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Title: Development of Nasal Allergen Challenge with Cockroach in Children with Asthma

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Abbreviations/Acronyms:

DSMB – Data and Safety Monitoring Board
HSV – Herpes Simplex Virus
ICAC – Inner City Asthma Consortium
NIH – National Institutes of Health
NIAID – National Institutes of Allergy and Infectious Diseases
NAC – Nasal Allergen Challenge
PCR – Polymerase chain reaction
SCIT – Subcutaneous immunotherapy
TNSS – Total Nasal Symptom Score

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ABSTRACT

Background: Nasal allergen challenge (NAC) could be a means to assess indication and/or an outcome of allergen-specific therapies, particularly for perennial allergens. NACs are not commonly conducted in children with asthma and cockroach NACs are not well established.

This study's objective was to identify a range of German cockroach extract doses that induce nasal symptoms and to assess the safety of cockroach NAC in children with asthma.

Methods: Ten adults (18-37 years) followed by 25 children (8-14 years) with well-controlled, persistent asthma and cockroach sensitization underwent NAC with diluent followed by up to 8 escalating doses of cockroach extract (0.00381 -11.9 mcg/mL *Bla g 1*). NAC outcome was determined by Total Nasal Symptom Score (TNSS) and/or sneeze score. Cockroach allergen-induced T cell activation and IL-5 production were measured in peripheral blood mononuclear cells.

Results: 67% (6/9) of adults and 68% (17/25) of children had a positive NAC at a median response dose of 0.120 mcg/mL [IQR 0.0380-0.379 mcg/mL] of *Bla g 1*. Additionally, three children responded to diluent alone, and did not receive any cockroach extract. Overall, 32% (11/34) were positive with sneezes alone, 15% (5/34) with TNSS alone, and 21% (7/34) with both criteria. At baseline, NAC responders had higher cockroach-specific IgE (p=0.03), lower cockroach-specific IgG/IgE ratios (children, p=0.002), and increased cockroach-specific IL-5-producing T lymphocytes (p=0.045). The NAC was well tolerated.

Conclusion: We report the methodology of NAC development for children with persistent asthma and cockroach sensitization. This NAC could be considered a tool to confirm clinically relevant sensitization and to assess responses in therapeutic studies.

Key Words: nasal allergen challenge, children, asthma, inner-city, cockroach allergy, allergic rhinitis

Clinical Trial Registration:

https://clinicaltrials.gov/ct2/show/NCT02710136?term=conac&rank=1

#NCT02710136

Key Message:

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Nasal allergen challenge with German cockroach extract is feasible and safe in children with well-controlled, persistent asthma.

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INTRODUCTION

Confirming clinical allergy to a perennial allergen or the efficacy of immunotherapy can be difficult because exposure varies greatly, no clear seasonality of exposure exists, and other allergens can confound the relationship between exposure and clinical manifestations. Nasal allergen challenge (NAC), an established test in the research setting, may offer an objective assessment of nasal airway allergen responsiveness.[1,2] NAC can also be used to evaluate the pathophysiology of allergic reactions in a readily accessible part of the respiratory tract.[1,2]

Cockroach sensitization and exposure have been established as a leading risk factor for morbidity in children with asthma living in low-income, US urban communities, and many other parts of the world.[3-8] Environmental approaches, eradication and avoidance, have been the focus for ameliorating the effects of cockroach exposure. However, these approaches may be unrealistic in the environment of a lower socioeconomic urban population.[9] Allergen immunotherapy with German cockroach extract is a potential alternative for treating cockroach allergy.

Three studies examining NAC with cockroach allergen have been reported, but did not provide clear data on measures of responsiveness, information on standardization of the procedure, or adequate safety and dosing information in children with asthma.[10-12] We conducted a study first in adults, then children, to develop a cockroach NAC protocol and assess its safety in children with asthma. Additionally, we conducted an initial evaluation of the T-cell epitope repertoire against cockroach allergens and we tested for NAC-induced changes in T cell responsiveness to cockroach in order to lay the groundwork for the evaluation of immunologic changes seen with this type of NAC.

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METHODS

Study Design

The Cockroach Nasal Allergen Challenge (CoNAC) study was a multi-center, open label study to determine the feasibility and safety of German cockroach NAC in cockroach sensitive adults (Stage 1, n=10) and children ages 8-14 (Stage 2, n=25) with persistent, well-controlled asthma (history of doctor-diagnosed asthma ≥ 1 year, daily inhaled corticosteroid equivalent to 100-500 mcg of fluticasone, $FEV_1 \geq 80\%$ predicted, Asthma Control Test score ≥ 20), and German cockroach sensitization (skin prick test wheal (SPT) ≥ 3mm and cockroach-specific IgE ≥0.35 kU_A/L). The data from Stage 1 were used to identify a dose range that was safe and elicited a clinical response determined by a Total Nasal Symptom Score (TNSS) ≥ 8 or a sneezing score of 3. The sneezing score threshold was implemented after 6 adult subjects completed the NAC to improve sensitivity of the responder criteria.[13] Based on Stage 1 results, the TNSS threshold for NAC response in Stage 2 was lowered to 6 to limit the severity of the reaction, shorten the study procedure, and decrease overall participant study burden; the sneezing score threshold was unchanged.

The protocol was reviewed for ethical compliance by a central institutional review board. Written informed consent was obtained from Stage 1 participants and Stage 2 participants' legal guardians. Stage 2 participants provided verbal (ages 8-11) or written (ages 12-14) assent.

Study Assessments

During the NAC, diluent control and up to 8 escalating intranasal doses (0.00381-11.9 mcg/mL *Bla g 1*) of German cockroach extract were administered as a nasal spray with a nasal drug delivery system (LMA/Teleflex, San Diego, CA), which delivers 0.1 mL per spray. For each dose, one spray was delivered in each nostril at the end of inspiration. Nasal symptoms, PEF, and peak nasal inspiratory flow (PNIF) were assessed at the beginning of the NAC and 10 minutes after each dose. The TNSS score was determined by tallying the results of four symptom categories, -- sneezing, runny nose, stuffy nose, and itchy nose; the participant graded each symptom on a scale of 0 to 3 (Appendix 1).[14] The sneeze score noted was derived from the total number of sneezes (Appendix 1). Participants could not undergo a NAC if their baseline TNSS was >3 or an individual symptom score was >1. Antihistamines, anticholinergic agents,

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cromolyn, systemic glucocorticosteroids, nasal decongestants, and nasal steroids were withheld prior undergoing to the NAC (Appendix 2). Blood was drawn before the NAC, 6-10 days post-NAC, and 30 days post-NAC. In a subset, additional blood was obtained 24-72 hours post-NAC. Dust from the participant's bedroom was collected and analyzed by ELISA for concentration of Bla g 1, 2 and 5.[15]

Adverse events were graded according to the National Cancer Institue (NCI) Common Terminology Criteria for Adverse Events (CTCAE) Version 4.03 (published June 14, 2010).[16] For local adverse events related to the NAC and to skin prick testing, for reduction in lung function (PEF), and for anaphylaxis study specific grading scales were utilized (Appendix 3).

German cockroach extract, peptide synthesis and T cell *in vitro* culture expansion

German cockroach extract was obtained from Greer (cat#XPB46D3A4, Lenoir, NC). For *in vitro* studies, German cockroach allergen-derived peptides were synthesized by A & A (San Diego, CA) as crude material on a 1-mg scale. Peptides were re-suspended in DMSO, and equal amounts of each peptide were pooled. T cell *in vitro* expansions were performed as previously described.[17] See Appendix 4 for details.

T cell response and antigen presenting cell activation assays

ELISPOT assays, T cell activation as measured by flow cytometry, as well as activation status and frequency of circulating antigen presenting cells were determined as previously described (see Appendix 4).[17-19]

Statistical methods

The probability of positive NAC response at each dose was estimated as the cumulative proportion of participants meeting at least one response threshold. Participants were assumed to have a NAC response for all doses beyond the dose at which threshold criteria were met. Exact 95% confidence intervals were estimated using the Clopper-Pearson method. SPT for cockroach and other allergens, cockroach-specific immunoglobulins, IL-5-producing T lymphocytes, and T cell activation were summarized by geometric mean and were compared between responders and

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non-responders with a Mann-Whitney U Test. IL-5-producing T lymphocytes and T cell activation before, 1 week after, and 1 month post-NAC were compared between responders and non-responders with a Wilcoxon test. Dust allergen levels were summarized as being below or above the lower limit of detection (yes/no) and compared between responders and non-responders with a Fisher's exact test. Activation and mobilization of antigen-presenting cells were compared pre- and post-NAC with a Wilcoxon matched-pairs signed rank test. Monocyte frequency and T cell activation were correlated separately pre- and post-NAC.

