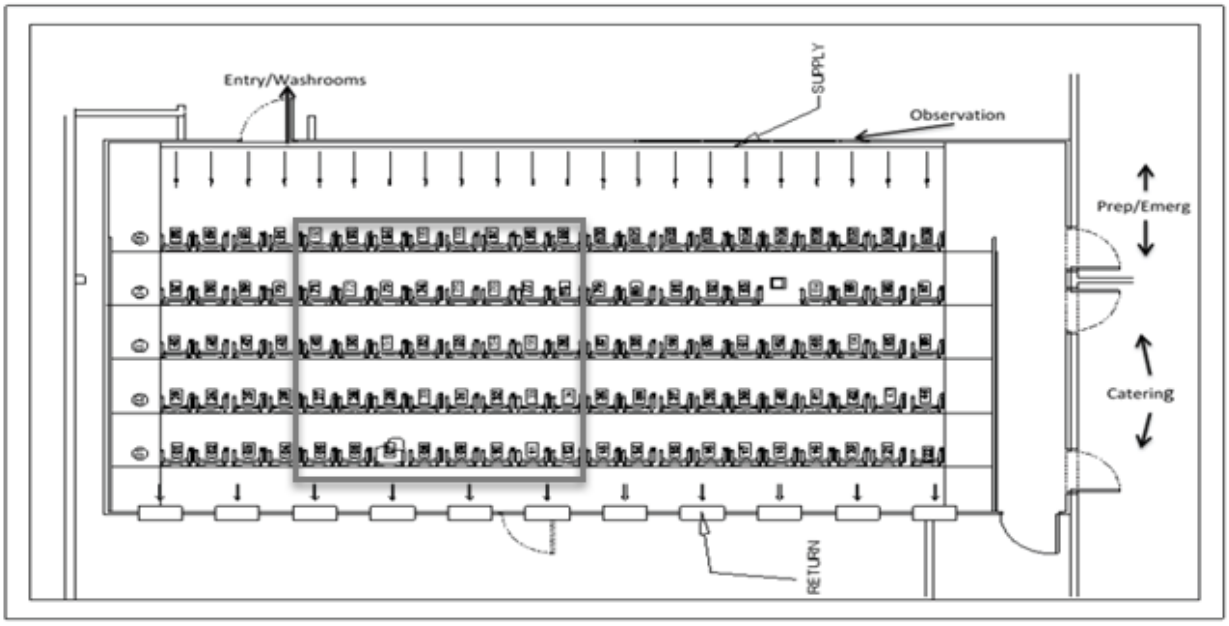
Human type 2 innate lymphoid cells disrupt skin keratinocyte tight junction barrier by IL-13

