

Cockroach sensitization mitigates allergic rhinoconjunctivitis symptom severity in patients allergic to house dust mites and pollen

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Background: Modifiers of symptom severity in patients with allergic rhinoconjunctivitis (AR) are imprecisely characterized. The hygiene hypothesis implicates childhood microbial exposure as a protective factor. Cockroach sensitization (C+) might be a proxy for microbial exposure.

Objective: We sought to determine whether C+ assayed by means of skin prick tests influenced AR symptom severity in controlled and natural settings.

Methods: Total symptom scores (TSSs) were recorded by 21 participants with house dust mite allergy (M+) in the natural setting and during repeated exposures of 3 hours per day to house dust mite allergen in an allergen challenge chamber (ACC). In M+ participants the peripheral blood and nasal cells were assayed for T-cell activation and transcriptomic profiles (by using RNA sequencing), respectively. Participants allergic to mountain cedar (n = 21), oak (n = 34), and ragweed (n = 23) recorded TSSs during separate out-of-season exposures to these pollens (any pollen sensitization [P+]) in the ACC; a subset recorded TSSs in the pollination seasons.

Results: The hierarchy of TSSs (highest to lowest) among M+ participants tracked the following skin prick test sensitization statuses: M+P+C- > M+P+C+ > M+P-C- > M+P-C+. In nasal cells and peripheral blood the immune/inflammatory responses were rapidly resolved in M+P+C+ compared with M+P+C- participants. Among those allergic to pollen, C+ was associated with a lower TSS during pollen challenges and the pollination season. After aggregated analysis of all 4 ACC

studies, C+ status was associated with a 2.8-fold greater likelihood of a lower TSS compared with C- status (odds ratio, 2.78; 95% CI, 1.18-6.67; *P* = .02).

Conclusions: C+ status is associated with mitigation of AR symptom severity in adults with AR. (*J Allergy Clin Immunol* 2015;■■■■:■■■■-■■■■.)

Key words: Cockroach sensitization, allergen challenge chamber, allergic rhinoconjunctivitis, T-cell activation, aeroallergens, house dust mite, skin prick test, pollen

Allergic rhinoconjunctivitis (AR) is the most frequent IgE-mediated disease, with its prevalence reaching 40% in some surveys.¹⁻³ Depending on sensitization status, symptoms can be precipitated by seasonal/outdoor aeroallergens (eg, pollen), perennial/indoor aeroallergens (eg, house dust mites [HDMs] and cockroaches), or both.^{4,5} Sensitization to the most common classes of aeroallergens is typically assessed by using skin prick test (SPT) responses.^{4,5} However, even among patients with sensitization to multiple aeroallergens, the severity of AR symptoms varies widely.^{4,5} Interindividual differences in AR symptom severity might relate to differences in aeroallergen concentrations in the environment. Another possibility is that even after controlling for concentration, sensitization to specific aeroallergens might modify the severity of AR symptoms elicited on exposure to another aeroallergen but not the sensitizing allergen. In other words, it is not the number of positive SPT responses *per se* but rather the presence of versus the lack of sensitization to specific aeroallergens that underpins interindividual variation in AR symptom severity.

We sought to determine whether sensitization to specific aeroallergens modifies AR symptom severity. To address this in the most controlled manner possible, we took advantage of 4 separate aeroallergen exposure studies previously conducted in an allergen challenge chamber (ACC). In these studies allergic adults volunteered for challenges to indoor or outdoor aeroallergens (HDMs, mountain cedar, Virginia live oak [VLO], and ragweed pollens).⁶⁻⁸ Confounding factors were mitigated by 2 conditions. First, specific concentrations of the aeroallergens were delivered in a highly controlled environment, which elicited symptoms highly consistent with those observed in natural settings.⁶⁻⁸ Second, studies were conducted when the concentration of tree pollens, a major inducer of AR symptoms in south Texas, was low. This strategy increased the likelihood that the symptoms elicited in the ACC were specifically related to the challenge aeroallergen and reduced the likelihood of confounding from exposure to other aeroallergens.^{6,7}

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Abbreviations used

ACC:	Allergen challenge chamber
AR:	Allergic rhinoconjunctivitis
C+:	Cockroach sensitization
GEE:	Generalized estimating equations
HDM:	House dust mite
ILC:	Innate lymphoid cell
M+:	House dust mite allergy
P+:	Any pollen sensitization
SPT:	Skin prick test
ssIgE:	Serum specific IgE
TLR:	Toll-like receptor
TSS:	Total symptom score
VLO:	Virginia live oak

Two sets of allergic individuals were evaluated. Study set 1 comprised participants who were allergic to HDM (M+) and challenged with HDM (*Dermatophagoides pteronyssinus*) in an ACC.⁸ Three end points were evaluated: (1) AR symptom severity; (2) levels of peripheral blood T-cell activation, including CD4/CD8 T-cell ratio, a known correlate of T-cell activation⁹⁻¹⁵; and (3) gene expression responses in nasal cells determined by means of RNA sequencing. We investigated whether these end points differed in study participants who had a positive versus negative SPT response to pollens, cockroach allergens, or both. Study set 2 comprised participants who underwent separate exposures to mountain cedar, VLO, or ragweed pollen (any pollen sensitization [P+]) in an ACC.⁶ In these participants we determined whether sensitization to cockroach influenced AR symptom severity.

Increasing evidence supports the hygiene hypothesis as a pathogenic basis for allergy, meaning that early-life exposure to certain allergens and bacteria might be associated with significant reductions in AR symptom severity and atopy.^{5,16-18} Recently, Palm et al⁵ revisited the tenets of the hygiene hypothesis. Although allergies are generally viewed as a mistargeted immune response that evolved to provide immunity to infectious agents, these authors posited that allergic immunity also has an important role in host defense against multiple noninfectious environmental insults.⁵ Therefore we postulated that cockroach sensitization (C+) might be a proxy for childhood exposure to infectious and other environmental agents. We determined whether C+ status was associated with (1) mitigation of AR symptom severity in participants of study sets 1 and 2 in both the ACC and natural settings and (2) dampened immune activation/responses in study set 1 participants.

METHODS**Study participants for challenge studies in the ACC**

The terms sensitization, sensitive, sensitivity, and reactivity are used interchangeably and refer to a positive SPT response (≥ 5 mm) to an aeroallergen. Allergic status or allergenicity refers to participants with a positive SPT response and symptoms on exposure to the specific allergen. Four separate groups of allergic participants were selected from a volunteer pool for the ACC studies conducted at the dates/months shown in Fig 1.⁶⁻⁸ We first excluded participants who were not sensitive to the aeroallergen with which they had volunteered to be challenged in the ACC. Among participants with a positive SPT response for the challenge aeroallergen, we excluded those without symptoms consistent with AR to the challenge aeroallergen in the natural setting (for inclusion into the HDM challenge study) and pollination

seasons (for inclusion into the pollen challenge studies). We also excluded those who did not meet the additional inclusion/exclusion criteria noted in Table E1 in this article's Online Repository at www.jacionline.org for the HDM challenge study⁸; similar inclusion/exclusion criteria applied to the pollen challenge studies.^{6,7}

To mitigate confounding in the HDM challenge study, we excluded those allergic to ragweed because the HDM study was conducted in the fall. Ragweed was present in the environment, but tree pollens were not. Thus sensitization status to aeroallergens other than the challenge aeroallergen did not influence participant selection. For example, when participants were challenged with HDM, each had both a positive SPT response to HDM and symptoms consistent with HDM allergy in their natural environment. Selection for the HDM challenge study was not predicated on allergenicity to cockroach or pollens. Similarly, selection for pollen challenge studies required allergenicity to the challenge pollen and was not influenced by SPT responses or allergenicity to other aeroallergens.

Study set 1 comprised 21 participants who were allergic to HDM and completed all the phases of the HDM challenge study (Fig 2, A).⁸ Participants in study set 1 were designated as M+ to reflect their allergenicity to HDM (Fig 1). Participants in study set 1 who were also allergic to pollens were designated as M+P+. Study set 2 included 3 separate groups of participants who were allergic to mountain cedar (n = 21), VLO (n = 34), and ragweed (n = 23) pollens, respectively. They were collectively designated as P+ to reflect their allergenicity to pollens. Symptom data collected in the mountain cedar, VLO, and ragweed pollination seasons were also available from 16, 17, and 23 participants, respectively, as described previously.^{6,7} All participants with a positive SPT response to cockroach were designated as C+. Symptom responsiveness of C+ participants to cockroach was unknown because this can only be confirmed by a challenge study with cockroach aeroallergen.

The IntegReview Institutional Review Board (Austin, Tex) approved the studies. Allergy medications were prohibited during the study period (see Table E2 in this article's Online Repository at www.jacionline.org). All participants provided written informed consent. Studies were conducted according to Good Clinical Practice standards.

