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Nasal and blood transcriptomic pathways underpinning the clinical response to grass pollen immunotherapy

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Abstract

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Background: Allergen immunotherapy (AIT) is a well-established disease-modifying therapy for allergic rhinitis, yet the fundamental mechanisms underlying its clinical effect remain inadequately understood.

Objective: The GRASS study was a randomized, double-blind, placebo-controlled trial of timothy grass allergic individuals who received 2 years of placebo (n=30), subcutaneous (SCIT) (n=27), or sublingual immunotherapy (SLIT) (n=27) and were then followed for 1 additional year. Here we used yearly biospecimens from the GRASS study to identify molecular mechanisms of response.

Methods: We utilized longitudinal transcriptomic profiling of nasal brush and peripheral blood mononuclear cell (PBMC) samples after allergen provocation to uncover airway and systemic expression pathways mediating responsiveness to AIT.

Results: SCIT and SLIT demonstrated similar changes in gene module expression over time. In nasal samples, alterations included downregulation of pathways of mucus hypersecretion, leukocyte migration/activation, and endoplasmic reticulum stress (log2 fold changes (logFC) −0.133 to −0.640, FDRs <0.05). Interestingly, we observed upregulation of modules related to epithelial development, junction formation, and lipid metabolism (logFC 0.104 to 0.393, FDRs <0.05). In PBMCs, modules related to cellular stress response and type 2 cytokine signaling were reduced by immunotherapy (logFC −0.611 to −0.828, FDRs <0.05). Expression of these modules was also significantly associated with both Total Nasal Symptom Score and Peak Nasal Inspiratory Flow responses, indicating important links among treatment, module expression, and allergen response.

Conclusion: Our results identify specific molecular responses of the nasal airway impacting barrier function, leukocyte migration activation, and mucus secretion, that are affected by both SCIT and SLIT, offering potential targets to guide novel strategies for AIT.

Trial Registration: ClinicalTrials.gov Identifier: NCT01335139, EudraCT Number: 2010–023536-16

Capsule Summary:

Using longitudinal transcriptomic assessments, we identified gene modules in the nasal epithelium and peripheral blood which are altered by allergen immunotherapy and could elucidate functional pathways and provide novel targets to guide treatment and improve therapeutic efficacy.

Keywords

Allergen immunotherapy; Sublingual immunotherapy; Subcutaneous immunotherapy; RNA sequencing; allergic rhinitis

INTRODUCTION

Allergic rhinitis (AR) is an IgE-mediated disease that affects an estimated 15–30% of the United States population[1]. AR increases the likelihood of developing asthma in both children and adults[2, 3], and is associated with negative impacts on patient quality of life, sleep, and work and school performance[4]. Allergen immunotherapy (AIT) is a disease-

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modifying treatment for AR and is an especially useful option for the roughly 60% of patients who do not respond to or cannot tolerate pharmacologic therapies[5]. For timothy grass pollen (TGP)-induced seasonal AR, the two AIT treatment modalities used clinically are subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SCIT is highly effective at improving clinical symptoms during the allergy season[6, 7]. SLIT offers similar efficacy while allowing for at home dosing, less frequent office visits, and minimal or no allergic reactions during administration, though mild local reactions are common and need for daily dosing can limit patient adherence[8, 9]. Past studies have shown AIT must be given for at least 3 years[8, 10] for sustained clinical benefit. In the Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy (GRASS) trial, 2 years of SCIT or SLIT were each compared to one another and to placebo. AIT provided significant improvement in response to a nasal allergen challenge (NAC) during therapy, but the effect was not sustained one year after treatment discontinuation[10]. GRASS provided a unique opportunity for mechanistic assessment of differential clinical responses to each therapy, which was the goal of the current analysis.