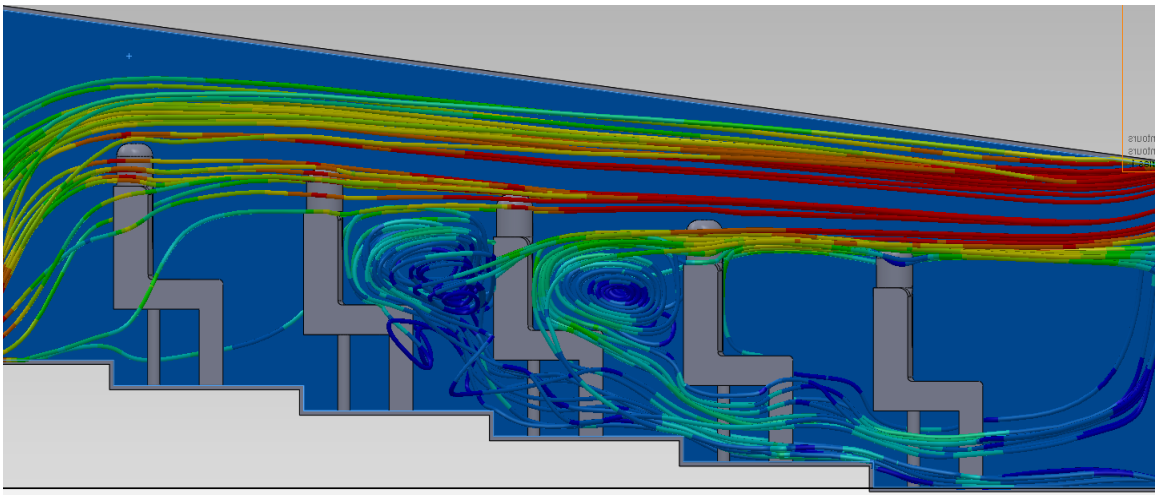
To the Editor,

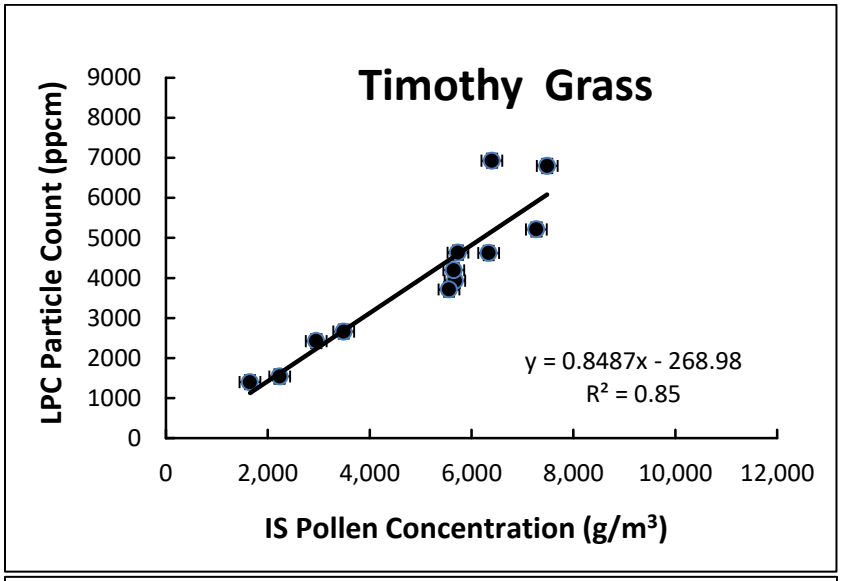
The largest organ of the human body is the epidermis, which consists mainly of keratinocytes (KCs) and serves as a physical barrier. Along with the stratum corneum, which is the outer surface of the epidermis, the tight junctions (TJs) located in the skin surface side of stratum granulosum are crucial for the integrity and function of the epidermal barrier.[1,2] TJs form a network of molecules that ensures a nonpermeable intercellular adhesion and seal the paracellular space in the epithelium, thus protecting the body from the penetration of invading microorganisms, pollutants, environmental toxins, and allergens.[3] Accordingly, skin KC TJ barrier dysfunctions permit the penetration of antigens, allergens, toxins, and pollutants through the surface of the epidermis to dermis and subdermal connective and fat tissues. Recent evidence suggests that TJ barrier dysfunction plays a key role not only in atopic dermatitis but also in asthma, allergic rhinitis, chronic rhinosinusitis, and colitis.[4] Other data from human and animal studies have shown that impairment of the skin barrier is an important mechanism in allergen sensitization.[4] Allergic sensitization triggers the first induction of innate immune responses by pathogen recognition receptors on epithelial cells and immune cells, such as innate lymphoid cells (ILCs), dendritic cells, macrophages, and mast cells, and leads to subsequent activation of type 2 immune responses.[4]