HDM and pollen challenge studies in the ACC

The HDM challenge study (study set 1) had 4 consecutive phases (Fig 2, A): (1) a 4-day run-in phase in the natural setting; (2) exposure to HDM allergen (*Dermatophagoides pteronyssinus*) for 3 hours each on 4 consecutive days (exposures 1-4, ACC-I phase); (3) a 38-day observation phase in the natural setting; and (4) 4 consecutive exposures (exposures 5-8) to HDM using conditions identical to those in ACC-I (ACC-II phase). Aeroallergen exposures in ACC-I and ACC-II commenced at 6 PM. Delivery of HDM powder and collection and quantification of HDM antigen in the ACC were performed as described previously⁸ and summarized in the Methods section in this article's Online Repository at www.jacionline.org. Mountain cedar, VLO, and ragweed challenge studies (study set 2) included separate out-of-season exposures in the ACC to these 3 pollens for 3 hours each on 2 (mountain cedar and VLO) or 4 (ragweed) consecutive days, as described previously (Fig 1).^{6,7}

Clinical end points in HDM and pollen challenge studies

In study set 1 each participant recorded nasal and ocular symptom scores on a Likert scale of 0 to 4 (see Table E3 in this article's Online Repository at www.jacionline.org). Total symptom scores (TSSs) were recorded as follows: (1) twice daily in the run-in and observation phases as reflective symptom scores; (2) before challenge with HDM (ie, baseline instantaneous TSS); and (3) every 30 minutes during exposure to HDM as instantaneous symptom scores.⁸ Nasal symptoms were congestion, itching, sneezing, and rhinorrhea; ocular symptoms were itching, tearing, and redness. TSSs ranged from 0 to 28. In study set 2 participants recorded reflective TSSs in the pollination seasons twice daily; the protocol for recording instantaneous TSSs in the ACC was similar to that of study set 1, except that the symptoms were graded on a Likert scale of 0 to 3, for a TSS range of 0 to 21 (see Table E4 in this article's Online

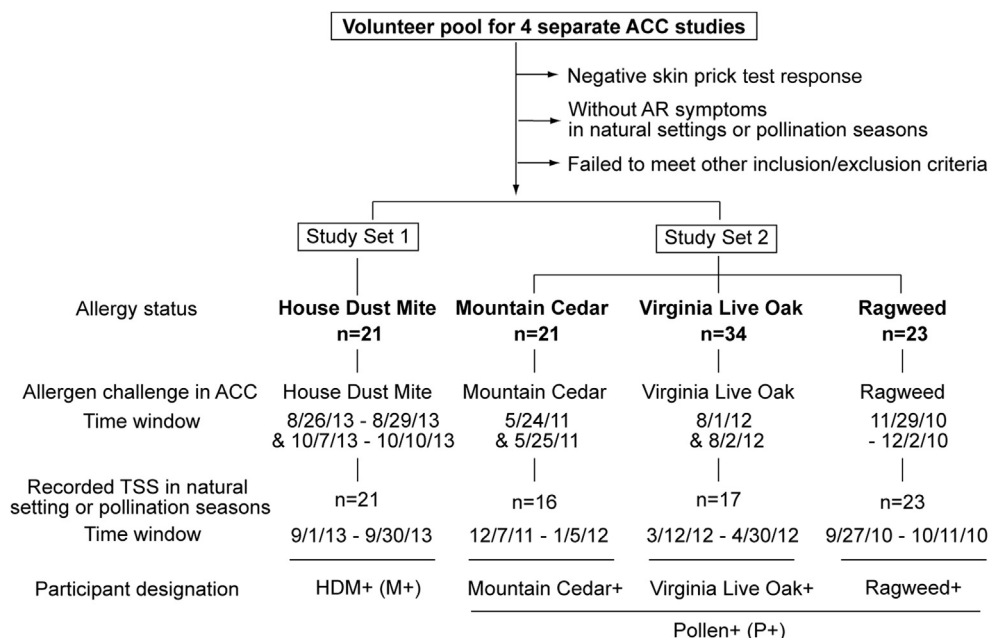


FIG 1. Selection of participants for study sets 1 and 2. Participants were selected based on SPT responses, allergic symptoms in the natural setting (for HDM challenge) and pollination seasons (for pollen challenge), and additional inclusion/exclusion criteria reported previously,⁶⁻⁸ as well as provision of consent for ACC exposure to the indicated aeroallergen. Participants of study sets 1 and 2 volunteered for exposure to HDM and the indicated pollens, respectively.

Repository at www.jacionline.org).^{6,7} Reasons for expanding the Likert scale from 0 to 4 in the HDM study were described previously.⁸ The baseline TSS was the TSS recorded before the start of each exposure. The maximum TSS was the maximum TSS recorded during the challenge. Δ TSS was the difference between the baseline and maximum TSS, which was used as a proxy for responsiveness to the aeroallergen in the ACC. Because we previously showed that TSSs follow a normal distribution,⁸ we used the mean TSS in the current studies.

Laboratory measures in the HDM challenge study

Three outcomes were assessed. First, markers of T-cell activation (CD69⁺HLA-DR⁺) on CD4⁺ and CD8⁺ T cells and the CD4/CD8 T-cell ratio (all from peripheral blood) were determined by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository. Second, serum specific IgE (ssIgE) levels for *D pteronyssinus* were measured by using a commercial ImmunoCAP assay (Thermo Fisher Scientific, Waltham, Mass) on samples collected before challenge with HDM in the ACC. Third, nasal brushings were used to collect nasal cells from M+ participants before and after (from the contralateral nare) exposures 1 and 4 in ACC-I (see the [Methods](#) section in this article's Online Repository). Total RNA from nasal cells was isolated for RNA sequencing, as described in the [Methods](#) section in this article's Online Repository. Samples from 13 M+ participants met quality control criteria for RNA-Seq.

Statistics

Flow cytometric data, including the CD4/CD8 T-cell ratio, were log₂ transformed. A linear generalized estimating equations (GEE) model (based on normal distribution) was used to compute the mean of the TSSs recorded in the ACC and natural settings and T-cell activation measures in participants stratified into subgroups according to their sensitization status. GEE models allowed consideration of correlations among multiple measurements obtained at different time points. The differences between the means of these parameters derived by using linear GEE methods was examined by using a *t* test. The Pearson correlation coefficient was used where appropriate.

Hierarchical clustering was performed by using Euclidean distances with average linkage to determine the association between cockroach sensitization and having higher versus lower TSSs during the ACC challenges. For these analyses, we used all the TSS measurements in study sets 1 and 2 during the ACC challenge studies. This analysis identified clusters of participants with higher versus lower TSSs in the ACC. Logistic regression models were then used to determine the association between C+ status and membership in high versus low TSS clusters. An aggregated (grouped) analysis using TSS data from study sets 1 and 2 was also performed, adjusting for membership in the specific ACC challenge study for which participants had volunteered.

Bioinformatic analyses

The bioinformatics analyses of RNA-Seq data are described in the [Methods](#) section in this article's Online Repository. We determined the relationship between sensitization status and changes in expression levels of genes representative of 6 pathways relevant to allergic manifestations: T_H1-, T_H2-, T-cell activation-, Toll-like receptor (TLR)-, interferon-stimulated gene-, and innate lymphoid cell (ILC)-related genes (genes are listed in [Table E5](#) in this article's Online Repository at www.jacionline.org).¹⁹⁻²⁶

Subgroup analyses

All subgroup analyses were prespecified. To determine associations between pollen and/or cockroach sensitization status with TSSs, M+ participants in study set 1 were stratified initially based on their pollen (ie, M+P+ vs M+P-) and cockroach (M+C+ vs M+C-) sensitization status and then based on their conjoint pollen and C+ status (ie, M+P-C-, M+P-C+, M+P+C-, and M+P+C+). Participants of study set 2 were stratified according to their C+ status.

RESULTS

Participants of study set 1 (HDM study)

Baseline characteristics of the 21 M+ participants who completed each phase of the HDM challenge study are shown

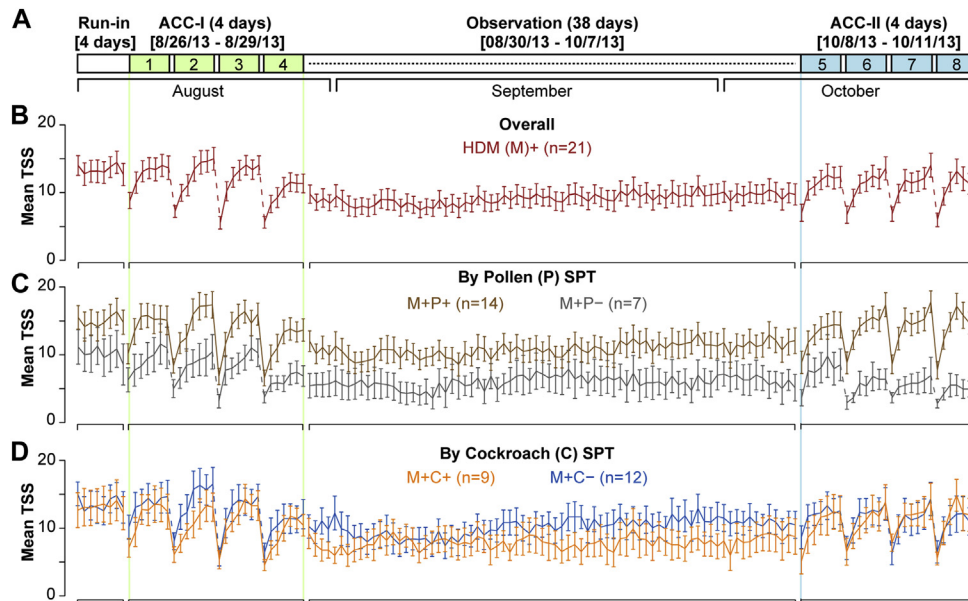


FIG 2. TSSs in participants of the HDM challenge study before and after stratification by pollen and cockroach SPT reactivity. **A**, Study phases and dates/months when the study was conducted. **B-D**, Mean TSSs ($\pm$ SEM) in M+ participants in the indicated study phases before (Fig 2, **B**) and after stratification by pollen (Fig 2, **C**) and cockroach (Fig 2, **D**) SPT reactivity. The 21 M+ participants were classified in Fig 2, **C**, as M+P+ (SPT response ≥ 5 mm for at least 1 pollen, $n = 14$) and M+P- (pollen SPT response < 5 mm, $n = 7$) and in Fig 2, **D**, as M+C+ (cockroach SPT response ≥ 5 mm, $n = 9$) and M+C- (cockroach SPT response < 5 mm, $n = 12$). TSSs recorded at baseline and then 30, 60, 120, 150, and 180 minutes after challenge with HDM in ACC-I and ACC-II are shown. + and -, Positive and negative SPT responses, respectively.