AIT is a treatment modality that leads to alterations in the immune response to allergen. Changes in allergen-specific memory T and B cell responses, induction of IgG4 and IgA antibodies, and alterations in mast cell and basophil responsiveness have been observed in previous studies of AIT[11–14]. The GRASS study showed that AIT additionally reduced allergen-induced cytokines in nasal secretions and Th2a cells in peripheral blood[15]. However, in GRASS as well as in other trials, these immunologic markers did not correlate well with the clinical responses to allergen exposure [16]. Moreover, previous studies on the effects of AIT in AR have not carefully assessed changes in the target organ, the nasal mucosa, in response to allergen exposure.

Increasing evidence suggests that disruption of epithelial barrier integrity plays a key role in the initiation or progression of allergic diseases[17]. Recent studies have shown that the expression of genes that promote epithelial barrier function is higher in non-allergic patients, which could help mitigate responses to allergen in those individuals[18]. It is therefore plausible that AIT could lead to changes in the expression of epithelial barrier-related genes as well as improved barrier function and integrity. A more complete understanding of the mechanisms of action of AIT is needed, including assessment of the airway molecular response. Such understanding is key to identifying biomarkers of efficacy that could indicate the duration of AIT needed on an individual basis. This could lead to improved approaches for both prevention and treatment of AR and associated diseases[19, 20].

In this study, we evaluated the effects of SCIT and SLIT on the response of the nasal epithelium, as our primary area of interest, as well as the peripheral immune system to experimental allergen exposure using longitudinal RNA-sequencing of both nasal epithelial brushings and peripheral blood mononuclear cells (PBMCs) throughout the course of therapy in the GRASS trial, and nasal epithelial samples collected from healthy non-atopic controls. Our goal was to use an unbiased transcriptomic approach to unveil mechanisms that link the molecular effects of SCIT and SLIT to improvements in clinical symptoms and nasal physiology after allergen exposure, as quantified by standardized parameters including the total nasal symptom score area under the curve (TNSS AUC; hereafter abbreviated

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TNSS) and the peak nasal inspiratory flow area under the curve (PNIF AUC; hereafter abbreviated PNIF).

METHODS

The GRASS trial was a randomized, double-blind, single-center, placebo-controlled, three-arm study. Details of the AIT protocol, clinical outcomes, and other study details have been described previously[10, 14, 15], and full details are available in the online repository. Briefly, eligible TGP allergic participants were randomized to one of three treatment arms in a 1:1:1 ratio (TGP SCIT: SLIT: Placebo) for a total of two years and followed for 1 additional year after treatment ended. Clinical endpoints were assessed at study baseline and each year thereafter at yearly NACs and included the 0–10 hour TNSS and 0–10 hour PNIF. Nasal brushings were collected 9 hours and PBMCs 6–7 hours after each NAC. The current study analyzed gene expression by RNA-sequencing in nasal brushings from 59 GRASS participants (SCIT n=20, SLIT n=19, Placebo n=20) in the per protocol clinical dataset and gene expression in PBMCs from all 84 participants in the per protocol dataset (SCIT n=27, SLIT n=27, Placebo n=30). An additional 17 non-atopic, non-rhinitic individuals were recruited in parallel with the GRASS participants to serve as a healthy control group for the nasal gene expression analysis following an identical nasal allergen challenge protocol. Finally, biopsy-obtained nasal mucosal tissue from 52 of those 59 GRASS participants at year 3 (SCIT n=19, SLIT n=16, Placebo n=17) was examined for epithelial integrity. See the online repository for details on biosample processing, RNA extraction, RNA sequencing, and statistical analyses.

RESULTS

Study overview and demographics

Table E1 summarizes demographic and clinical characteristics of the 59 per protocol GRASS study participants with available nasal brush samples as well as 17 non-atopic healthy controls (HCs) used for comparison. Only 59/84 GRASS participants had nasal brush samples because ethical approval for sampling was obtained part way through the first year of study enrollment. These 59 individuals were statistically similar in terms of demographic, clinical, and response characteristics to the full cohort. Within the brush sample subgroup, ethnicity, age, sex, wheal size, and specific IgE levels to TGP were not significantly different between the 3 study treatment arms. None of the non-atopic healthy controls had a measurable skin prick test (SPT) response to TGP nor to house dust mite, cat, or birch pollen, and all had specific IgE levels to TGP ≤ 0.02 kU/L. Compared to GRASS study participants with nasal brush samples, non-atopic healthy controls were older (mean age: 46.5 vs 34.7 years, $p<0.001$) and were comprised of more females (76.5% vs 35%, $p=0.002$). PBMC samples were available from all 84 GRASS participants and are reported herein.