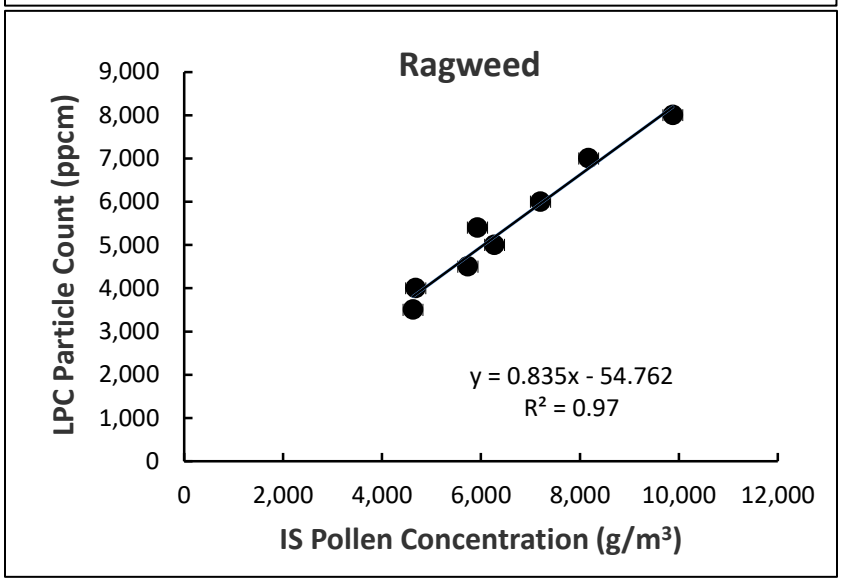
Innate lymphoid cells are preferentially enriched in barrier tissues such as the skin, lung, and intestine and play important roles in the maintenance of tissue homeostasis, regulation of immune responses between epithelium and microorganisms, and sensitization to allergens.[5] Among ILC subsets, type 2 innate lymphoid cells (ILC2s) reside in the barrier surfaces and contribute to lung inflammation and asthma development.[6] They enable the immune system to respond quickly to environmental antigens on epithelial surfaces. Mechanisms of skin and mucosal barriers differ, and skin shows a stronger barrier both with filaggrin, loricrin, involucrin located in the stratum corneum, and TJs located in the stratum granulosum. We have recently demonstrated that ILC2s facilitate bronchial epithelial barrier disruption via downregulation of the TJ barrier through IL-13 in asthma.[7] Although ILCs are relatively well studied with regard to their role in the development of skin diseases such as atopic dermatitis and psoriasis, several major questions remain to be addressed concerning whether the epidermal TJ barrier is influenced by ILC2s.

In the present study, we first investigated the role of ILC2s in the regulation of TJ function of human KCs. We cultured normal human epidermal keratinocytes (NHEKs) with ILC2s at air-liquid interface (ALI), and transepithelial resistance (TER) was measured as a readout for barrier integrity (See Online Supporting Information). Data analysis was performed using 2-tailed Mann-Whitney *U* test or analysis by variance (ANOVA), followed by Tukey's post hoc multiple comparison. A P value of <0.05 was considered significant. ILC2s were cocultured with NHEKs and TER was measured before and after 12, 24, 48, 72, and 96 hours. We found that after 48 hours of coculture with ILC2s a significant decrease in TER was observed (Figure 1A). To further investigate whether ILC2s


Entry/Washrooms
SUPPLY
Observation
Prep/Emerg
Catering
RETURN




Timothy Grass
LPC Particle Count (ppcm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
2,000
4,000
6,000
8,000
10,000
12,000
IS Pollen Concentration (g/m³)
y = 0.8487x - 268.98
R² = 0.85


Ragweed
LPC Particle Count (ppcm)
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
0
2,000
4,000
6,000
8,000
10,000
12,000
IS Pollen Concentration (g/m³)
y = 0.835x - 54.762
R² = 0.97

Supplemental material

Description of the ACT

The ACT (Figure S1) is 16m wide by 8m deep (area 133 m^2) and contains adjustable seating for up to 100 subjects in a series of elevated rows (Figure S1). Chair locations and configurations can be easily customized to suit specific test requirments. The raised floor provides improved visibility of each subject's face from the observation room and also achieves an uninterrupted allergen flow path to each subject's face. To avoid disturbance of allergen, test subject movement is kept to a minimum by of 3 large TV screens providing entertainment during testing.