RESULTS

Clinical Findings

The 10 adult participants had a median age of 22.5 years (range 18.0-37.0) and were predominantly female (70%); the 25 pediatric participants had a median age of 12.0 years (range 8.0-14.0) and were predominantly male (64%) (**Table 1**). Most of the participants were Black (adult 100%; pediatric 72%). The median baseline TNSS was 0 for adults and 1 for children. Baseline characteristics, including asthma medication use, are shown in Table 1.

Sixty-seven percent of adults had a positive NAC based on meeting the TNSS threshold or sneeze score (**Appendix 5; Appendix 6)**. The median response dose in the adult portion of the trial was 0.120 mcg/mL [IQR 0.0380–0.379] Bla g 1 (**Appendix 5**).

Eighty percent (20/25) of the children responded to the NAC, including 28% (7/25) based on sneeze count alone, 28% (7/25) on TNSS alone, and 24% (6/25) meeting both criteria (**Figure 1; Appendix 5; Appendix 7)**. Three children responded to the diluent on TNSS alone and did not receive any of the cockroach allergenic extract. The median response dose in the pediatric portion of the trial was the same as in adults (**Figure 1; Appendix 5**). None of the Stage 1 and Stage 2 participants with a negative NAC had a TNSS higher than a 4 (Appendix 6 and 7).

In general, the PNIF results tended to reflect the symptoms experienced by the participant in both the adult and pediatric participants (Appendix 6 and 7).

Overall there were twelve adverse events related to the NAC; eleven were grade 1. Headache was most frequently reported with an incidence of 20% in adults and 8% in children (**Table 2**).

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One adult had a PEF decline of 20.5% from baseline during the NAC that resolved without treatment after the challenge was stopped (**Appendix 6, participant 9**). There were no reports of asthma symptoms worsening during the NAC or 24-hours post-NAC.

There was one serious adverse event (SAE) (**Table 2**). The SAE was a reactivation of infranasal Herpes Simplex Virus in an adult that occurred three days post-NAC, which resolved without sequelae. There were no instances of anaphylaxis related to the NAC in the study.

In Vitro Parameters

NAC responders (to a cockroach-containing dose) had significantly higher levels of cockroach-specific IgE (median 7.0 kU/L vs. 1.2 kU/L; p=0.03) and lower cockroach-specific IgG/IgE ratios (0.5 vs. 5.7; p=0.002) compared to non-responders **(Figure 2**). Cockroach SPT, specific-IgG, and house dust cockroach allergen levels were not significantly different between responders and nonresponders, although SPT tended to be larger in responders (p=0.06) (**Figure 2; Appendix 8**). When comparing other allergen sensitizations, SPT to cat (p=0.008), dog (p<0.001) and Alternaria (p=0.056) were smaller in responders (**Appendix 9**).

NAC-positive participants had significantly more IL-5 producing cells than NAC-negative participants **(Figure 3A**). However, there were no significant differences in overall activated T cells between groups **(Figure 3B)**. Cockroach-specific IL-5 production and T cell activation were assessed both one week and one month post-NAC and showed no consistent pattern of CR-specific T cell response modulation at either time point **(Appendix 10)**.

Monocytes, plasmacytoid dendritic cells (pDCs) and basophils were identified by flow cytometry in blood acquired pre- and 48 hours post-NAC in a subset of participants (n=6). Significant increases in surface FcεRIα expression (monocytes and basophils), cell frequency (monocytes and pDCs), and expression of surface MHC-Class II (HLA-DR; monocytes and pDCs) were observed post-NAC. CD14 expression was also increased on monocytes post-NAC. These findings were also observed in samples from two participants who reacted to challenge with the diluent alone (uncolored symbols in **Appendix 11**).

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A significant correlation was observed between antigen-specific *ex vivo* T cell activation and monocyte frequency both pre- and post-NAC (R^2= 0.67, p=0.04; R^2= 0.8, p=0.015, respectively) (**Appendix 12**). No correlations were observed for IL-5 production (data not shown).

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DISCUSSION

This study assessed whether NAC with German cockroach extract can induce nasal responses in adults and children with cockroach sensitization and well-controlled, persistent asthma, to identify the dosing range and to assess the safety of this procedure. The reason for involving children was because cockroach sensitization has been strongly associated with asthma in this age group. Furthermore, establishing the cockroach NAC in children was a pre-requisite for us to proceed with a clinical trial where we would test the effectiveness of cockroach allergen immunotherapy (https://clinicaltrials.gov/ct2/show/NCT03541187?term=cockroach&recrs=ab&cond=Asthma+in+Children&cntry=US&draw=2&rank=1) in this population. [3, 20]

As with some other perennial allergens, it is difficult for cockroach-allergic patients to link symptoms to exposure and consequently the clinical significance of allergic sentitization demonstrated by the presence of serum specific IgE or positive skin prick testing is not clear. With acute delivery of allergen to the nasal passages, induction of symptomatology may be a stronger indicator that an individual is allergic and a candidate for allergen-specific immunotherapy. Also, reduction in the NAC response may be a good clinical outcome surrogate, but this requires investigation.

We designed this study to identify a narrow range of doses, refine the threshold definition, and assess safety in adults before engaging children. The NAC reached a threshold of nasal symptoms in 76% (26/34) of participants; 9% (3/34) responded to the administration of diluent alone, leaving 68% (23/34) who responded to cockroach extract. Additionally, 26% (9/34) did not respond to the challenge suggesting that, in the case of German cockroach, allergic sensitization alone may not be reliable enough for the selection of individuals who should participate in an allergen immunotherapy study. NAC responsiveness did not differ between adults and children, but only children had diluent responses possibly reflecting a higher degree of nonspecific nasal reactivity or increased sensitivity to the relatively unpleasant aspects of the challenge.

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In both adults and children, the PNIF results tended to be concordant with the direction of the symptoms, but, in several instances, the reductions in PNIF were either too small or absent when the symptomatic responses were clear (**Appendix 6 and 7**). In addition, we encountered technical difficulties conducting PNIF in the pediatric population leading to increased burden and longer time periods to complete a NAC, indicating that the pros and cons of adding PNIF as part of a NAC in a pediatric population should be carefully considered.

Data from the adults did not help reduce the number of allergen doses, but they did allow us to adjust the threshold TNSS score from 8 to 6 to reduce the burden of the procedure in children and to include sneezing as another indicator of positive response.[21] Although recent NAC studies use the TNSS as the primary outcome of the challenge,[22, 23] it became obvious in adults that several participants responded to the NAC primarily with sneezing and little or no other symptoms. A threshold based on sneezing alone has been used previously, when NAC was used to elucidate the mechanisms of allergic rhinitis.[13] In our study, the NAC response based on sneezing score threshold was similar in adult and pediatric participants.

Overall, no participants with a negative NAC had a TNSS higher than a 4. In both study stages, individuals with negative NAC, would either have a flat dose-response or would experience mild symptom elevation which would flatten thereafter showing no dose-response (**Appendix 6 and 7**). Given this pattern, we do not believe that false negative results were observed. Additionally, while three participants (two adults and one child) had a maximum TNSS of 5 after receiving all 8 doses, these three participants met the criteria for a positive response based on the sneeze score, highlighting the importance of adding this cut-off for a positive NAC as the sneezing score converted the NAC to "positive" and prevented false negative results **(Appendix 6 and 7)**.

Despite the large body of evidence supporting the safety of NAC, safety was of utmost consideration in this study. One SAE related to the NAC occurred in the adult group with a participant experiencing an infranasal reactivation of herpes simplex virus (HSV). Because of the unusual presentation and the originally unknown nature of the reaction, this participant was hospitalized as a precaution, which led to the characterization of the event as a SAE. The study clinicians felt the impetus for the reactivation may have been nasal discharge and manipulation

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of the nasal and infranasal area as there was no theoretical reason for the cockroach extract to have triggered the reactivation of HSV. PCR testing of the allergen extract used for the NAC was negative for HSV. Future studies may wish to consider HSV as an exclusion criterion for NAC.