TNSS and PNIF measurements

The 0–10 hour TNSS and 0–10 hour PNIF were reanalyzed for the 59 participants with nasal brush samples. This showed that SCIT and SLIT led to improvements in both TNSS and

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PNIF after TGP NAC, which peaked at year 2. However, clinical improvements were not sustained at year 3 as no significant differences in TNSS or PNIF were observed comparing the 3 treatment groups at year 3 (Fig. E1). These findings were equivalent to the results obtained in the complete intention to treat and per protocol populations, which have been previously reported[10, 21].

Nasal gene expression signal

RNA-sequencing was performed on nasal brush samples obtained after the NAC at study baseline and each year thereafter. We identified a total of 2237 genes differentially expressed due to SCIT, SLIT, or placebo treatment, and/or related to TNSS (FDR<0.1; Fig. E2) through linear modeling contrasting gene expression at years 1, 2, or 3 relative to year 0 within each group, or testing for association with TNSS among all samples (see Supplementary Methods for full details). We conducted a modular network analysis to uncover biological pathways altered by grass pollen AIT; these genes grouped into 24 distinct coexpression modules by WGCNA (modules 1.N-24.N) (see Supplementary Methods for full details). These 24 GRASS study modules were analyzed in parallel with 8 previously published allergen-response modules identified as differentially expressed due to allergen challenge (modules 1.C-8.C)[22] (Fig. E3). Constituent genes in each module are listed in Table E2 and functional enrichment terms for each module in Table E3/ Supplemental File 1. These modules were used to compare the longitudinal transcriptional responses among the 3 GRASS study treatment groups and then to compare to non-allergic healthy controls.

SCIT and SLIT each led to significant changes in the expression of multiple nasal gene modules at years 1 and 2 relative to year 0, whereas in the placebo group, no modules reached a level of statistically significant change at either of those time points (Figs. 1A, 2A–C, E4A, FDR<0.05). Figures 2A–C depict the magnitudes of change in module expression and the level of statistical significance of those changes from baseline to year 2, and Figure 1A and Table E4 show the results for years 1, 2, and 3. Both active Figures 2A–C treatment groups demonstrated similar overall changes in module expression (Fig. E3, E4B), which peaked at year 2. Changes in module expression over time were similar when SCIT was compared to SLIT (Figs. E3, E5) and there were no statistical differences in any of the modules when SCIT was directly compared to SLIT.

Notably, modules that changed significantly with SCIT and SLIT also showed significant associations with TNSS and PNIF and thus coincided with improvement of the clinical response to the NAC, as assessed by equivalent weighted linear modules. Modules that were decreased by AIT were positively associated with TNSS and negatively associated with PNIF. Similarly, modules that increased with AIT were negatively associated with TNSS and positively associated with PNIF (Figs. 1B, 2D,E and Table E4). Specifically, at year 2, 5/32 modules (1.N, 10.N, 15.N, 16.N, and 3.C) were significantly decreased in both SCIT and SLIT groups, but not changed in placebo and were significantly positively associated with TNSS and significantly negatively associated with PNIF (Table E4, FDRs<0.05). The changes in expression of these modules, with 1.N and 3.C serving as representatives, are shown in Figures 3A, B and the overall expression levels of all 5 modules in Figure