The walls and ceiling of the ACT are low-maintenance polyester panels resistant to particulate buildup. The floor is ceramic tile with epoxy grout for mold resistance. Pollen is injected into air supply ducts where it is mixed with the humidity- and temperature-controlled ventilation air and delivered through selected vanes at the front of the room. The air supply ducts are divided into 4 zones so that each section of the facility can be used for different antigens. Pollen-laden air exits the ACT through return air ducts behind the subjects. The return air is HEPA-filtered to remove the pollen and returned for recirculation at the front of the room where it picks up new pollen grains. The ACT is maintained at a slight positive pressure to ensure that no other allergens enter the room during testing. A windowed observation and control room allows surveillance of the subjects and communication with the staff in the ACT.

At the end of a study, the ventilation system removes airborne pollen within 10 to 15 minutes of shutting off the pollen generator. The ventilation ducts are cleaned using pulsed compressed air and inspected to be certain the pollen is removed. Any areas containing pollen are washed with

water and dried. After cleaning, pollen-free air is blown through the ventilation ducts for several hours and air samples are taken to ensure that no pollen remains. The ACT is washed down to remove residual pollen on the surfaces including the chairs. Excess water is removed with dry cloths and exhaust fans are run overnight to remove any remaining moisture. After cleaning, floor and wall samples are taken to validate the removal of pollen from the room. During a study, pollen grains are captured on the rods of Impact Samplers (IS) which are inspected to ensure that only the pollen of interest is being aerosolized.

Technical Validation Studies

Modeling and simulation

Computational Fluid Dynamics (CFD) analysis was used to optimize the air and particle flow in the distribution system and the ACT. CFD uses numerical analysis to generate solutions for fluid flows with or without interactions with solid surfaces. Iterative CFD computer modeling using a commercial software program (SolidWorks, Dassault Systèmes SOLIDWORKS Corp., Waltham, Massachusetts, USA) was used to simulate the transport of suspended particles (pollen) within the ACT in order to determine the optimal configuration of the delivery system, the room geometry and the airflow. In the simulations, these parameters were varied systematically. The analysis was used to characterize allergen concentrations between 1000 and 5000 grains/m^3 and size variations from 10 to 60 µm.

CFD analysis indicated that air flow needed to be high enough to permit pollen delivery to the back of the room but predicted there would be a recirculation of pollen dropping to the floor and flowing to the front of the room (Figure S2). A smoke pencil which emits a visible vapor was used to verify these airflows and to adjust the ventilation to minimize them. As indicated in the simulation illustrated in Figure 2, at the appropriate ventilation, eddies circulate below the breathing zone of the subjects.

Measurement of pollen concentrations

Because pollen levels were measured using both Laser Particle Counters (LPC) and Impact Samplers (IS) and an LPC was used to control the level of pollen in the ACT, a calibration was done to align the two methods of measurement. The LPCs were calibrated by comparing the pollen counts from the IS to the output of the LPC over a range of pollen levels. The pollen concentration was adjusted in a stepwise fashion and when the LPC readings had stabilized, a 15-minute pollen sample was collected using the IS. The average LPC particle count over that 15-minute period was compared to the average pollen count from the two rods of the impact sampler. The relationship between LPC and IS pollen counts is illustrated in Figure S3 for grass (panel A) and ragweed (Panel B). The relationship is linear and the correlation coefficient between the two is excellent.