The most common adverse event was mild headache. Concerns that the NAC could trigger bronchospasm in participants with persistent asthma were largely unrealized. No asthma symptoms related to the NAC were reported during the NAC or within 24-hours post-NAC. This supports the general experience with other allergens that NAC is low risk in children and specifically in children with asthma,[24-28] although caution is still advised. In particular, NAC in adults or children with severe or uncontrolled asthma has not been tested.

NAC responders had significantly elevated indicators of allergen-specific immunity. Although it is important to emphasize that the NAC response can not be reliably predicted with these parameters, the elevated cockroach sIgE, and the low sIgG/sIgE ratio in the pediatric participants support the relationship between a strong systemic, allergen-specific immunologic process and the airway response to allergens and are consistent with prior studies demonstrating that asthma in children is associated with low allergen-specific IgG/IgE ratios to cat and dust mite.[29, 30]

Similarly, T cell IL-5 production in response to a cockroach allergen-derived peptide pool measured at baseline after *in vitro* culture was significantly higher in responders compared to non-responders. However, cockroach allergen-induced *ex vivo* T cell activation pre-NAC was not predictive of NAC response, suggesting that an *in vitro* culture step coupled with measurement of functional T cells may be a better indicator of clinical reactivity compared to direct *ex vivo* antigen-specific T cell activation.

No changes were detected in cockroach-specific T cell responses between pre-NAC and 7 or 30 days post-NAC. It is possible that the effects of a local allergen provocation on circulating lymphocytes are too small to detect, or the kinetics of changes did not match the timepoints we chose.

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The activation and mobilization of pDCs, monocytes and basophils observed post-NAC, although not specific to NAC responders, indicates a link between environmental airway mucosal stimuli and systemic innate immune responses. This is consistent with results of previous studies, where both allergen challenge and administration of intranasal live viral vaccines have been shown to similarly activate systemic innate immune responses.[1, 31, 32.] However, activation and mobilization of monocytes and pDCs was observed in two participants who reacted to challenge with the diluent alone, suggesting these changes may not be allergen-specific, but could be induced by mucosal irritation or some other stimulus associated with the NAC.

There are two limitations of this pilot trial. First, we did not test non-cockroach sensitized individuals. We reasoned that, provocation of cockroach non-allergic controls may only be necessary if every cockroach sensitive participant was to have a positive response to the NAC. However, 8 adults and children did not show any evidence of reactivity after receiving all 8 consecutive doses of the cockroach allergen (**Appendices 6 and 7)**. Additional support for our decision not to challenge cockroach non-allergic controls comes from the fact that we found a significant relationship between cockroach-specific IgE and NAC responsiveness suggesting that IgE sensitization to cockroach allergen played an important role in defining the NAC response and that the NAC response was not secondary to non-specific reactivity.

Second, while the dosing range was based on only one allergen, *Bla g* 1, cockroach extracts are not standardized and their content in other allergens, together with the study participants' pattern of specific cockroach allergen sensitization, may play a role in determining *in vivo* responsiveness.[33] Although matching the sensitization pattern of a study participant with the allergen content of the provocation extract will be very difficult, knowledge of these parameters may help better predict NAC responsiveness in future studies.

In conclusion, we established a NAC model utilizing German cockroach extract that can be safely performed in children with well-controlled, persistent asthma. We have shown that not all sensitized individuals respond to the NAC and, although NAC responders appear to have higher sensitization based on IgE and skin prick testing, these evaluations are not adequate to identify

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who will develop nasal symptoms upon exposure. Next steps should be to test whether German cockroach NAC may serve as a tool in selecting appropriate candidates for and assessing responses to allergen-specific immunotherapy.

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Table 1 – Demographics

		Adult	Pediatric
		N=10	**N=25**
Site – n (%)	Cincinnati	3 (30)	6 (24)
	Dallas	2 (20)	5 (20)
	Denver	2 (20)	7 (28)
	New York	0 (0)	3 (12)
	Washington, DC	3 (30)	4 (16)
Age in Years	Mean (SD)	26.0 (7.5)	11.2 (1.9)
	Median (Min – Max)	22.5 (18 - 37)	12 (8 - 14)
Gender – n (%)	Female	7 (70)	9 (36)
	Male	3 (30)	16 (64)
Ethnicity – n (%)	Hispanic or Latino	0 (0)	6 (24)
	Not Hispanic or Latino	10 (100)	19 (76)
Primary Race – n (%)	Black	10 (100)	18 (72)
	White	0 (0)	6 (24)
	Unknown	0 (0)	1 (4)
Baseline Total Nasal Symptom	Mean (SD)	0.43 (0.65)	1.04 (1.06)
Score	Median (Min – Max)	0 (0 - 2)	1 (0 - 3)
Inhaled Corticosteroid Medication	100 mcg Fluticasone daily	0 (0)	1 (4)
Equivalent– n (%)	200 mcg Fluticasone daily	1 (10)	9 (36)
	500 mcg Fluticasone daily	5 (50)	9 (36)

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	500 mcg Fluticasone daily + LABA	4 (40)	6 (24)
Allergic Rhinitis – n (%)	Has History of Rhinitis	4 (40)	12 (48)
	Has Ongoing Rhinitis	3 (30)	9 (36)

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Table 2 – Adverse Events

	Adult	**Pediatric**
	N=10	**N=25**
Number of Participants with at Least One Event	4 (40%)	8 (32%)
Most Frequent Adverse Event- Headache	2 (20%)	2 (8%)
Number of Adverse Events	9	12
Adverse Events Related to the Nasal Allergen Challenge	6 (67%)	6 (50%)
Serious Adverse Events Related to the Nasal Allergen Challenge	1	0

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FIGURE LEGENDS

Figure 1 – Pediatric Response to Nasal Allergen Challenge with German Cockroach Extract. Error bars represent 95% confidence limits.

Figure 2 – German CR Skin Prick Test Wheal, CR-specific IgE, and Pediatric CR-specific IgG and IgG/IgE in NAC-positive versus NAC-negative Participants. Bars show the geometric mean with 95% CI.

Figure 3 – German Cockroach-Specific IL-5 (A) and T Cell (B) Activation for Combined Adult and Pediatric Populations. Bars shown the geometric mean ± 95% confidence intervals.

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Appendices for "Development of Nasal Allergen Challenge with Cockroach in Children with Asthma"

Appendix 1: Total Nasal Symptom Score (TNSS)

Appendix 2: Length of time to withhold medications prior to the Nasal Allergen Challenge

Appendix 3: Study Specific Grading for AEs (tables replicated from the protocol version 5.0)

Appendix 4: Laboratory methods

Appendix 5: Adult and Pediatric NAC Response

Appendix 6: Individual Adult NAC Responses.

Appendix 7: Individual Pediatric NAC Responses

Appendix 8: German Cockroach Dust Allergen Levels by NAC Response for Combined Adult and Pediatric Populations

Appendix 9: Baseline Inhalant Allergen Skin Prick Test Wheals by NAC Response for Combined Adult and Pediatric Populations

Appendix 10: German Cockroach Specific NAC Induced Modulation of IL-5 Production (A) and T-cell Activation (B) Over Time.

Appendix 11: Activation and Mobilization of Blood Antigen Presenting Cells 48 Hours After NAC.

Appendix 12: Correlation of % Monocytes and HLA-DR Expression with German Cockroach Specific T Cell Activation**.**

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Appendices for "Development of Nasal Allergen Challenge with Cockroach in Children with Asthma"

Appendix 1: Total Nasal Symptom Score (TNSS)*

Category	Measurement	**Points**
Sneezing **Sneeze Count = __**	none	0
	1-2	1
	3-4	2
	5 or more	3
Runny nose	None - my nose feels fine and not runny at all	0
	Mild - my nose is a little runny; I might be able to blow a little snot or mucous out	1
	Moderate - my nose is very runny; I could definitely blow out some snot or mucous	2
	Severe - my nose is extremely runny; I could blow out a lot of snot or mucous	3
Stuffy nose	None - my nose feels fine; I am not at all stuffy or congested	0
	Mild - my nose feels a little stuffy	1
	Moderate - my nose feels very stuffy; it is hard to breathe through my nose	2
	Severe - my nose is completely stuffy; I cannot even breathe through my nose	3
Itchy Nose	None - my nose does not itch at all	0
	Mild - my nose feels a little itchy	1
	Moderate - my nose feels very itchy	2
	Severe - my nose is extremely itchy	3
TNSS		/12

- The verbiage in the traditional TNSS was modified for a pediatric population.