in Table I. Mean age was 41 years, with Hispanic women representing 57% of participants. The median (interquartile range) number of positive SPT responses per participant was 3 (interquartile range, 3-5), which is inclusive of the SPT responses for HDM, M+ participants manifested sensitivity to mountain cedar (67%), VLO (33%), and pecan (14%) pollens and cat allergen (14%). Forty-three percent were also sensitized to cockroach allergen (Table I and see Table E6 in this article's Online Repository at www.jacionline.org). Thus among the M+ participants, the top 2 sensitization aeroallergens were pollens (P+) and cockroach (C+). Only 62% of M+ participants had detectable ssIgE specific for *D pteronyssinus*; the basis for this finding has been discussed previously.⁸ The mean baseline peripheral eosinophil level was 1.9%.

TSSs in the 4 study phases of the HDM challenge study

Fig 2, **B**, shows the overall trajectory of the TSSs in M+ participants during the 4 study phases before stratifying them according to their pollen or cockroach SPT sensitization status. M+ participants were responsive to HDM challenge in both ACC-I and ACC-II. Baseline instantaneous TSSs recorded before the start of exposures 1 and 5 were lower than the reflective TSSs recorded during the natural settings that immediately preceded these exposures (ie, the run-in and observation periods, respectively). As discussed previously,⁸ we attributed this lower TSS to 3 factors: (1) differences in how the TSS was scored in the natural versus ACC settings (reflective vs instantaneous scoring, see the Methods section in this article's Online

TABLE I. Characteristics of 21 M+ participants in study set 1 (HDM challenge study)

Age (y), mean (SD)	41.1 (8.7)
Male sex, no. (%)	6 (28.6)
Ethnicity, no. (%)	
White	7 (33.3)
Hispanic	12 (57.1)
African American	2 (9.5)
No. of positive SPT responses of 17 aeroallergens tested, median (IQR)	3 (3-5)
Positive SPT response to any pollen (P+), no. (%)	14 (66.7)
Mountain cedar +, no. (%)	14 (66.7)
C+, no. (%)	9 (42.9)
M+/P+/C+, no. (%)	7 (33)
M+/P+/C-, no. (%)	7 (33)
M+/P-/C-, no. (%)	5 (24)
M+/P-/C+, no. (%)	2 (9.5)
IgE specific to HDM (Der p 1), ng/mL (range)	0.6 (0-2.4)
Peripheral eosinophils (%), mean (SD)	1.9 (1.2)

+ and -, Positive and negative SPT responses, respectively; IQR, interquartile range.

Repository); (2) increased emphasis/education by research staff in the ACC on accurate recording of the TSS; and (3) the controlled environment in the ACC.

Clinical symptoms by sensitization status in the HDM study

Sex, age, ethnicity, eosinophil counts, and ssIgE levels to *D pteronyssinus* were not consistently associated with TSSs in the natural setting or ACC phases (see Table E7 in this article's Online Repository at www.jacionline.org). We next determined

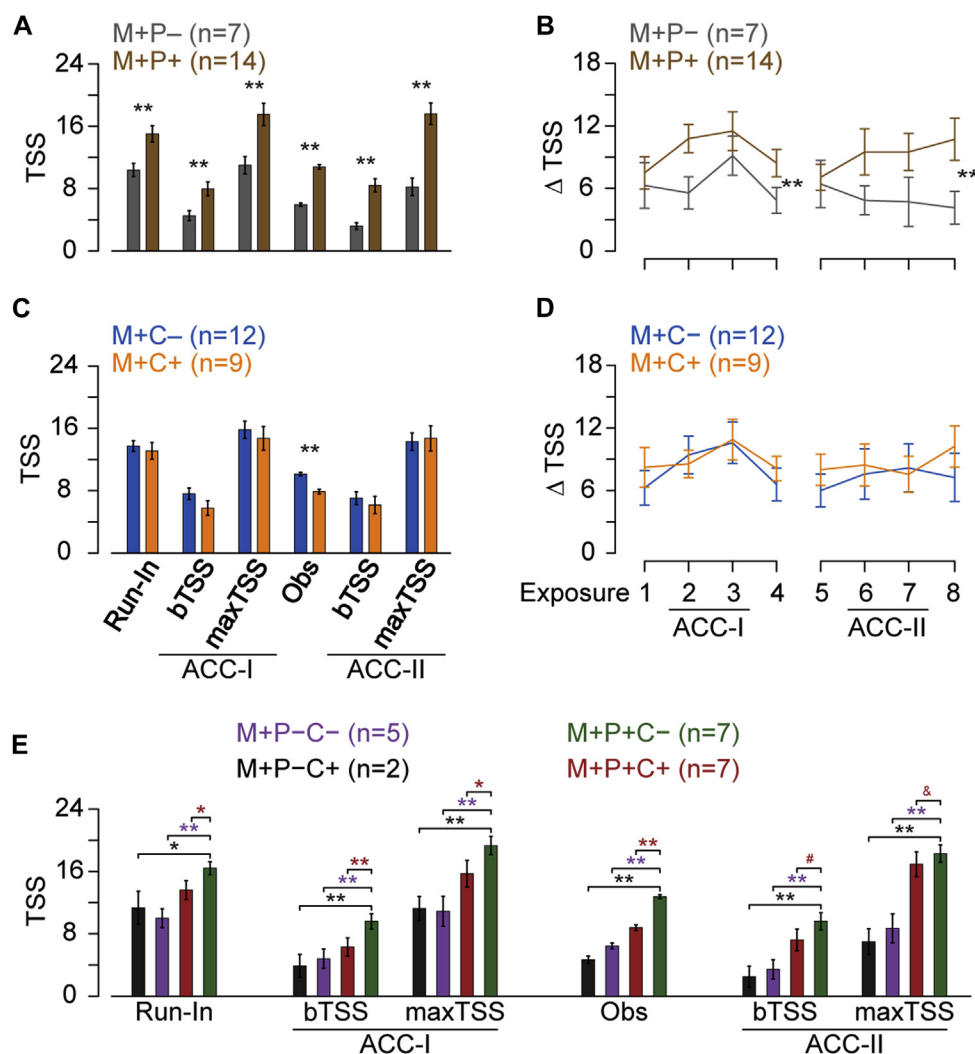


FIG 3. TSSs recorded during HDM exposure according to pollen and/or cockroach SPT reactivity. **A**, TSSs in the indicated study phases in M+ participants stratified by pollen SPT reactivity. **B**, Mean change in TSS (Δ TSS) in M+ participants stratified by pollen SPT reactivity in ACC-I and ACC-II. **C** and **D**, Same as Fig 3, **A** and **B**, except participants were stratified by cockroach SPT reactivity. **E**, TSSs in M+ participants stratified by both pollen and cockroach SPT reactivity. Fig 3, **A**, **C**, and **E**, show mean TSSs ($\pm$ SEM) from all TSSs in the run-in and observation (*Obs*) study phases and baseline (*bTSS*) and maximum (*maxTSS*) TSSs recorded during ACC-I and ACC-II. + and -, Positive and negative SPT responses, respectively. * $.01 < P < .05$, ** $P < .01$, # $P = .09$, and & $P = .39$. The reference group in Fig 3, **E**, is made up of participants with M+P+C- sensitization status.

the TSSs of M+ participants according to their SPT sensitization status to either pollens or cockroach (Figs 2, C and D, and 3). Compared with M+P- participants, M+P+ participants manifested (1) higher TSSs in each of the study phases (Figs 2, C, and 3, A) and (2) increased responsiveness to HDM challenge (Fig 3, B). M+C+ and M+C- status associated with similar (1) TSS values except for a lower TSS in the observation phase (Figs 2, D, and 3, C) and (2) responsiveness to HDM in the ACC (Fig 3, D).

However, after conjoint stratification by both pollen and cockroach sensitization status, in general, the hierarchy of TSS values (highest to lowest) in the ACC and the natural settings (run-in and observation phases) was as follows: M+P+C- (green), M+P+C+ (red), M+P-C- (purple), and M+P-C+ (black; Fig 3, E). Overall, among M+P+ and

M+P- participants, C+ status was associated with lower TSSs, and the protective effect of C+ status was most prominent among M+P+ participants.