Distribution of pollen within one zone

In order to measure grass pollen levels in the area designated for pollen studies, Impact Samplers (IS) were set up at 6 points in a T-shaped configuration with 3 IS across Row 1 and 1 IS in each of rows 2, 3 and 4 behind the middle IS. Ten-minute pollen samples were taken every 30 minutes over 2.5 hours. Table S1 illustrates the variability in the pollen counts both over time (illustrated by the SD) and at different positions. The percent deviation from the mean at each sampling point ranged from 4 to 22% and the deviation of the individual values from the overall mean of 4,000 g/m^{3}was 16%. This is comparable to the ±20% reported by Jacobs *et al.* [1].

Ragweed concentrations averaged over 15-minutes were measured with Laser Particle Counters (Table S2). Means and SD are provided for front-to-back and side-to-side concentrations. The variability was lower from side-to-side than from front-to-back likely because the distance is shorter. The mean (SD) for the entire zone was 4622 ± 578 ppcm.

For ragweed, the percent deviation was 10.7% and is lower than seen for grass pollen possibly because it does not take into account the variation that would occur with time. In any case, these counts provide a quite detailed assessment of the variation in participant exposure..

Position	**Row 1**	**Row 2**	**Row 3**	**Row 4**

Right IS	4368 (867)			
Middle IS	3363 (492)	5039 (585)	4302 (236)	3333 (338)
Left IS	3597 (176)			

Table S1: Distribution of grass pollen in a single zone in the ACT. Values are g/m^3 mean (SD) for each IS over the time period. The mean (SD) for all samples was 4000 (801) g/m^3. Abbreviations: ACT, allergen challenge theatre; IS, Impact Sampler; g/m^3, grains per cubic meter.

Position	**Row 1**	**Row 2**	**Row 3**	**Row 4**	**Row 5**	**Mean (SD)**
Right LPC	4554	5166	4802	4238	3625	4477 (523)
Middle LPC	4947	5430	5508	4622	3988	4899 (559)
Left LPC	5401	4804	4229	3852	4163	4490 (550)
Mean (SD)	4967 (346)	5133 (257)	4846 (523)	4237 (315)	3925 (224)	
Overall mean (SD) all LPC data points						4622 (578)

Table S2: Distribution of ragweed pollen in a single zone in the ACT. The mean (SD) for all samples is 4622 (578) ppcm. Abbreviations: ACT, allergen challenge theatre; LPC, laser particle counter; ppcm, particles per cubic meter.

Clinical validation studies

Clinical validation studies were performed for both grass and ragweed. Written informed consent was obtained before any study evaluations were done. Each study population consisted of healthy male and female adults, 18 to 65 years of age, with a clinical diagnosis of seasonal allergic rhinitis, a positive skin prick test to the pollen being studied ($\geq$3mm) and nasal symptoms during that pollen season in the previous two years. Exclusion criteria included asthma with the exception of mild intermittent asthma treated by short-acting β-agonists, structural nasal defects or nasal polyps, an infection of the upper airways within 2 weeks prior to the screening visit, pregnancy, a history of anaphylactic reactions and allergen desensitization

within the previous 2 years. Subjects were not allowed to take allergy medications during the study with the exception of rescue medication (cetirizine, 10 mg) given at the end of the allergen challenge to treat nasal symptoms if required and olopatadine to treat ocular symptoms. The studies were performed in the winter (grass) or early spring (ragweed) to avoid concomitant exposure to other seasonal allergens.

In the ACT, subjects wore protective gowns and caps. They were provided with paper forms on which to register their symptom scores. When the driving LPC which controlled the pollen distribution and was positioned 5 feet above floor level demonstrated low and stable values indicating dust settling, pollen was blown into the study area through ducts located across the top of the front wall. Air flow was adjusted to obtain pollen counts of 4000 grains/m^3 as measured by the LPC and confirmed by impact samplers (IS) set at face level. Four IS were installed, one in the center of each row and measurements taken every 30 minutes.

Subject-reported symptom scores were obtained using a 4-point scale (0 to 3) for none, mild, moderate and severe, respectively. Nasal symptoms (congestion, rhinorrhea, sneezing and itch) and ocular (watery and itchy eyes) symptom scores were recorded prior to pollen exposure and every 30 minutes during each challenge. Total nasal (TNSS) and total ocular (TOSS) symptom scores were calculated later.