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Appendix 2: Length of time to withhold medications prior to the Nasal Allergen Challenge

Medication	**Length of Abstention**
Antihistamines (1st and 2nd generation)	5 days
Anticholinergic agents	24 hours
Cromolyn	8 hours
Nasal decongestants	24 hours
Nasal steroids	14 days
Systemic glucocorticosteroids	21 days

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Appendix 3: Study Specific Grading for AEs (tables replicated from the protocol version 5.0)

Table 12.3.1a: Grading of Local Reactions to Study Procedures

Grade	**1**	**2**	**3**	**4**
Nasal allergen challenge	Requiring antihistamines, decongestants or nasal steroids as rescue medication	Requiring oral steroids as rescue medication	Requiring a visit to a health care provider for treatment.	Life threatening and/or requiring hospitalization (e.g. upper airway obstruction)
Skin testing	Interfering with usual daily activities or sleep, but requiring no medication other than topical corticosteroids or antihistamines.	Interfering with usual daily activities or sleep and requiring oral steroids.	Requiring a visit to a health care provider for treatment	Not applicable

Table 12.3.1b: Grading of PEF Declines Occurring During the Challenge Visit

Grade	**1**	**2**	**3**	**4**	**5**

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[1]**Peak Expiratory Flow**	79 - 75% of baseline[2] PEF value	74 - 70 % of baseline[2] value	69 - 50 % of baseline[2] value	< 50 % of baseline[2] value	Death

[1] If spirometry is performed because of a PEF less than 80% of baseline and the FEV1 is ≥ 85% of baseline value then the decline in PEF will not be considered an AE.

[2] Baseline refers to the PEF value (best of 3 efforts) prior to the initiation of the NAC.

Table 12.3.1c: Grading System of Severity of Anaphylaxis**

Grade	Defined By	Grade
1. Mild (skin & subcutaneous tissues, GI, &/or mild respiratory[1])	Flushing, urticaria, periorbital or facial angioedema; mild dyspnea, wheeze or upper respiratory symptoms; mild abdominal pain and/or emesis	1. Mild (skin & subcutaneous tissues, GI, &/or mild respiratory[1])
2. Moderate (mild symptoms + features suggesting moderate respiratory, cardiovascular or GI symptoms)	Marked dysphagia, hoarseness, and/or stridor; SOB, wheezing & retractions; crampy abdominal pain, recurrent vomiting and/or diarrhea; and/or mild dizziness	2. Moderate (mild symptoms + features suggesting moderate respiratory, cardiovascular or GI symptoms)
3. Severe (hypoxia, hypotension, or neurological compromise)	Cyanosis or SpO2 < 92% at any stage, hypotension, confusion, collapse, loss of consciousness; or incontinence	3. Severe (hypoxia, hypotension, or neurological compromise)

[1]During the Nasal Allergen Challenge, respiratory symptoms, upper or lower, will ONLY be considered as part of an anaphylaxis event if they appear together with other systemic symptoms, as described in the Table 12.3.1c, but not if they appear alone.

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**Adapted from: Brown SG. Clinical features and severity grading of anaphylaxis. JACI. 2004;114:371-6.

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Appendix 4: Laboratory methods

T cell *in vitro* culture expansion

For *in vitro* expansions, PBMCs were thawed and cultured in RPMI 1640 (Omega Scientific, Tarzana, CA) supplemented with 5% human AB serum (Gemini Bio-Products, West Sacramento, CA) at a density of 2×10^6 cells per mL in 24-well plates and stimulated with German cockroach extract at 10 µg/mL. Cells were kept at 37°C, 5% CO_2, and additional IL-2 (10 U/mL; ThermoFisher, San Diego, CA) was added every 3 days after initial antigenic stimulation. On day 14, cells were harvested and screened for reactivity against cockroach-specific peptide pools and extract.

Dual ELISPOT assays

The production of IL-5 post-stimulation was analyzed by ELISPOT as described previously.[1] Briefly, plates (Millipore, Bedford, MA) were coated with 10 µg/ml anti-human IL-5 (clone TRFK5; Mabtech, Cincinnati, OH). Cells were incubated at a density of 1×10^5/well either with peptide pools (2 µg/ml), extract (10 µg/ml), PHA (10 µg/ml), or medium containing 0.25% DMSO (corresponding to the percentage of DMSO in the pools/peptides) as a control. After 24 h, cells were removed, and plates were incubated with 2 µg/ml biotinylated anti-human IL-5 Ab (1:50 dilution) (Mabtech, Cincinnati, OH) at 37°C. After 2 h, spots were developed by incubation with Alkaline-phosphatase-Complex (Vector Laboratories, Burlingame, CA) and then visualized by applying the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories) according to the manufacturer's instructions. Spots were counted by computer-assisted image analysis (KS-ELISPOT reader, Zeiss, Munich, Germany). Each assay was performed in triplicate. The level of statistical significance was determined with a Student *t* test using the mean of triplicate values of the response against stimulus versus the response against the DMSO control. Criteria for peptide pool positivity were 100 spot-forming cells (SFCs)/10^6 PBMC, $p \leq 0.05$, and a stimulation index (SI) ≥ 2, whereas criteria for individual peptide positivity were ≥20 SFC/10^6 PBMC, $p \leq 0.05$, and an SI ≥ 2.

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T cell *ex vivo* activation assay

PBMC were thawed and cultured in RPMI 1640 (Omega Scientific) supplemented with 5% human AB serum (Gemini Bio-Products, West Sacramento, CA) overnight for 24 h with 2 mg/ml of German cockroach epitope megapool. As a positive control, cells were stimulated with 10 µg/ml PHA and medium alone was used as negative control. After 24h stimulation, cells were harvested, washed and stained with a panel containing fixable viability dye eFluorV500 and CD8/CD14/CD19-V500 for exclusion, CD4-APC780, CD3-AF700, CD25-APC, Ox40-BV421, and PDL-1-PE (ThermoFisher, San Diego, CA). After staining, cells were washed and acquired on a BD LSR-2 flow cytometer (BD, San Diego, CA). Analysis was performed using Flowjo 10.4 software (Ashland, OR). Activated T cells were gated as live cells, $CD8/CD14/CD19^-$ $CD4^+CD3^+CD25^+Ox40^+$. Data shown is expressed as percent of stimulated cells after subtraction of background (percent cells activated in response to medium stimulation).

Flow cytometry of antigen presenting cells

To determine the impact of NAC on the activation status and frequency of circulating antigen presenting cells, blood samples were obtained in a subset of participants (6 participants at the Dallas site) before and 48 hours after the NAC. PBMCs purified by density centrifugation were then stained as previously described[2] with the following fluorochrome-conjugated anti-human antibodies: CD14-Pacific Blue, HLA-DR APC-Cy7, Lineage-FITC mixture, CD123-PeCy5 (BD Biosciences, San Diego, CA) and FcεRIα-PE (eBiosciences, San Diego, CA), acquired on a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Flowjo, Ashland, OR). Monocytes were defined as CD14-positive, HLA-DR-positive cells. pDCs were identified as lineage-negative, HLA-DR-positive, CD123-positive cells and basophils as lineage-negative, HLA-DR-negative, CD123-positive cells. The mean fluorescence intensity of FcεRIα (on monocytes, pDCs and basophils), HLA-DR (on monocytes and pDCs), and CD14 (on monocytes) was determined by flow cytometry and subsequently converted to Molecules of Equivalent Soluble fluorochrome (MESF) using the Ultra Rainbow Calibration kit (BD Biosciences, San Diego, CA). Monocyte, pDC and basophil frequencies were calculated as percentages of respective cells type identified within forward scatter versus side scatter-based live gates.[2]

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Appendix 5: Adult and Pediatric NAC Response