T-cell activation markers by sensitization status in the HDM study

Among M+ participants, there was an inverse relationship between CD4/CD8 T-cell ratios and the proportion of CD69⁺HLA-DR⁺CD8⁺ T cells ($r = -0.56$, $P < .01$; see Fig E1 in this article's Online Repository at www.jacionline.org). A weaker inverse association was observed with CD69⁺HLA-DR⁺CD4⁺ T cells (data not shown). Although M+P+ participants had significantly higher levels of activated CD4⁺ and CD8⁺ T cells compared with M+P- participants

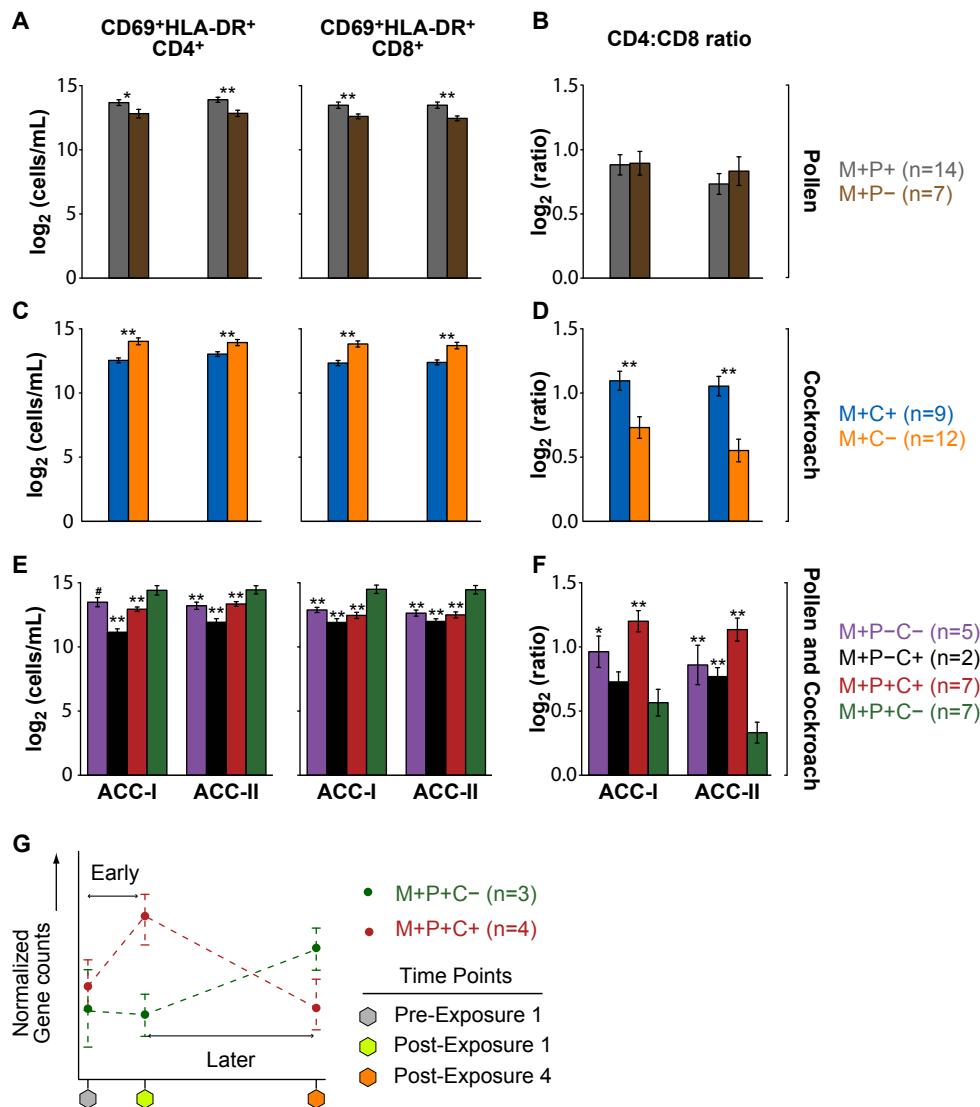


FIG 4. T-cell activation, CD4/CD8 T-cell ratio, and RNA transcriptomic expression pattern in M+ participants by pollen and C+ status. Shown are mean log₂ values ($\pm$ SEM) of CD69⁺HLA-DR⁺CD4⁺ and CD69⁺HLA-DR⁺CD8⁺ T cells (**A**, **C**, and **E**) and CD4/CD8 T-cell ratios (**B**, **D**, and **F**) in participants categorized by their SPT status to pollens (Fig 4, **A** and **B**), cockroach (Fig 4, **C** and **D**), or both (Fig 4, **E** and **F**). Data were derived before and after the first and final exposure to HDM in ACC-I (exposures 1 and 4) and ACC-II (exposures 5 and 8). *.01 < *P* < .05, **.01 < *P* < .01, and #.05 < *P* < .15. The reference group in Fig 4, **E** and **F**, is made up of participants with the M+P+C- sensitization status. + and -, Positive and negative SPT responses, respectively. **G**, Overall expression patterns of genes in the 6 pathways analyzed in nasal cells collected before and after HDM challenge in participants with M+P+C+ and M+P+C- sensitization status. Also shown is the schema for analyzing changes in gene expression that occurred early versus later by using RNA-Seq data available at 3 time points (see key below). Circles indicate means of the normalized gene expression values, and error bars indicate SEMs. Details of changes in expression levels of genes in the 6 pathways or gene systems are shown in Fig E2.

(Fig 4, **A**), we observed no differences in CD4/CD8 ratios between participant groups (Fig 4, **B**).

M+C+ participants had significantly lower levels of activated CD4⁺ and CD8⁺ T cells and higher CD4/CD8 T-cell ratios compared with M+C- participants (Fig 4, **C** and **D**). After conjoint analysis, M+P+C- participants had the highest proportion of activated T cells and the lowest CD4/CD8 ratios compared with all other participants (Fig 4, **E** and **F**). These differences were also evident at baseline (ie, before the HDM challenge study [data not shown]).

RNA expression profiles in nasal cells by sensitization status in the HDM study

RNA sequencing results obtained from nasal cells of M+P+C- and M+P+C+ participants were evaluated for temporal changes in expression levels of genes in 6 immune pathways by comparing changes between postexposure 1 versus pre-exposure 1 (designated as early response) and separately between postexposure 4 versus postexposure 1 (designated as later response; Fig 4, **G**). The few M+P-C+ participants (*n* = 2) precluded comparisons between M+P-C+ and

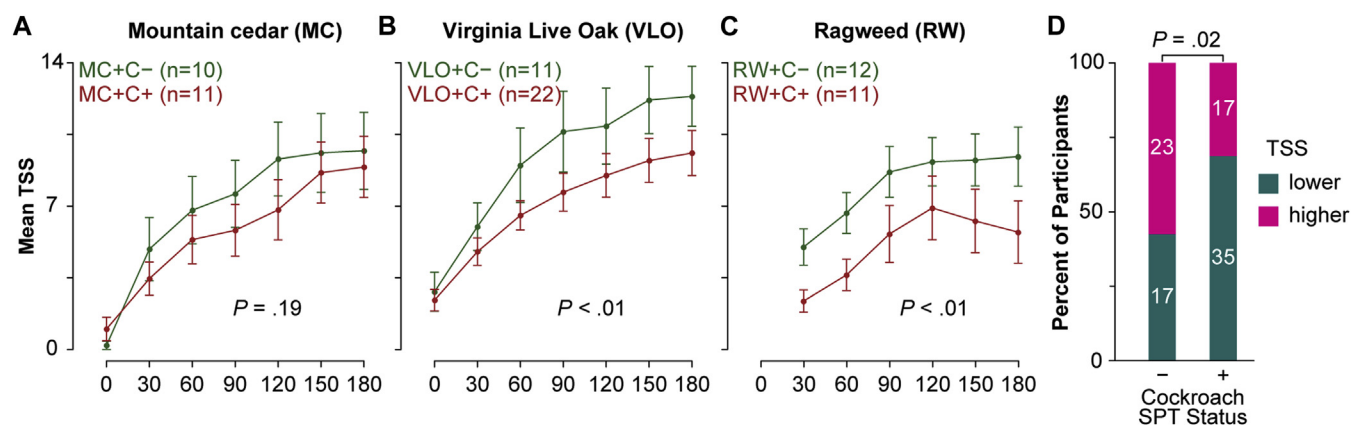


FIG 5. Association of cockroach sensitivity and TSS responses in 4 different ACC challenge studies. **A-C**, Mean ($\pm$ SEM) TSSs recorded in 3 separate groups of participants who volunteered for out-of-season exposures to mountain cedar (Fig 5, A), VLO (Fig 5, B), or ragweed (Fig 5, C) in the ACC; TSSs were stratified according to C+ status. TSS data are from the challenge study conducted on the second day for each pollen. Baseline TSS data were unavailable for the ragweed exposure study. **D**, Proportion of participants who were sensitive (+) versus not sensitive (-) to cockroach and belonged to the cluster associated with higher versus lower TSSs after challenge to an aeroallergen in the ACC (logistic regression results are shown in Fig E5). The results are derived by a pooled analysis and comprised of participants who were M+P+ and exposed to HDM and allergic to mountain cedar, VLO, or ragweed and exposed to these pollens.

M+P-C- participants. Fig 4, G, shows the general temporal pattern of the expression levels of representative genes in each of the 6 gene pathways in M+P+C- and M+P+C+ participants. Fig E2 in this article's Online Repository at www.jacionline.org shows the temporal pattern observed in each pathway. Gene expression levels increased early in M+P+C- participants, followed by a decrease. In contrast, in M+P+C+ participants the early response was muted, followed by increased gene expression levels. The most prominent changes occurred in genes associated with ILCs, T_H2 responses, and TLR-related genes (see Fig E2). This temporal pattern was not observed in 3 different sets of randomly selected genes (see Fig E3 in this article's Online Repository at www.jacionline.org).

Cockroach sensitization as a determinant of responsiveness to aeroallergens

Participants who were allergic to mountain cedar, VLO, and ragweed and were C+ manifested lower TSSs after challenge with those 3 pollens in the ACC (Fig 5, A-C). A similar association with C+ status was also observed during some periods in the natural setting (see Fig E4).