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E6. Module 1.N is a large network of 462 genes enriched for leukocyte function, in particular T cell immunity (including genes *PTPRC*[CD45], *CD2*, *CD3E*, *CD4*, *CD80*, *CD86*, *CD44*) and also with specific enrichment for genes related to type 2 inflammation (*IL4R*, *CCL24*, *FCER2*, *ALOX5*, *ALOX5AP*, *IL1RL1*, *IKZF3*) (Fig. 3E). Module 3.C is a previously defined NAC responsive module representing a network of 96 genes related to mucin production and prostaglandin synthesis. In a previous study, we showed that allergen provocation increased the expression of module 3.C, and expression was positively associated with TNSS and negatively associated with PNIF[22]. In our current study, the relationship of 3.C to TNSS and PNIF is consistent with our previous results. Functionally relevant genes in this network include secretory products of goblet and serous cells, *MUC5AC* and *LYZ*, the transcriptional activator *FOXA3*, which induces goblet cell metaplasia, and the three trefoil factors (*TFF1, TFF2, TFF3*), secretory proteins that can interact directly with MUC5AC protein. The remaining 3 modules (10.N, 15.N, 16.N) each contain components of ER-stress/stress-response pathways, and together form a network of 119 genes including multiple heat shock protein family members (*HSPD1, HSPH1, HSPA1A, HYOU1*) and associated co-chaperones (*FKBP4, BAG3, CHORDC*).

A reverse pattern was observed in 10/32 modules (2.N, 5.N, 6.N, 7.N, 9.N, 11.N, 18.N, 21.N, 4.C, 6.C). These modules were significantly increased in both SCIT and SLIT groups but not in placebo at year 2, inversely associated with TNSS, and positively associated with PNIF (Fig. 1B, 2D,E and Table E4, FDRs<0.05). The changes in expression of these modules, with 2.N and 6.N as examples, are shown in Figures 4A, B, and the overall expression levels of all of these modules in Figure E7. Module 2.N is a network of 148 genes enriched for mRNA processing genes that also contains *HES1*, a transcription factor important for differentiation and maturation of epithelial cells. Modules 5.N, 6.N, 7.N, 9.N, 11.N, 18.N, 21.N each contain molecules related to epithelial barrier functions and are significantly enriched for pathways related to both epithelial integrity (e.g. *EGFR, EPHA1, KFT7, KRT2, KFT4, JUP, TJP2, MKL2, MYO5B*) and lipid metabolism (e.g. *PLCD1, PLCD3, PLBD1, APOE, LIPH, LYPLA1*); given their similar patterns in the data and similar functional enrichment, we have depicted their genes within a single network (Fig. 4E). Module 4.C is a NAC responsive module that we have previously identified[22] composed of a network of 61 genes related to the negative regulation of transcription. Our previous study demonstrated that the expression of this module decreased following allergen challenge, and expression was negatively associated with TNSS and positively associated with PNIF. The relationship of 4.C to TNSS and PNIF in our current study was consistent with our previous results.

At year 3, similar to how the clinical responses reverted to baseline (pretreatment) levels, the majority of modules that had been differentially expressed at the earlier time points returned towards their baseline levels of expression. However, both the module enriched for mucin production/prostaglandin synthesis (3.C) and the module enriched for type 2 inflammation (1.N) remained decreased in SCIT and SLIT groups, but a modest decrease in the placebo group was also seen (Fig. 3A,B), which may represent a regression to the mean in the placebo group.

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Nasal gene expression in healthy non-atopic controls

We next compared the expression of the post-NAC modules changed by AIT to the expression levels seen in 17 non-atopic healthy control participants that had received a corresponding NAC with TGP with grass pollen extract. The analyses were adjusted for age and sex given the differences in these variables between GRASS study participants and non-atopic healthy controls. Of the 5 modules discussed above that were decreased by SCIT and SLIT and related to TNSS and PNIF, 4 modules (1.N, 15.N, 16.N, and 3.C) started at levels higher in allergic individuals and decreased to levels that were similar to those observed in healthy controls who had received the same nasal allergen challenge; these include the modules enriched for type 2 inflammation and mucin production/prostaglandin synthesis noted above (Figs. 3C,D, E6)Only module 10.N, enriched for stress-response genes, was similar in expression to HCs and trended towards levels lower than HCs during SCIT and SLIT (years 1 and 2) (Fig. E6).