	Total N[2]	Met[1] TNSS ≥ 6, 8 Only N	Met TNSS ≥ 6, 8 Only Cum. N	Met Sneeze Score = 3 Only N	Met Sneeze Score = 3 Only Cum. N	Met Both Thresholds N	Met Both Thresholds Cum. N	Primary Outcome Met Either Threshold N	Primary Outcome Met Either Threshold Cum. N	Primary Outcome Met Either Threshold Cumulative Probability
Adult Participants										
Dose 0 - 0 mcg/mL	10	0	0	0	0	0	0	0	0	0.00 (0.00, 0.31)
Dose 1 - 0.00381 mcg/mL	10	0	0	0	0	0	0	0	0	0.00 (0.00, 0.31)
Dose 2 - 0.01204 mcg/mL	10	1	1	0	0	0	0	1	1	0.10 (0.00, 0.45)
Dose 3 - 0.0380 mcg/mL	9	0	1	1	1	0	0	1	2	0.22 (0.03, 0.60)
Dose 4 - 0.120 mcg/mL	9	0	1	1	2	1	1	2	4	0.44 (0.14, 0.79)
Dose 5 - 0.379 mcg/mL	9	0	1	1	3	0	1	1	5	0.56 (0.21, 0.86)
Dose 6 - 1.20 mcg/mL	9	0	1	0	3	0	1	0	5	0.56 (0.21, 0.86)
Dose 7 - 3.78 mcg/mL	9	0	1	0	3	0	1	0	5	0.56 (0.21, 0.86)
Dose 8 - 11.9 mcg/mL	9	0	1	1	4	0	1	1	6	0.67 (0.30, 0.93)
Pediatric Participants										
Dose 0 - 0 mcg/mL[3]	25	3	3	0	0	0	0	3	3	0.12 (0.03, 0.31)[3]
Dose 1 - 0.00381 mcg/mL	25	0	3	1	1	0	0	1	4	0.16 (0.05, 0.36)
Dose 2 - 0.01204 mcg/mL	25	0	3	0	1	0	0	0	4	0.16 (0.05, 0.36)
Dose 3 - 0.0380 mcg/mL	25	1	4	2	3	1	1	4	8	0.32 (0.15, 0.54)
Dose 4 - 0.120 mcg/mL	25	0	4	3	6	1	2	4	12	0.48 (0.28, 0.69)
Dose 5 - 0.379 mcg/mL	25	1	5	0	6	3	5	4	16	0.64 (0.43, 0.82)
Dose 6 - 1.20 mcg/mL	25	2	7	0	6	0	5	2	18	0.72 (0.51, 0.88)
Dose 7 - 3.78 mcg/mL	25	0	7	0	6	1	6	1	19	0.76 (0.55, 0.91)
Dose 8 - 11.9 mcg/mL	25	0	7	1	7	0	6	1	20	0.80 (0.59, 0.93)

[1] The TNSS threshold was 8 for adult participants and 6 for pediatric participants.

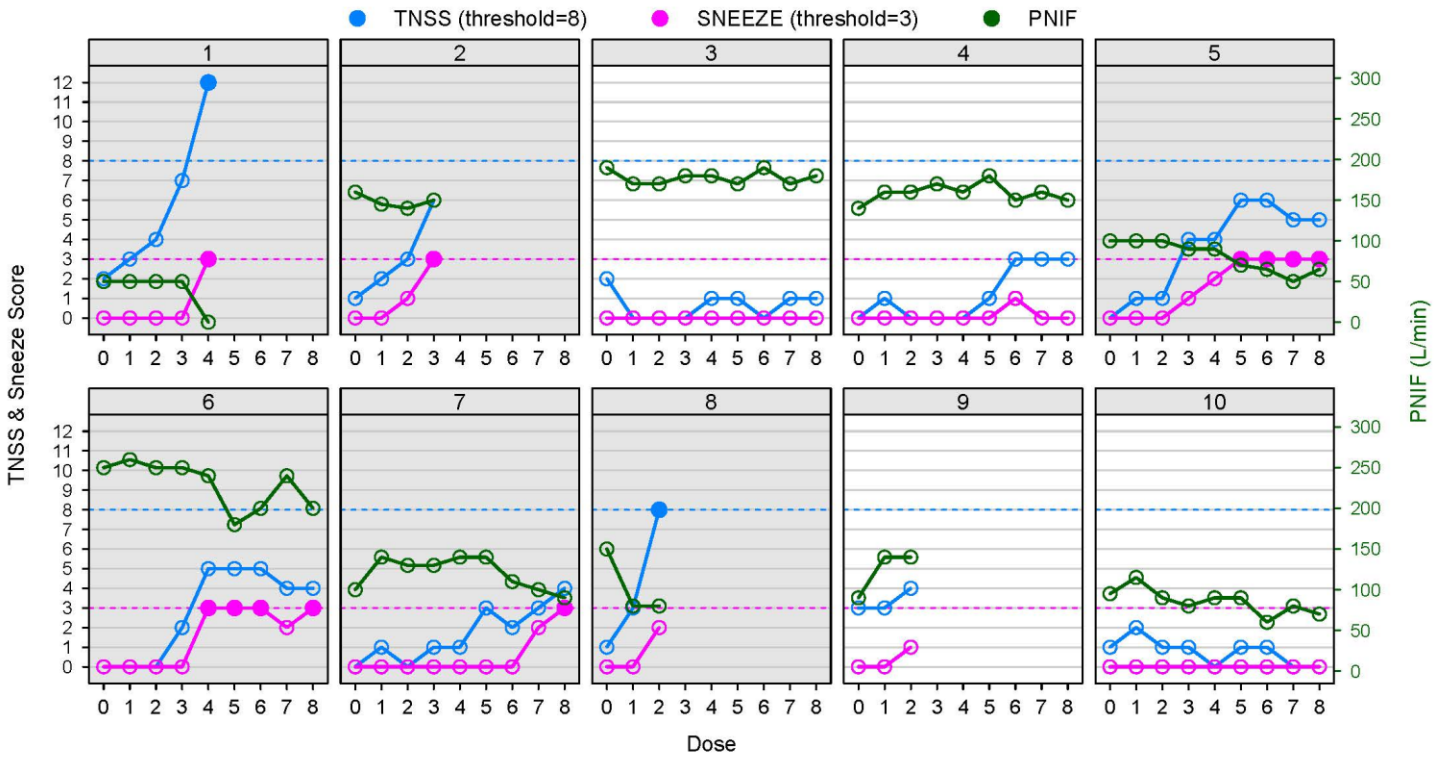
[2] One adult participant stopped the NAC after dose 2 due to a 20% peak flow decline. This participant did not meet either response criteria and was excluded from the denominator of response percent calculations for doses not received.

[3] Dose 0 responders met the pre-specified TNSS threshold for positivity, so they are included in the tally of those participants that achieved the primary outcome, despite not having received German cockroach extract.

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Appendix 6:

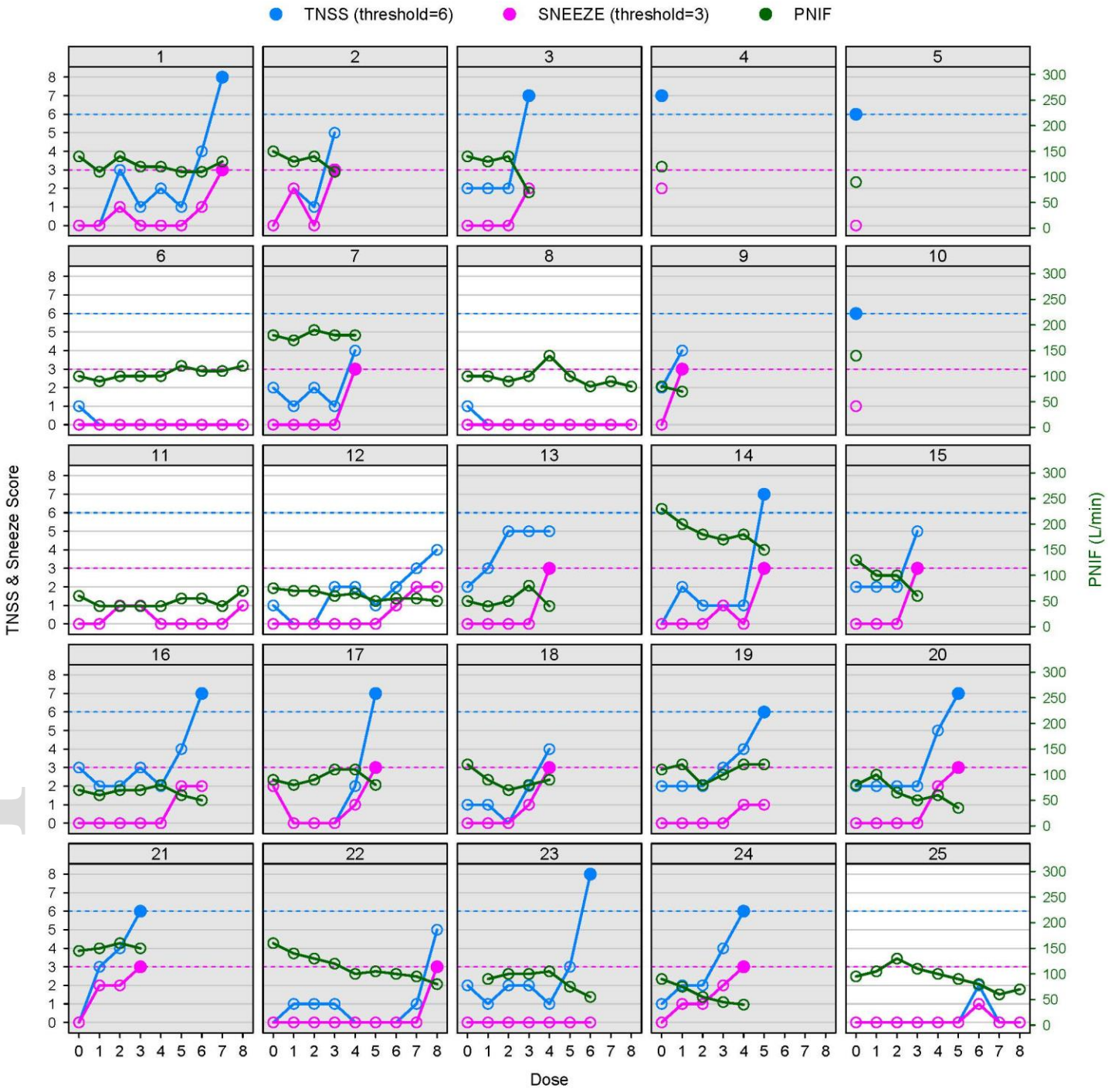


Individual Adult NAC Responses. Closed and open dots indicate when each threshold was and was not reached, respectively. Grey indicates participants meeting either TNSS>=8 or sneeze score=3. Participant 9 did not complete the challenge because of a peak flow decline.

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Appendix 7:



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Individual Pediatric NAC Responses. Closed and open dots indicate when each threshold was and was not reached, respectively. Grey indicates participants meeting either TNSS>=6 or sneeze score=3.

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Appendix 8: German Cockroach Dust Allergen Levels by NAC Response for Combined Adult and Pediatric Populations

		NAC+		NAC-		
	Total N	**Above LLD n (%)**	**Below LLD n (%)**	**Above LLD n (%)**	**Below LLD n (%)**	**P-Value**
Bla g 1 ng/g	28	6 (30%)	14 (0%)	0 (0%)	8 (100%)	0.14
Bla g 2 ng/g	28	3 (15%)	17 (85%)	0 (0%)	8 (100%)	0.54
Bla g 5 ng/g	28	0 (0%)	20 (100%)	0 (0%)	8 (100%)	0.54
Any of Bla g 1,2, or 5 ng/g	28	6 (30%)	14 (70%)	0 (0%)	8 (100%)	0.14

Note: LLD = Lower Limit of Detection.

Note: NAC+ excludes diluent responders.

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Appendix 9: Baseline Inhalant Allergen Skin Prick Test Wheals by NAC Response for Combined Adult and Pediatric Populations

		NAC+			NAC-			
Wheal (mm)	**Total N**	**Geometric Mean (SD)**	**95% CI**	**Median (Min, Max)**	**Geometric Mean (SD)**	**95% CI**	**Median (Min, Max)**	**P-Value**
German cockroach	32	6.3 (1.4)	(5.3, 7.4)	7.0 (3.0, 13.0)	4.8 (1.4)	(3.4, 6.6)	4.0 (3.0, 11.0)	0.062
Alternaria tenuis	25	0.6 (2.2)	(0.1, 1.3)	0.0 (0.0, 9.5)	2.8 (2.9)	(0.3, 10.6)	5.5 (0.0, 8.5)	0.056
American cockroach	24	2.3 (2.5)	(1.1, 4.3)	3.3 (0.0, 8.5)	4.2 (2.4)	(1.1, 11.9)	5.5 (0.0, 11.0)	0.311
Aspergillus fumigatus	24	0.3 (1.6)	(0.0, 0.6)	0.0 (0.0, 2.5)	0.0 (1.0)	--	0.0 (0.0, 0.0)	0.431
Bermuda grass	17	1.7 (3.2)	(0.2, 5.0)	0.0 (0.0, 13.5)	1.9 (3.3)	(-0.2, 9.1)	2.0 (0.0, 10.0)	0.959
Cat	24	1.6 (2.8)	(0.6, 3.3)	1.3 (0.0, 11.0)	8.0 (1.4)	(5.5, 11.5)	7.3 (5.5, 13.0)	0.008
Der f	26	2.0 (2.4)	(0.9, 3.5)	3.5 (0.0, 9.5)	3.4 (2.0)	(1.3, 7.4)	4.0 (0.0, 6.5)	0.227
Der p	25	2.5 (2.4)	(1.3, 4.4)	3.5 (0.0, 13.0)	2.6 (2.5)	(0.5, 7.5)	4.0 (0.0, 10.0)	0.914
Dog	24	0.2 (1.6)	(-0.1, 0.5)	0.0 (0.0, 4.5)	5.5 (1.4)	(3.6, 8.1)	5.5 (3.0, 8.5)	<0.001
Eastern 8 tree mix	25	1.6 (2.9)	(0.5, 3.3)	0.0 (0.0, 13.5)	2.6 (2.7)	(0.3, 9.2)	5.3 (0.0, 7.0)	0.588
Juniper	18	1.2 (2.6)	(0.2, 3.0)	0.0 (0.0, 7.5)	1.8 (3.2)	(-0.2, 8.5)	3.0 (0.0, 9.0)	0.581
K-O-T grass mix	25	1.5 (3.1)	(0.4, 3.2)	0.0 (0.0, 16.0)	2.9 (3.0)	(0.2, 11.2)	5.0 (0.0, 12.0)	0.419
Mouse	25	1.0 (2.4)	(0.3, 2.0)	0.0 (0.0, 7.5)	2.0 (3.3)	(-0.2, 9.5)	2.8 (0.0, 9.5)	0.427
Ragweed mix	24	0.8 (2.5)	(0.2, 1.9)	0.0 (0.0, 10.0)	1.6 (2.9)	(-0.1, 6.9)	2.5 (0.0, 7.0)	0.490
Rat	24	0.5 (2.2)	(0.0, 1.2)	0.0 (0.0, 9.5)	0.6 (2.1)	(-0.3, 2.5)	0.0 (0.0, 3.5)	1.000

Note: NAC+ excludes diluent responders.

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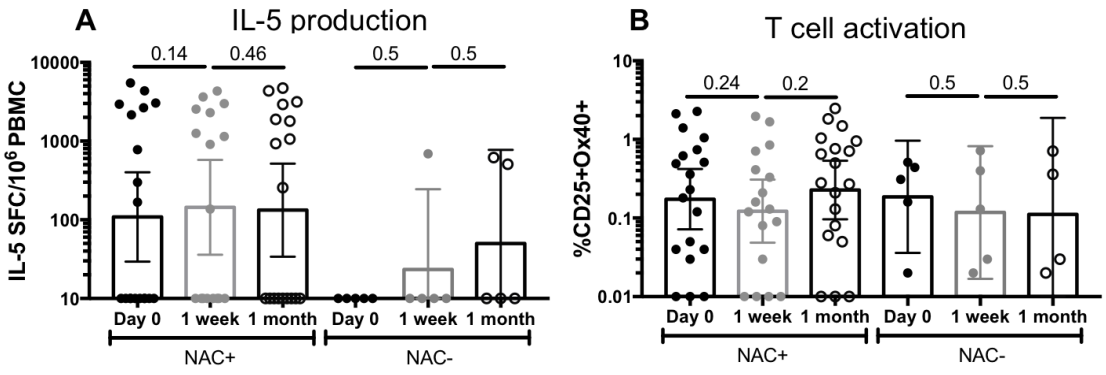
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Note : The geometric mean was calculated as the exponential of the arithmetic mean of log wheal size. Since log of zero is undefined, the contant 1 was added to each wheal value prior to computing the log wheal size and subtracted from the mean after exponentiation.