Hierarch clustering dichotomized participants in study sets 1 and 2 into 2 broad clusters with higher versus lower TSSs in the ACC ($P < .01$, see Fig E5 in this article's Online Repository at www.jacionline.org). In study sets 1 and 2 those who were also C+ compared with those who were C- were more likely to be in the cluster associated with a lower TSS in the ACC (see Fig E6 in this article's Online Repository at www.jacionline.org). An aggregated analysis of study sets 1 and 2 demonstrated that C+ status was associated with a nearly 2.8-fold higher likelihood of membership to the cluster with lower TSSs (odds ratio, 2.78; 95% CI, 1.18-6.67; $P = .02$; Fig 5, D, and see Fig E6).

DISCUSSION

M+P+ participants experienced greater AR symptom severity after exposure to HDM allergen in the ACC compared with

M+P- participants, indicating that allergy to pollens magnifies the symptoms related to HDM exposure in M+ participants. However, further stratification of M+P+ and M+P- participants by C+ status revealed that the hierarchy of the TSS (higher to lower) after HDM exposure was as follows: M+P+C- > M+P+C+ > M+P-C- > M+P-C+. A similar hierarchy of TSSs was also observed in the natural setting. The consistency of these observations in a controlled setting (ACC) and natural environment suggests that in those allergic to HDM, the sensitization pattern to heterologous aeroallergens provides a basis for wide interindividual differences in the severity of AR symptoms. Specifically, symptom severity to HDM is magnified in participants with allergenicity to pollens, whereas C+ status associates with a mitigation in symptom severity, especially in those allergic to pollen. These findings affirm our study hypothesis.

Participants who were allergic to pollen and were also C+ manifested reduced AR symptom severity during separate out-of-season challenges to 3 different pollens. Furthermore, C+ status was also associated with reduced AR symptom severity in each pollination season. A pooled analysis of all participants allergic to pollen in the 4 challenge studies indicated that C+ status was associated with a nearly 2.8-fold greater likelihood of having lower AR symptom severity after challenge with an aeroallergen in the ACC.

To understand the mechanistic basis for the reduced AR symptom severity associated with C+ status, we conducted immunologic and whole gene expression studies in participants of the HDM challenge study. Among M+ participants, C+ compared with C- status was associated with higher levels of activated T cells and lower CD4/CD8 T-cell ratios in the peripheral blood. The highest levels of T-cell activation and the lowest CD4/CD8 T-cell ratios were in M+P+C- participants relative to all other participants. We focused on peripheral blood T-cell activation because it is a well-documented determinant of disease severity in atopy^{27,28} and immunologic/inflammatory diseases.^{19,29,30} We also focused on the CD4/CD8 T-cell ratio as a readily measureable biomarker of T-cell activation.⁹⁻¹⁵ An

inverted CD4/CD8 T-cell ratio (<1.0) is associated with an immune risk phenotype characterized by increased morbidity and mortality and is correlated with increased levels of T-cell activation.^{9-15,31} In asthmatic adults the CD4/CD8 T-cell ratio is inversely correlated with bronchial hyperactivity,²⁷ and a lower CD4/CD8 ratio has been observed before and during the natural allergy season in those with atopic allergies.²⁸ Consistent with these prior findings, we also observed an inverse correlation between levels of activated CD8⁺ T cells and CD4/CD8 ratios.

Nasal cells of participants with M+P+C− versus M+P+C+ sensitization status had different temporal expression patterns of genes involved in immune responses. On challenge with HDM in the ACC, M+P+C+ status was associated with an early robust immune/inflammatory response followed by a rapid end of this response, despite repeated intervening HDM challenges. In contrast, M+P+C− sensitization status was associated with a muted inflammatory response to HDM challenge. Instead of a resolution, as with M+P+C+ status, immune/inflammatory responses increased or persisted with continued exposure.

Among the pathways tested, the most significant differences were in the T_H2-, TLR-, and ILC-related genes, which have been implicated in cockroach allergy or atopy in general.³²⁻³⁴ In other inflammatory settings (eg, HIV infection) there is growing evidence that it is not the initial increase in activation that contributes to ongoing disease pathogenesis but whether activation is resolved promptly.^{35,36} Thus the temporal gene expression patterns we observed might contribute to the differences in the symptom severity observed between the study groups.

The basis for the mitigating effects on AR symptom severity associated with C+ status is undetermined. Our results might invoke the hygiene hypothesis of allergy, which asserts that the cleaner environment related to modernization decreases the immune system's exposure to infectious agents, resulting in increased incidence of allergy.^{5,16-18,37,38} Thus C+ status might be a proxy for early-life exposure to not only cockroach but also possibly microorganisms and other environmental agents. These exposures might skew the immune response such that during subsequent exposures to other aeroallergens (eg, pollens), C+ subjects manifest reduced clinical responsiveness and T-cell activation. Supporting this contention, exposure to cockroach, mouse, and cat allergen during an infant's first year of life had a strong inverse relationship with recurrent wheezing 2 years later.¹⁶

In summary, our results underscore that the studies conducted within an ACC have utility for identifying pathogenic determinants that might underpin high versus low clinical responses to aeroallergens in a nonconfounded manner. Cockroach sensitization in polyallergic subjects influences AR disease severity in an ACC and natural settings. Lower AR symptom severity associated with cockroach sensitization might relate to lower T-cell activation in the periphery and a gene expression pattern that connotes rapid termination of immune/inflammatory pathways induced by aeroallergen exposure.

These observations have broad implications. Accounting for the overall aeroallergen sensitization status of participants in clinical trials might help mitigate confounding. This is because misallocation of trial participants in the intervention versus placebo arm with sensitization profiles that associate with symptom promotion (eg, M+P+C−) versus mitigation (eg,

M+P+C+) might artificially skew statistical analyses. The same concern applies to investigations of immune correlates associated with AR severity.

Clinical implications: The pattern of allergen sensitization, as assessed by SPT reactivity, influences the symptom severity and immune responses of patients with AR. Balancing the sensitization status of patients with AR across the treatment versus placebo arms in clinical trials might mitigate confounding.

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I. Study participants:

87 participants were studied herein, and their sources are summarized below. The characteristics of the house dust mite (HDM) sensitive participants (M+) are described previously and summarized in **Table 1**. The characteristics of the participants in the mountain cedar, Virginia live oak (VLO) and ragweed allergen challenge studies are as reported previously^{E1}.

Derivation of participants studied.			
	ACC*	Natural setting	Comments
House dust mite study	21	21	Derived from ^{E2}
Mountain cedar study	21	16	Derived from ^{E1}
Virginia live oak study	34	17	Derived from ^{E1}
Ragweed study	23	23	Derived from ^{E1}
*Total number of participants was 87, as some participants volunteered for more than one pollen challenge study.			

II. Computation of Total symptom scores (TSS) for HDM challenge study

At the initial screening visit, a Medical Event Calendar was dispensed to each participant to monitor adverse events and concomitant medications. A Subject Run-in Diary was dispensed to participants for recording reflective nasal and ocular symptoms on a Likert scale of zero to four (scale noted in **Table E3**), twice daily for four days and once in the morning before the start of the ACC-I phase. A similar diary was dispensed for recording of symptoms in the Observation Phase. Nasal symptoms comprised congestion, itching, sneezing, and runny nose. Ocular symptoms comprised itching, tearing, and redness. Reflective scores are indicative of the highest score for each individual symptom experienced at any point during the previous 12-hour period. The sum of the individual symptom scores was used to compute the *reflective* total symptom score (rTSS). A Likert scale was also used for the instantaneous scoring during the

ACC exposures. The *instantaneous* total symptom score (iTSS) is the sum of the highest score for each individual symptom within a 10 minute interval on a Likert scale of zero to four (**Table E3**). In our previous studies that involved pollen challenges^{E1,E3}, we used a TSS scoring system on a 4-point Likert scale (0-3, **Table E4**). The rationale for increasing the scoring scale to a 5-point Likert scale was described previously^{E2}.

III. Computation of total symptom scores for pollen challenge studies

Reflective TSS was recorded during the pollination seasons and ACC in a manner similar to the HDM study. The only difference was that a TSS scoring system on a 4-point Likert scale was utilized (0-3; **Table E4**).

IV. Delivery of allergens in the ACC

Delivery of HDM: The methods for seating of participants, delivery of HDM powder, collection of HDM and quantification of Der p1 antigen levels by ELISA (EL-DP1; Indoor biotechnologies) are as described previously^{E2}. The allergen-dispersal system was activated at predetermined settings to deliver the purified, mite body powder (Greer Laboratories, Lenoir, NC). The Der p1 content in the *D. pteronyssinus* mite body powder (Lot# RMB82M (18950)) was 1,266 µg/G and the Der p2 content was 441 µg/G. The system was calibrated to deliver between 70 to 100 ng/m³ of HDM into a vacant chamber prior to the beginning of the study, and the average HDM delivery was 92 ng/m³ (Ref ^{E2}). This dose was based on preliminary and published studies^{E4-E8} and as discussed previously^{E2}.

Delivery of mountain cedar, Virginia live oak and ragweed: Defatted VLO pollen (mean $\pm$ SD, $10,000 \pm 2000$ grains/m³), defatted short RW pollen (3500 ± 500 grains/m³), and non-defatted MC pollen ($12,500 \pm 2500$ grains/m³) were delivered by a computer-controlled feeder and monitored at counting stations as described previously^{E1}.