For the grass pollen study, 63 subjects were screened to achieve 31 participants (17 male, 14 female). Those selected for the study came back for two 3-hour challenges separated by 1 day. One subject did not return for the second challenge so analyses were performed on data from the 30 subjects who completed both challenges.

In the ragweed study, 76 subjects were screened and 48 eligible subjects underwent a 3-hour screening challenge. Thirty-three subjects achieving a peak TNSS ≥ 5 were selected for two

subsequent 4-hour challenges 2 days apart. Demographic data for both studies is provided in (Table S3).

The primary purpose of these studies was to assess the reproducibility of the allergic response in the ACT. For the grass study, this was done by comparing the TNSS change from baseline to end of challenge (ΔTNSS) on the two days. For the ragweed study, the average TNSS over the last hour (180 to 240 minutes) for each challenge was compared.

Safety information

Although subjects were excluded if they had asthma other than mild intermittent asthma treated with an inhaled beta agonist, they were instructed to contact the clinic or seek emergency care if they had a late-phase reaction. No late phase reactions were reported by the subjects.

Grass Study: Seven subjects reported 8 adverse events of which 1 was suspected to be related to the study. The related AE was headache. Not suspected AEs were: sinus infection in 1 subject, common cold in 2 subjects, pain related to pneumonia in 1 subject and neck pain 2 subjects. One SAE of pneumonia was reported outside the study period and was unrelated to the study.

Ragweed Study: Seven subjects reported 9 adverse events of which 4 were suspected related to the study. The related AEs were: Epistaxis (nosebleed) in 3 subjects and profuse diaphoresis (sweating) in 1 subject. Not suspected AEs were chest congestion, diaphoresis, vaginitis and epigastric pain.

		Grass Study N=31	Ragweed Study N=33
Gender	Male	17	19

	Female	14	14
Age (years)			
	Mean (SD)	39 (11.2)	36 (14.2)
	Median	40	29
	Min	19	19
	Max	60	65
Race (m)			
	Caucasian	28	28
	Black	0	1
	Oriental	1	2
	Other	2	2
Smoking status			
	Non-smoker	22	23
	Ex-smoker	8	7
	Smoker	1	3
Smoking cessation (years)	n=8		
	Mean (SD)	11 (8.4)	15 (12.6)
	Median	7	12
	Min	4	1
	Max	30	30
Symptom duration			
	Mean (SD)	23 (11.8)	17 (11.0)
	Median	20	14
	Min	5	3
	Max	47	48
Reported sensitization, n (%)			
Pollen alone		17 (55)	11 (33)
Pollen +PAR		14 (45)	22 (67)

Table S3. Demographic data for study participants. PAR- perennial allergic rhinitis (animals, dust)

References:

1. Jacobs RL, Ramirez DA, Andrews CP. Validation of the biogenics research chamber for Juniperus ashei (mountain cedar) pollen. *Ann Allergy Asthma Immunol.* 2011;107(2):133-138.

Figure Legends:

Figure S1. The Allergen Challenge Theatre (ACT). Subjects enter from a gowning area at the upper left and are observed through a large window at the upper right. Top arrows indicate pollen entry; bottom arrows, pollen exit through HEPA filters. Box indicates subject seating area for pollen validation studies. Catering and an emergency treatment room are on the right.

Figure S2 Results of CFD simulation of pollen transport in the ACT. Air flow is sufficient to permit pollen delivery to the back of the room but there is some recirculation of pollen which drops to the floor and flows to the front of the room. Pollen at the back of the room is drawn into exhaust vents in the wall behind the subjects.

Figure S3: The LPC-IS relationships for grass and ragweed are linear over a wide range of concentrations, highly correlated and allowed adjustment of the controlling LPC output to ensure correct pollen concentrations in the ACT.