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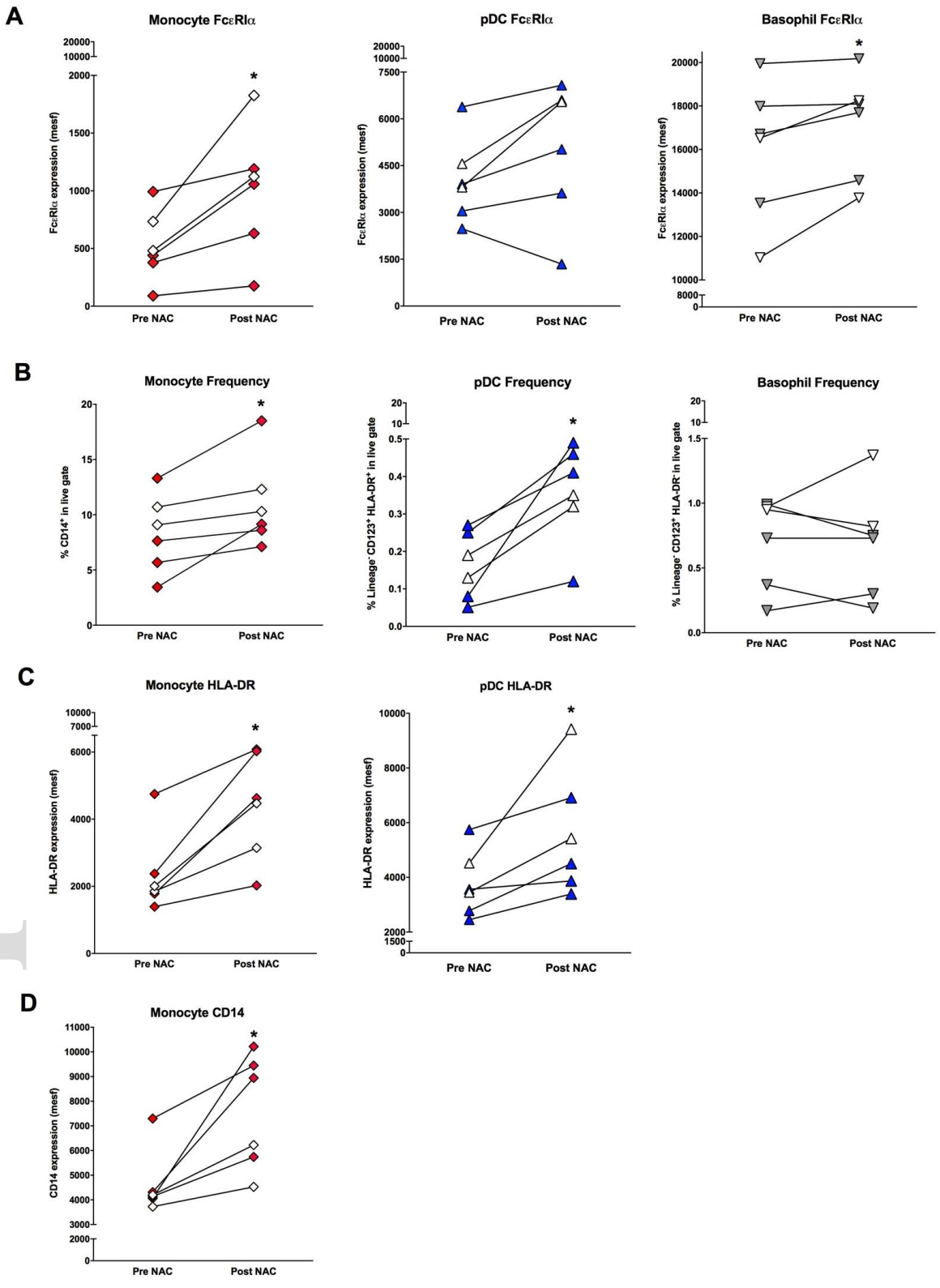
Appendix 10:



German Cockroach Specific NAC Induced Modulation of IL-5 Production (A) and T-cell Activation (B) Over Time.

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Appendix 11:



Activation and Mobilization of Blood Antigen Presenting Cells 48 Hours After NAC.

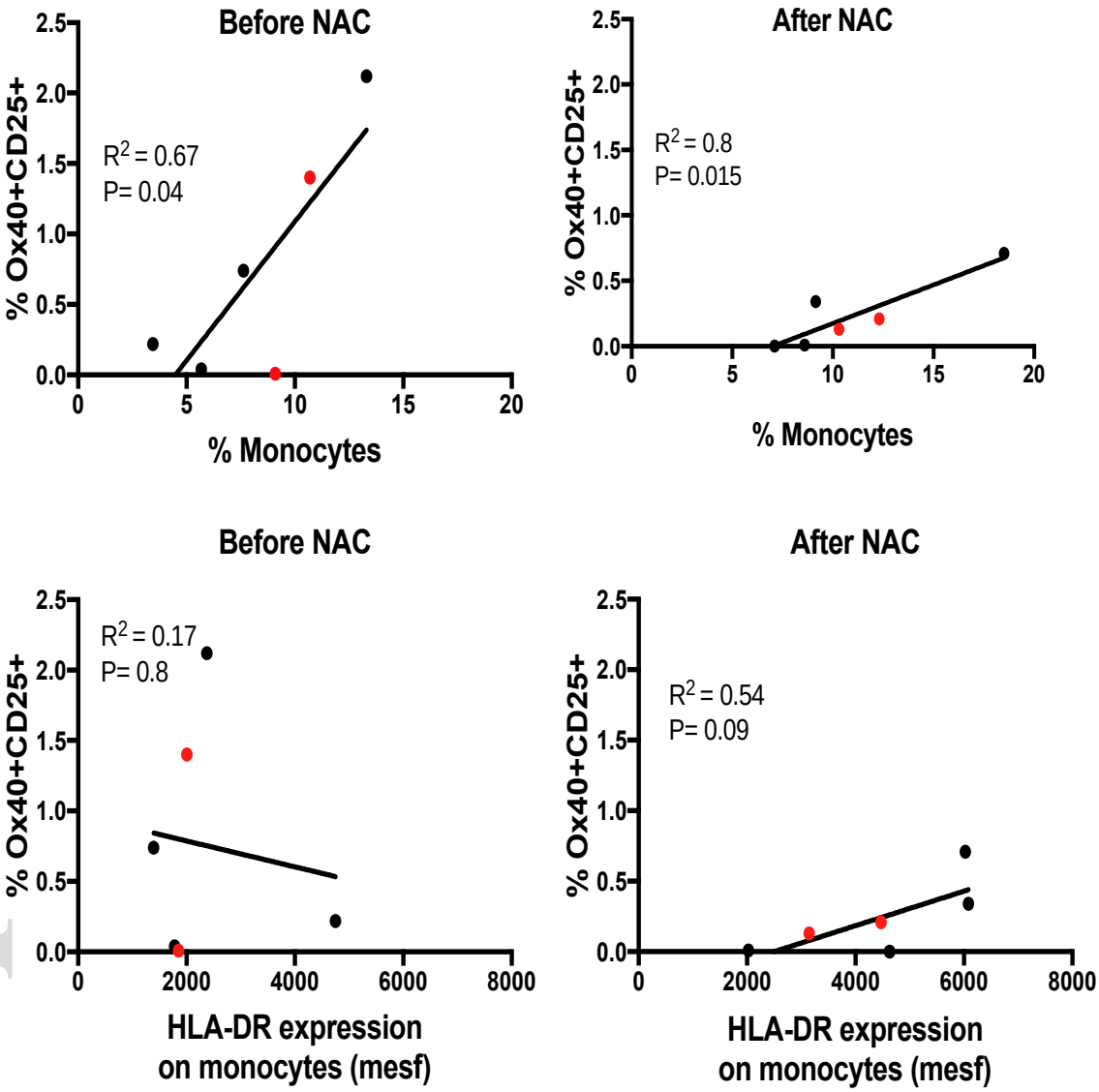
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Uncolored/colored symbols represent diluent/cockroach allergen responders respectively.