V. Medications during and prior to the study

Participants were allergy-drug free during the study periods of each of the ACC challenges as described previously^{E2}. **Table E2** shows medications that were prohibited and were not to be taken during the study except as prescribed by the investigator as a rescue medication.

VI. Laboratory markers

Flow cytometry: Anti-coagulated peripheral blood collected from M+ participants (100 μ L) was stained for markers of T-cell activation in CD4+ and CD4- cells. The following antibodies were used according to manufacturer's directions: CD3 V500, CD4 PE-Cy7, CD69 PE and HLA-DR APC (BD Biosciences). CD3+CD4- T-cells were classified as CD8+ T-cells. After 20 minutes of staining at room temperature, cells were washed with FACS buffer, centrifuged and decanted. Subsequently, blood was lysed with BD FACS lysing solution following manufacturer's instructions and washed twice with FACS buffer. Finally, cells were re-suspended in FACS buffer and read in a BD FACSCanto II. Data was analyzed using FlowJo v8.8.7.

ssIgE levels specific to *D. pteronyssinus*. During the screening visit peripheral

eosinophil counts and serum-specific IgE (ssIgE) specific to *D. pteronyssinus* (assayed by Thermo Fisher Scientific by ImmunoCAP assay) were determined in all participants. A ssIgE specific to HDM ≥ 0.35 kU/L was defined as detectable. ELISA was used to determine the Der p1 antigen content in the HDM delivered in the ACC.

VII. RNA-seq (whole genome RNA sequencing)

Samples: Nasal cells were collected by nasal brushings from participants in ACC-I, before and after the conclusion of exposure 1 (pre- and post-exposure 1, using contralateral nares) and at the conclusion of exposure 4 (post-exposure 4). Nasal cells for RNA-seq were not available pre-exposure 4.

Nasal cell acquisition and RNA isolation: RNase- and DNase-free 4N6FLOQSwabs™ (Life Technologies) were used for nasal mucosal brushing. Each participant was asked to wipe the nose before brushing the nostril. Nasal cells were collected by brushing with a sterile swab inside the nostril using circular movements. Subsequently, swabs were placed into a microcentrifuge tube containing RNA lysis solution (Qiagen) supplemented with Beta-mercaptoethanol (1:100) (Sigma). Samples were immediately refrigerated and then stored at -80°C until the time of isolation of total RNA isolation. If the collected swab showed evidence of hemorrhage, the sample was excluded.

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase I digestion according to the manufacturer's instructions. RNA quantity and purity was determined by spectrophotometry (260/280=1.8-2.0) (Nanodrop) and integrity (RIN) was determined using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano assay

(Agilent Technologies, Palo Alto, CA). Samples with RIN ≥ 7.0 were selected for RNA sequencing.

Library Preparation and Sequencing. Double stranded cDNA library was prepared starting from 1 μg of total RNA input according to the TrueSeq RNA v2 sample preparation kit protocol (Illumina, San Diego, CA). Briefly, mRNA was selected using poly-T oligo-attached magnetic beads and then fragmented. First and second cDNA strands were synthesized and end-repaired. Multiplexed adaptors were ligated after 3'-adenylation. Double stranded cDNA templates were enriched by PCR. Libraries were validated using a DNA High Sensitivity assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified by a Kapa Library quantification kit (Kapa Biosystems, Woburn, MA).

Libraries were clustered using the Illumina cBot (Illumina, San Diego, CA) and then paired-end sequenced (2 x 101 bp) on an Illumina HiSeqTM 2000 (Illumina, San Diego, CA). Base calling and quality filtering were performed using the CASAVA v1.8.2 (Illumina, San Diego, CA) pipeline. Sequences were aligned and mapped to the UCSC hg19 build of the *Homo sapiens sapiens* genome (from Illumina igenomes) using tophat v2.0.1^{E9}. Gene-counts for 23,239 unique, well curated genes were obtained using HTSeq framework v0.5.3P3 (<http://www-huber.embl.de/users/anders/HTSeq/doc/history.html>). Gene matrix was imported in the statistical package R v2.15.3 and genes with a value of 0 across all three samples (pre- and post-exposure 1 and post-exposure 4) were removed, and the remaining 0's were

changed to 1's. Gene counts were normalized, and dispersion values were estimated using the R package, DESeq v1.10.1^{E10, E11} and the lower quartile (<25%) of genes that had low expression in most samples were removed, leaving a total of 15,673 genes for further analyses.

Th1 and Th2 genes: Th1 (n=147) and Th2 (n=577) specific genes were derived from cross-sectional comparisons of RNA-seq expression data obtained from a previous study on T-cell polarization performed in our laboratory [unpublished work]. In that study, activated T-cells (stimulated by anti-CD3 and anti-CD28 antibodies) were cultured under polarizing conditions towards Th1 (using rIL-2m rIL-12, anti-IL4, anti-IL10) and Th2 (rIL4, anti-IFNG, anti-IL10) *in vitro* for 14 days. Of these genes, 139 Th1 genes and 552 Th2 genes overlapped with the quality filtered dataset from the HDM study. The list of these genes is in **Table E7**.

TCA, ILC and ISG genes: T-cell activation (TCA) related genes (n=58), genes that broadly relate to innate lymphoid cells (n=30) and interferon stimulated genes-related genes (n=379) were derived from literature database searches^{E12-E19}. Of these, 44 TCA-related; 20 ILC-related and 338 ISG-related genes overlapped with the quality filter dataset from the HDM study. The list of these genes is in **Table E7**.

TLR genes: Approximately 110 genes belonging to the Toll-like receptor (TLR) signaling pathway were derived from SABiosciences website [http://www.sabiosciences.com/rt_

pcr_product/HTML/PAHS-018A.html]. 76 TLR genes overlapped with the quality filtered dataset from the HDM study. The list of these genes is in **Table E7**.

Random sets of genes: To determine the specificity of the findings, three sets of randomly selected genes (n=200 each) were analyzed. The list of these genes is in **Table E7**.

Bioinformatics: A linear generalized estimating equations (GEE) model based on the Gamma distribution was used to plot the expression pattern and also test if the mean change in normalized expression values for all of the genes included in a gene/pathway groups (Th1, Th2, TCA, TLR, ISG, ILC, and the 3 random groups) between pre-exposure 1 vs. post-exposure 1 and post-exposure 1 vs. post-exposure 1 in ACC-I differs between participants with M+P+C+ versus M+P+C– sensitization status. A *P*–value < 0.05 threshold was used to determine significant difference between the groups.

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Legends to ONLINE REPOSITORY figures:

Figure E1. Correlation between the CD4:CD8 T-cell ratio and CD8+CD69+HLA-DR+ T-cells. These laboratory parameters were collected at pre- and post- exposure 1 and day 4 of ACC-I of the HDM exposure study (study set 1 participants). The trend line, Pearson correlation r (rho value) and P value are shown. Pearson's correlation coefficient was used because $\log_2$ transformation provides normally distributed data.

Figure E2. Expression patterns of genes in defined immune pathways in nasal cells collected before and after HDM challenge in participants with the M+P+C+ and M+P+C- sensitization status. (A) Schema for analyzing changes in gene expression that occurred early versus later using RNA-Seq data available at three time points (see key below). The circles indicate the mean of the normalized gene expression values and the error bars indicate SEM. (B-G) Changes in expression levels of genes (n) in the following pathways or gene systems: Th1 ($n=139$), Th2 ($n=552$), T-cell activation (TCA; $n=44$)-, Toll-like receptor (TLR; $n=76$)-, Interferon stimulated genes (ISG; $n=338$)-, and Innate lymphoid cells (ILC; $n=20$)-related pathways, derived by RNA-Seq. The genes in each group are listed in **Table E7**. The values within the panels indicate the $\log_2$ of the ratio of changes between the M+P+C+ and M+P+C- groups that occurred early or later. A positive versus negative value indicates a relatively higher or lower change (Δ), respectively, in gene expression levels in participants with M+P+C+ compared with M+P+C- (reference) sensitization status, when comparing the early or later changes. The color of the values indicates the significance threshold of these comparisons and they are shown below (e.g. red denotes $P<0.01$). A linear generalized estimating equations (GEE) model based on the Gamma distribution was used to test if the mean change in normalized expression for genes in the indicated pathways differs between pre- vs. post

ACC exposure 1 and post-exposure 1 vs. post-exposure 4 in participants with M+P+C+ versus M+P+C– sensitization status.

Figure E3. Expression patterns of randomly selected genes in nasal cells collected before and after HDM challenge in participants with M+P+C+ and M+P+C– sensitization status. Note: This figure schema is similar to **Figure E2** except the data are from three different random sets of genes (n=200 each, listed in **Table E7**). The legend is similar to that for Figure E2.

Figure E4. TSS in the natural pollination seasons according to cockroach sensitivity. Mean ($\pm$ SEM) TSS in the natural season of (A) Mountain cedar (MC), (B) Virginia live oak (VLO) and (C) ragweed (ragweed) in participants stratified according to their cockroach (C) SPT reactivity. The corresponding pollen counts are shown in brown shadow. + and – refer to positive versus negative SPT reactivity to the indicated allergen, respectively.

