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SUPPORTING INFORMATION

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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

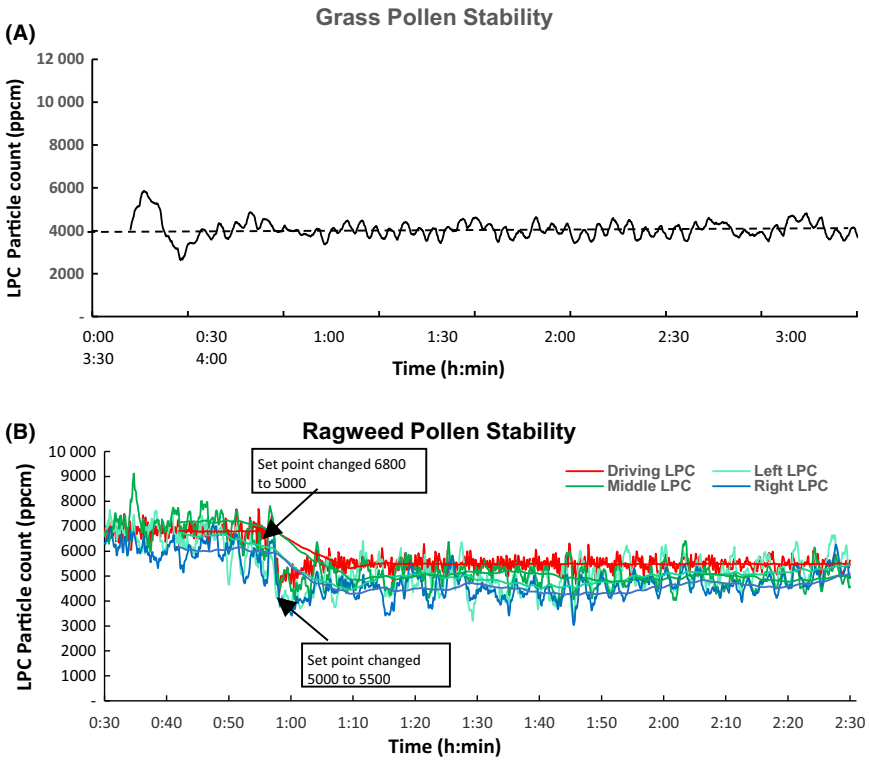

FIGURE 1 Panel A, Grass pollen concentration over 4 h, the dashed line represents the pollen setpoint. Panel B, Ragweed pollen concentrations over a 2-h period. One LPC was designated as the driving LPC (red line), and the others were placed at the right (blue), middle (green), and left (aqua) in the 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.

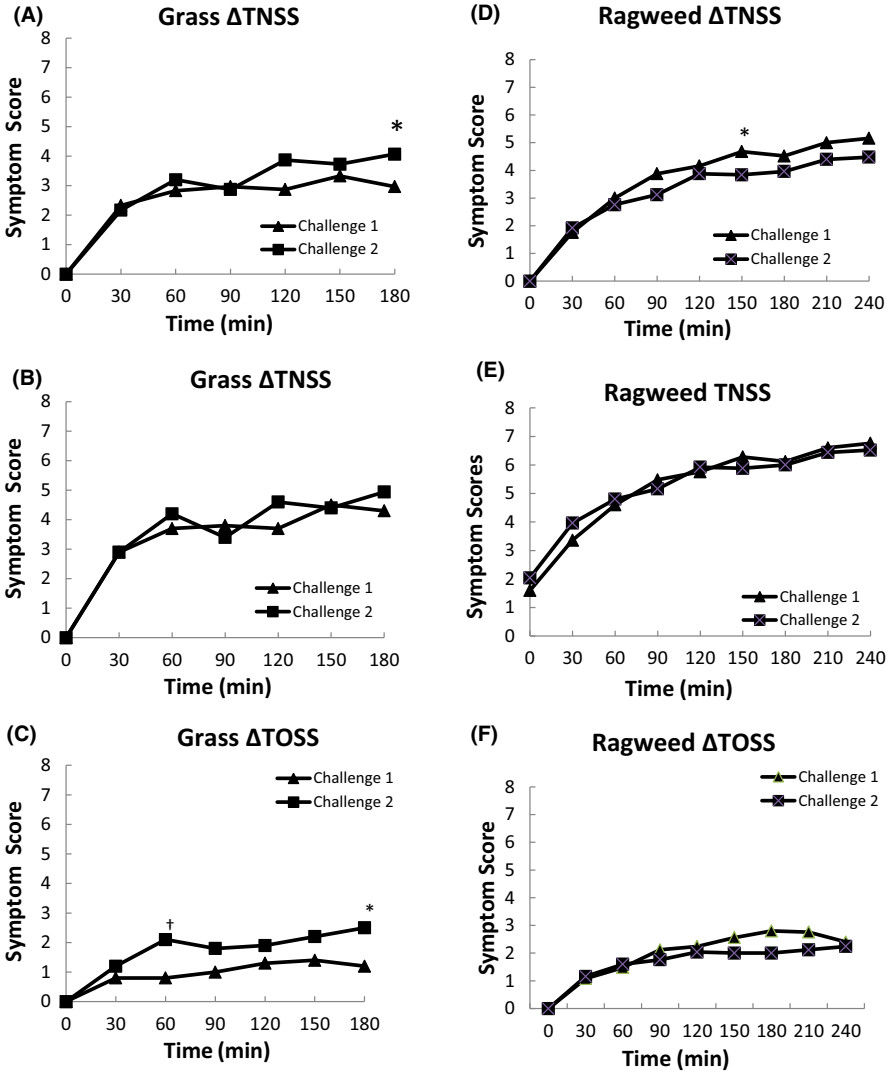

FIGURE 2 Nasal and ocular symptom scores during grass (A, B, and C) and ragweed challenges (D, E, and F). Panel A: nasal symptoms expressed as change from baseline in response to grass challenge in all subjects. Panel B: Nasal symptoms in a subset of 17 subjects who achieved a TNSS score ≥ 5 in Challenge 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

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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