Of the 10 modules that were increased by SCIT and SLIT and related to TNSS and PNIF, 8 of these modules (5.N, 6.N, 9.N, 11.N, 18.N, 21.N, 4.C, 6.C) started at levels lower in allergic individuals following nasal allergen challenge and increased towards those observed in healthy control levels after the same allergen exposure (Figs. 4D, E7); these include the modules enriched for epithelial integrity and lipid metabolism. In contrast, 2.N, which was enriched for mRNA processing genes, was similar in expression to healthy controls and increased to levels higher than healthy controls during SCIT and SLIT (years 1 and 2) (Fig. 4C). Module 7.N showed overlapping levels with healthy controls at all time points.

Nasal mediation analysis

Causal mediation analysis was performed to understand which among the module expression changes showed the strongest causal link to the observed changes in TNSS outcome. Here a potential mediator is defined as explaining a meaningful portion of the treatment effect originally ascribed to therapy alone. Of the modules decreased by AIT, modules 10.N and 16.N (both enriched for stress-response genes) significantly mediated the effect of SCIT therapy on TNSS. Decrease in expression of modules 10.N and 16.N accounted for 38.4% (bootstrap p=0.004; FDR=0.043) and 35.2% (bootstrap p=0.002; FDR = 0.032), respectively, of the observed decrease in TNSS for SCIT and 32.3% (bootstrap p=0.014; FDR=0.085) and 28.2% (bootstrap p=0.006; FDR=0.085), respectively, of the observed decrease in TNSS for SLIT across all 3 years of the study. Among the upregulated modules, 2.N (enriched for mRNA processing genes) showed a near significant mediating effect, accounting for 30.0% (bootstrap p=0.008; FDR=0.064) of the observed increase in TNSS for SCIT and 27.0% (bootstrap p=0.002; FDR=0.064) of the observed increase in TNSS in SLIT across all 3 years of the study (Fig. 5). Interestingly, modules 10.N and 2.N were also those modules seen to decrease or increase, respectively, to levels outside the range of HCs during treatment (Figs. 4C, E6, E7).

Nasal biopsies and epithelial integrity

Nasal biopsies were obtained 9±2 hours after the NAC at year 3 in a subset of 52 individuals and were used to evaluate the integrity of the nasal epithelial barrier at the end of study. An intact brush border (epithelial score of 4) was seen predominantly in individuals treated

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with SCIT or SLIT (10 individuals) as opposed to placebo (1 individual), which was statistically significant (p=0.03). Epithelial integrity score showed a significant association with module 3.C expression measured at the same time point; module 3.C is enriched for mucus hypersecretion genes and lower module expression was related to a more intact nasal epithelium (p=0.01) (Fig. E8A,B).

PBMC gene expression signal

RNA-sequencing was performed on RNA extracted from PBMC samples that were collected pre-and 6–7 hours post-NAC at study baseline and each year thereafter. A total of 697 differentially expressed genes were identified and used to generate 11 unique PBMC modules. Enrichment terms for each of the modules can be found in Table E3/Supplemental File 1. The SCIT and SLIT groups had significant decreases in the expression of multiple PBMC gene modules at years 1 and 2 relative to year 0, which were not significant in the placebo group, with maximal reductions occurring at year 2 (Figs. 6A–C, E9, E10, Table E5). Two of the modules significantly decreased by AIT (Fig. 7A, B) were also significantly positively associated with TNSS values and negatively associated with PNIF values (Fig. 6D, E, Table E5). Like in the nasal samples, SCIT and SLIT groups demonstrated overall similar changes in differential module expression at each time point (Figs. S11, S12). Module 4.B is a network of 42 genes significantly enriched for cytokine mediating signaling molecules and specifically appears to reflect IL-4/IL-13 signaling (Fig. 7C). Module 11.B is a network of 15 genes significantly enriched for molecules related to cellular stress response and cytokine stimulus (Fig. 7D). Both of these modules were also significantly upregulated in post-NAC compared to pre-NAC samples (Fig. E13, Table E6).