Figure E5. Clustering analysis for pollen+ participants in the HDM, Mountain cedar, Virginia Live Oak, and Ragweed challenge studies. (A-H) Panels A-D depict the hierarchical clustering strategy employed to categorize the indicated groups of allergic participants into groups based on their TSS. The allergic participants clustered into two large color-coded groups. Panels E-H shows the mean TSS with SEM during the ACC studies. The data in panels E-H shows that the two clusters tracked higher

versus lower TSS (pink versus blue). Results are for 4 challenges with HDM in panel E, and 2 challenges each with mountain cedar, VLO and ragweed pollens in panels F, G, H, respectively. A linear GEE model was used to determine the mean TSS across all time points in the ACC and then a t-test was used to compare the TSS between the clusters ($P < 0.01$ for each of the 4 comparisons). (I-L) TSS in the natural setting or pollination season in participants who clustered into the high vs. low TSS groups in the ACC as shown in panels A-H. Results for TSS recordings in the observation period in the HDM study (I), and mountain cedar (J), VLO (K) and ragweed (L) pollination seasons. The mean of the morning and evening TSS measurements were used in the analyses. The P values for the comparison in panels I to L were <0.01 , <0.01 , 0.06 , <0.01 , respectively.

Figure E6. Association between cockroach sensitization status and membership to clusters of participants with high versus low responses following exposure to indoor (HDM) or outdoor aeroallergens in the ACC. Data shown are the odds ratio, 95% confidence intervals, and p-values obtained by logistic regression analyses in which we determined whether cockroach sensitization status predicted membership to the cluster that associated with lower TSS values in each of four studies separately, and overall (combination of the 4 studies). The overall analysis was a pooled analysis of the four separate studies, and was computed using a logistic GEE model that adjusted for study (same as in **Figure 5D**).

Table E1. Inclusion/Exclusion Criteria for HDM positive individuals.**Inclusion Criteria: HDM positive (M+)**

1. Male or female, 18 to 70 years of age.
2. History of AR to HDM in their natural environment.
3. Positive response by prick skin test to *D. pteronyssinus* of ≥ 5 mm.

Exclusion Criteria:

1. Female subject who is pregnant or lactating.
2. Have any significant medical illness that may interfere with the study.
3. Have any abnormalities on physical exam that might interfere with the study.
4. Had a respiratory and/or bacterial sinusitis infection during the past 14 days prior to the Screening visit.
5. Current medical history of pulmonary disease requiring daily drug therapy, or asthma requiring treatment more than 2 times per week.
6. Has participated in a trial with an investigational drug in the last thirty days.
7. History of rebound nasal congestion from extended use of topical decongestants.
8. History of nasal polyps, nasal septal perforation, or significant nasal tract malformations noted on physical exam.
9. Current alcohol or drug abuse or history of the same in the past three years.
10. Use of disallowed medications.
11. Immunotherapy within 90 days of Screening Visit.
12. Subjects with documentation of a positive skin prick test (more than 3.0 mm larger than that produced by the negative control) to any confounding seasonal allergen that might cause clinically relevant symptoms through the duration of the study.
13. Subjects with a skin prick test 5 mm greater than the negative control of perennial antigens (animal dander, cockroach and mold) which cannot be avoided during the study and/or which through medical history might cause clinically relevant symptoms throughout the duration of this study.
14. Subjects with a positive skin prick test response to ragweed pollen.
15. FEV1.0/FVC $\leq 80\%$ of predicted.

HDM, house dust mite; AR, allergic rhinoconjunctivitis; FEV 1.0, forced expiratory volume in one second; FVC, forced vital capacity

Table E2. Prohibited medications during the HDM study period

Disallowed medications for study duration	Required withholding interval prior to study
Topical/Oral/Nasal decongestants	10 days
Ocular allergy preparations	10 days
Short-acting antihistamines (nasal and ocular)	5 days
Long-acting antihistamines (nasal and ocular)	10 days
Over-the-Counter cough and cold preparations or sleep medications	10 days
Airozan® (OTC food supplement /diet leukotrienes)	7 days
Cromolyn, nedocromil, or Iodoxamine (intranasal, ocular, or oral)	14 days
Leukotriene or 5-LO inhibitors	14 days
Inhaled/Oral/Intranasal anticholinergics	14 days
Tricyclic antidepressants	14 days
Monoamine oxidase inhibitors	14 days
Inhaled/systemic/intranasal corticosteroids	30 days
Allergen immunotherapy	90 days
Azoles, anti-fungals	30 days
Immunosuppressive drugs	60 days

Table E3. Likert Scale of Symptom Scores for HDM study

0 = absent	No sign/symptom evident
1 = mild	Sign/Symptom clearly present, but minimal awareness; easily tolerated
2 = moderate	Awareness of sign/symptom; bothersome, but tolerable
3 = severe	Definite awareness of sign/symptom; difficult to tolerate but does not interfere with the activities of daily living
4 = very severe	Difficult to tolerate and interferes with the activities of daily living

Table E4. Likert Scale of Symptom Scores for studies with mountain cedar, Virginia live oak and ragweed

0 = absent	absent
1 = mild	Sign/Symptom clearly present, but minimal awareness; easily tolerated
2 = moderate	Awareness of sign/symptom; bothersome, but tolerable
3 = severe	Definite awareness of sign/symptom; difficult to tolerate but does not interfere with the activities of daily living

Table E5, List of genes used for RNA-Seq analyses: File contains the list of 139 Th1, 552 Th2, 44 TCA, 76 TLR, 338 ISG, 20 ILC-related as well as the three sets of 200 randomly selected genes used for analyses in **Figure E2** and **Figure E3**. Note, this Table E5 is in a separate excel table. TCA, T-cell activation; TLR, Toll-like receptor; ISG, Interferon stimulated genes; ILC, Innate lymphoid cells.

Table E6. Skin-prick test responses in 21 M+ participants*

Allergen	M+ participants
Mountain Cedar	14 (67%)
Virginia Live Oak	7 (33%)
Fall (Cedar) Elm	2 (10%)
Pecan Pollen	3 (14%)
Arizona Ash	0
Ragweed, Short	0
Ragweed, Mixed	0
Spiny Pigweed	0
Bermuda Grass	0
Timothy Grass	0
Mold Mix I **	0
Mold Mix II ***	0
Dog Epithelium	0
Cat Hair	3 (14%)
<i>D. pteronyssinus</i>	21 (100%)
<i>D. farinae</i>	18 (86%)
German Cockroach	9 (43%)

*≥5mm wheal was defined as a positive SPT response.

**Mold mix I contains equal parts of *Alternaria*, *Cladosporium sphaerospermum*, *Drechslera*, *Aspergillus niger*, and *Penicillium notatum*.

***Mold mix II contains equal parts of *Curvularia*, *Fusarium*, *Aureobasidium*, *Mucor*, and *Rhizopus*.

Table E7. Association between demographic features and serum-specific IgE for HDM with TSS in study set 1.

Variables	Run-in		Maximum TSS ACC-I		Δ TSS ACC-I		Observation		Maximum TSS ACC-II		Δ TSS ACC-II	
	Slope	P	Slope	P	Slope	P	Slope	P	Slope	P	Slope	P
Gender	-5.51	0.104	-5.57	0.086	-2.65	0.259	-0.85	0.762	-3.59	0.291	-0.91	0.757
Age	0.27	0.136	0.06	0.722	0.03	0.825	0.08	0.606	0.08	0.666	-0.03	0.835
Race:												
Hispanic	4.55	0.180	4.32	0.439	1.18	0.579	2.09	0.430	2.09	0.548	2.04	0.479
African American	-1.70	0.761	1.76	0.594	8.18	0.032	-5.06	0.260	1.48	0.799	5.64	0.250
%Eosinophil	2.49	0.099	1.89	0.191	0.07	0.945	1.00	0.418	1.61	0.286	-0.20	0.878
Specific IgE	0.08	0.903	1.28	0.048	0.70	0.145	0.24	0.678	1.01	0.140	0.34	0.576

The average of following TSS recordings were used in this table: (i) 9 TSS measurements in the Run-In phase, derived from morning and evening measures during the 4 days prior to ACC-I and a single measure in the morning of the first challenge in ACC-I; (ii) the four maximum TSS values from ACC-I, derived from each of the four daily challenges with HDM; (iii) the four Δ TSS measurements from each of the daily challenges in ACC-I; (iv) the 77 TSS measurements from the observation phase, derived from morning and evening measures for 38 days and the morning of first challenge in ACC-II; (v) the four maximum TSS values from ACC-II, derived from each of the four daily challenges with HDM; and (vi) the four Δ TSS measurements from each of the daily challenges in ACC-II. The associations conducted using linear regression with the indicated variables. With regards to gender, the slope indicates the mean difference between males and females, with females as the reference. With regards to race, the slope indicates the mean difference between either Hispanics and Caucasians or African Americans and Caucasians, with Caucasians as the reference.

Figure E1.

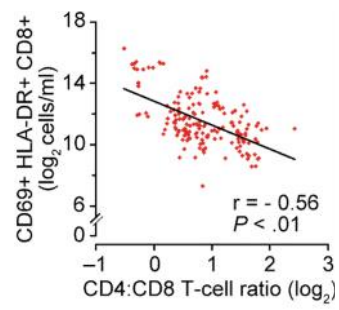


Figure E2.