Appendix 12:



Correlation of % Monocytes and HLA-DR Expression with German Cockroach Specific T Cell Activation. Diluent responders are shown in red.

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Figure 1

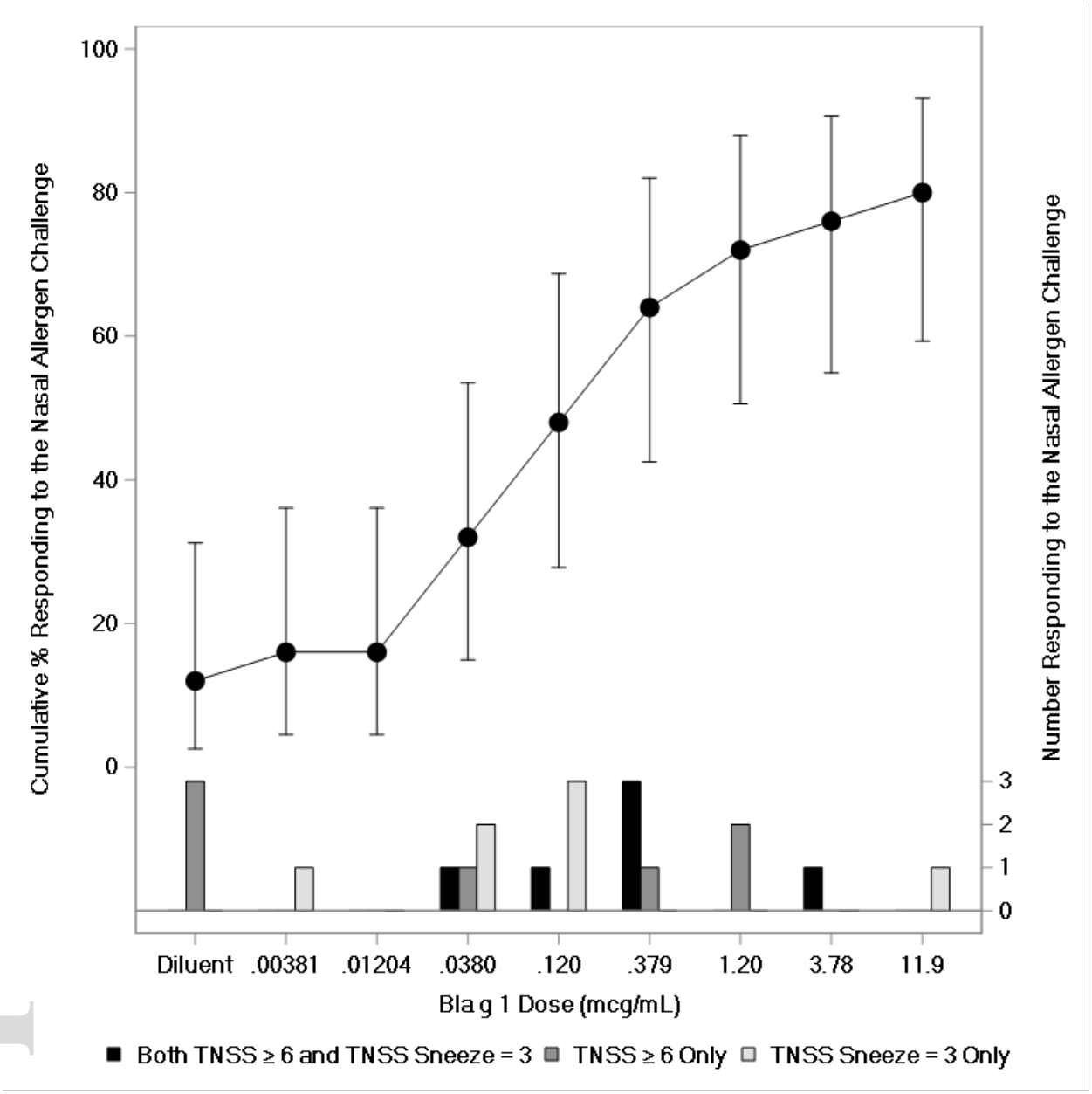


Figure 1

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Figure 2

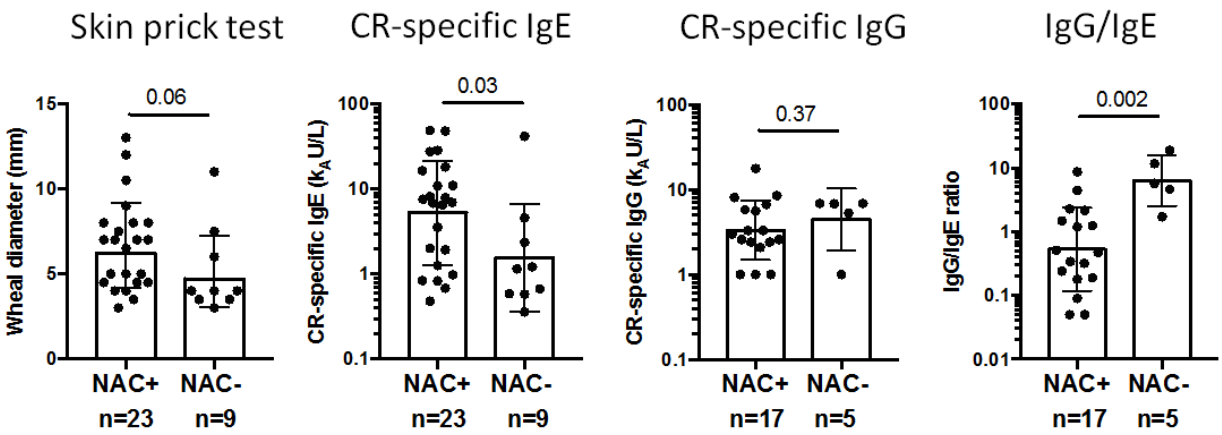


Figure 2

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Figure 3

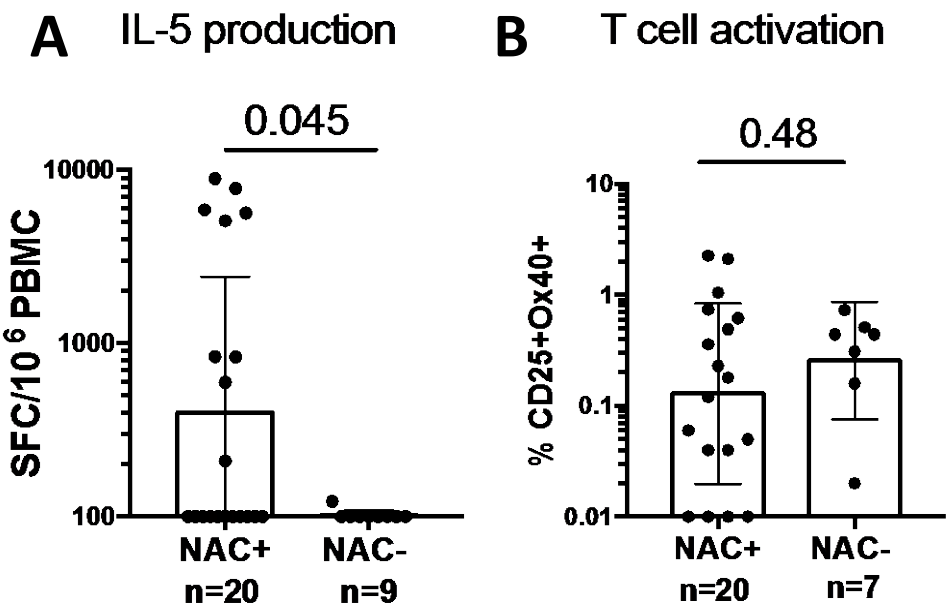


Figure 3

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