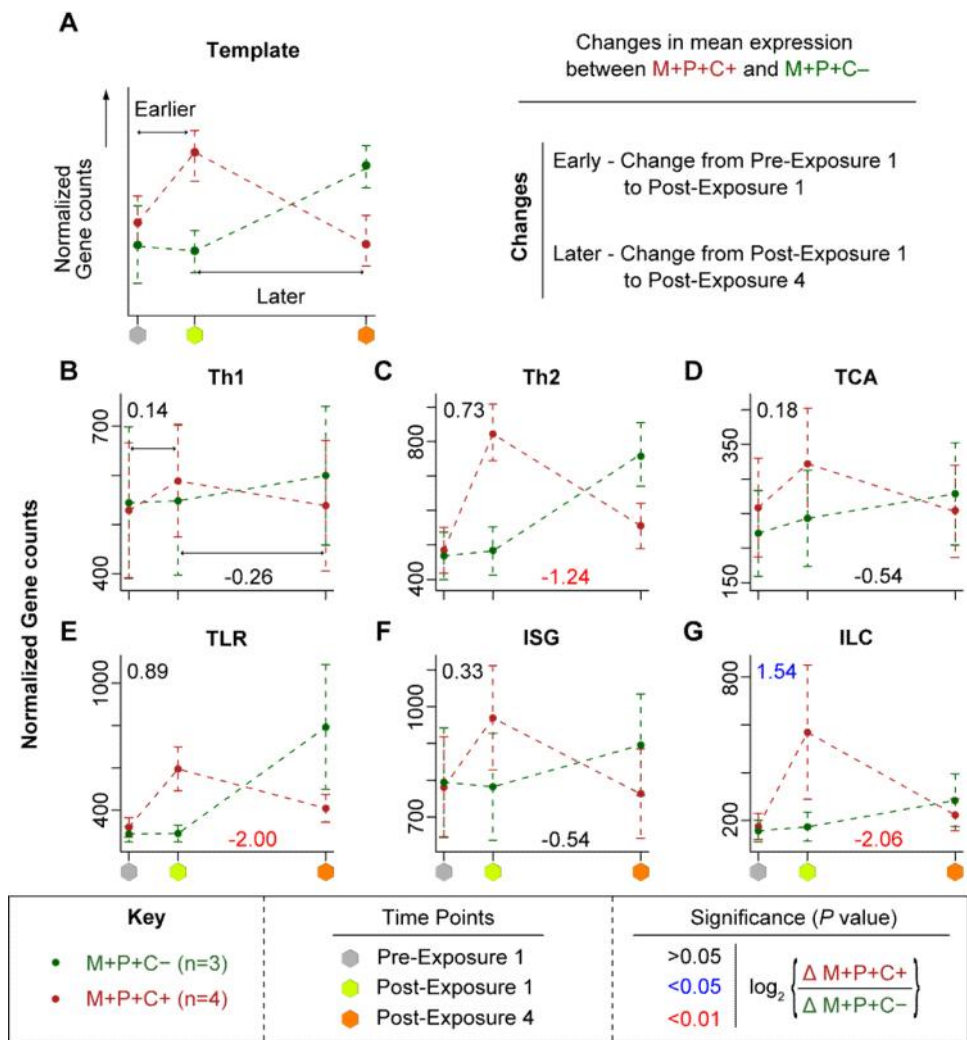


Figure E3. .

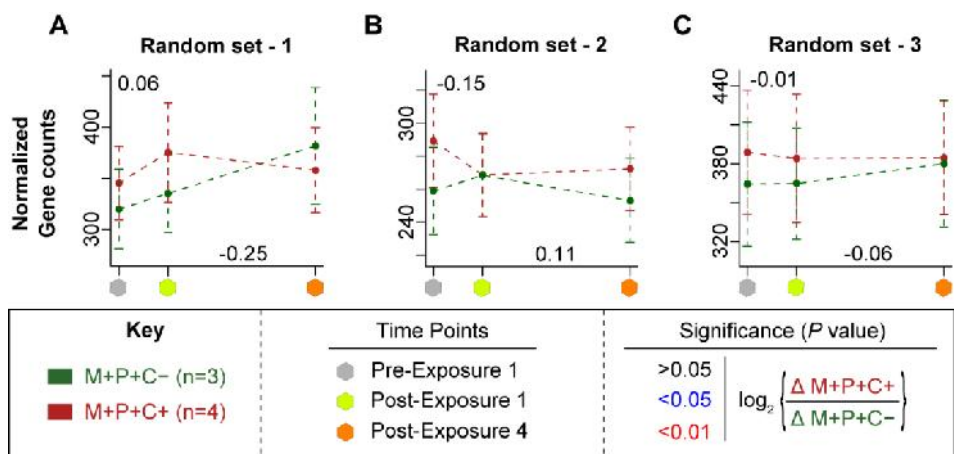


Figure E4..

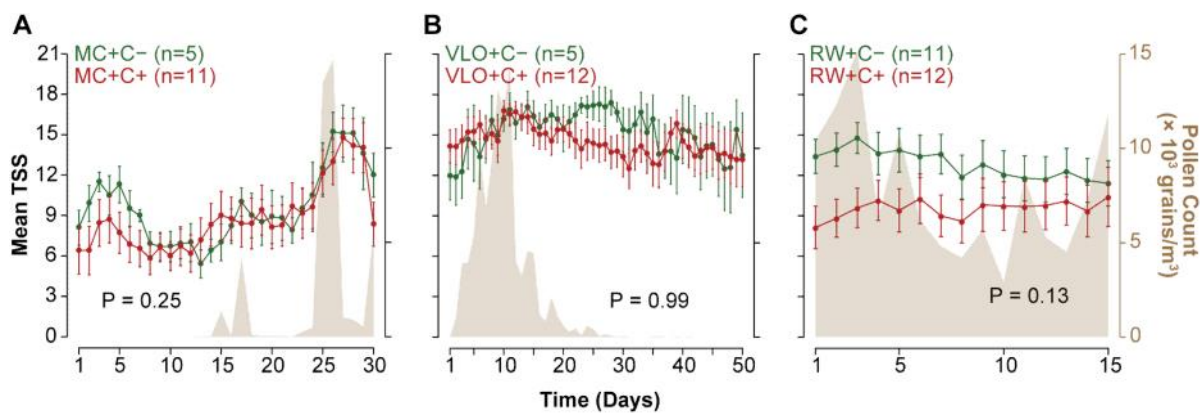


Figure E5..

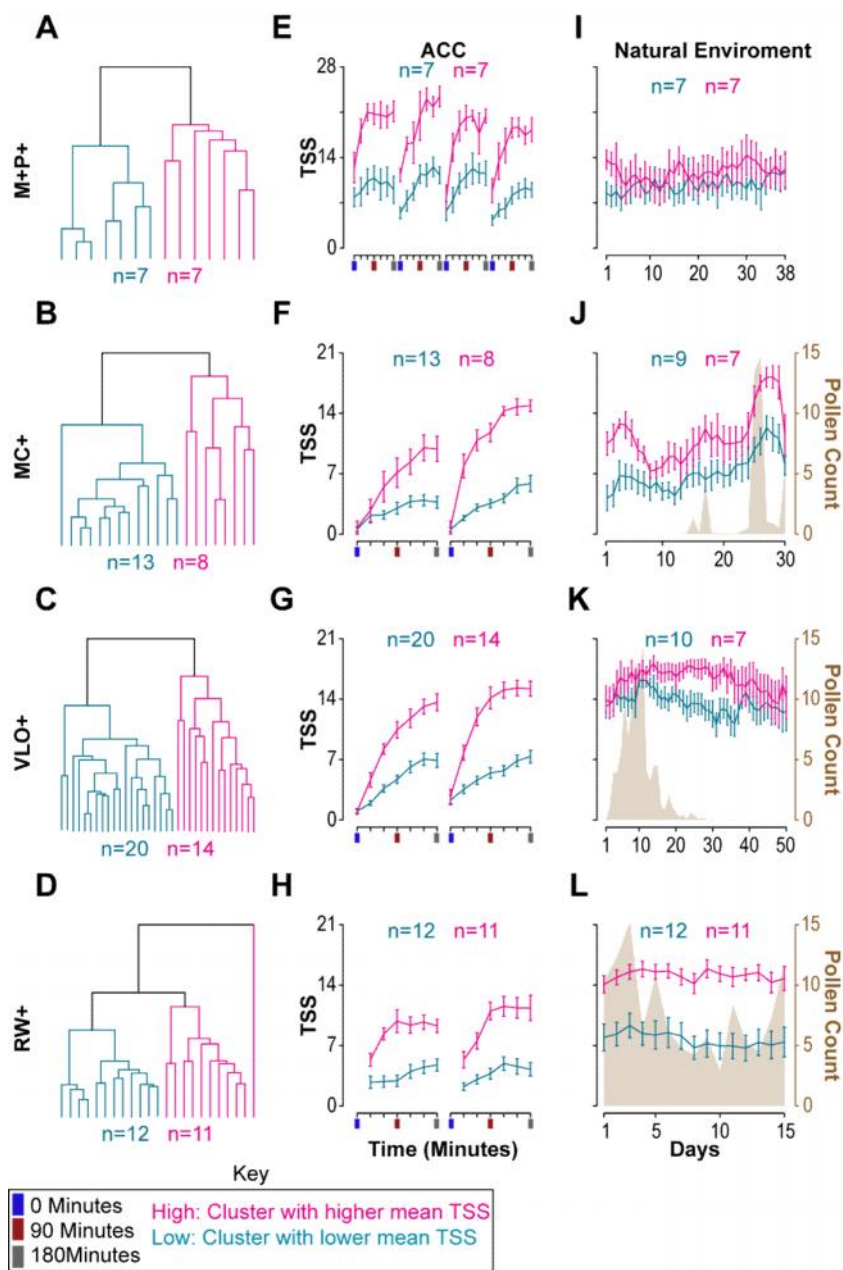


Figure E6..