PBMC mediation analysis

Decrease in expression of module 11.B demonstrated near significant causal mediation of AIT on TNSS values, accounting for 21.4% of the observed treatment effect in SCIT (bootstrap p = 0.008; FDR=0.088) on TNSS and 15.1% of the observed treatment effect in SLIT (bootstrap p = 0.066; FDR=0.286) across all years compared to placebo (Fig. 8). Module 4.B did not show any significant causal mediation effect.

DISCUSSION

AIT is an effective treatment option for patients with AR, but the precise mucosal and immunologic mechanisms responsible for clinical improvement are uncertain[11]. In the context of an AIT clinical trial, we observed a multifaceted change in transcriptional networks attributable to AIT in both longitudinal nasal and blood samples, which, importantly, showed direct association with two measurements of clinical responsiveness, TNSS and PNIF.

Perhaps not surprisingly, with both SCIT and SLIT, we observed significant decreases in the NAC-provoked expression of elements of type 2 immunity. This was observed both at the level of the nasal mucosa (module 1.N) and in PBMC gene expression (module 4.B). Downregulation of type 2 immune responses by AIT, including reductions in circulating Th2A cells and nasal type 2 cytokines, have been shown in previous work[12, 15]. However,

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in this study, the gene module decreases attributable to AIT involved a much larger array of interconnected immune pathways beyond what we might have anticipated of canonical type 2 inflammation. The decreased modules included 5 nasal modules, 1.N, 3.C, 10.N, 15.N, 16.N, and 2 PBMC modules, 4.B and 11.B.

The functions represented in the largest nasal module, 1.N, were broadly characterized by Gene Ontology terms including immunity and lymphocyte chemotaxis, and its decrease appears to reflect changes in mucosal associated immune cells resultant from AIT. Multiple specific immune functions are found within this module including aspects of T cell migration and signaling, such as the T cell receptor signaling pathway and several components of type 2 inflammation including enrichment for the FcεRI signaling pathway, IL-4 production, and leukotriene biosynthesis. This module also contains a large set of innate immunity genes including those involved in IFN, IL-1, and complement signaling demonstrating a close correlation of these innate pathways with T cell signaling in the nasal mucosa and relationships to the severity of the allergic response.

Module 3.C on the other hand, reflects changes in the mucosal epithelium itself that were decreased by AIT, specifically functions of MUC5AC production and prostaglandin synthesis. In our previous study[22], we showed that allergen provocation increased the expression of this module, and that expression was positively associated with TNSS and negatively associated with PNIF. In our current study, the relationships of 3.C to TNSS and PNIF are consistent with those past results and we have now demonstrated that the module is decreased by AIT. Notably, the expression of 3.C with AIT decreased towards a value similar to that of post-NAC non-allergic HCs, showed some persistent change at year 3, and was associated with an increase in the epithelial integrity histologically at year 3. We thus hypothesize that this MUC5AC pathway is upregulated secondary to impaired epithelial integrity in AR, as has been observed in other diseases, and plays an important role in allergen provoked symptoms[23, 24].

Modules 10.N, 15.N, and 16.N each contain components of ER stress and stress-response pathways. ER stress plays an important role in many diseases and has been seen as an important pathway in asthma indicative of multiple inflammatory phenotypes including type 2 inflammation, mucus secretion, and airway remodeling[25, 26]. To our knowledge, this is the first demonstration of its role in allergic rhinitis in humans, though this is consistent with data from animal models[27]. Interestingly, two of these modules, 10.N and 16.N, had a significant causal mediation effect statistically linking AIT to improvement in TNSS, suggesting they play a proximal role in the efficacy of AIT. Thus, we could hypothesize that allergen-induced ER stress represented by these modules can act as the biological upstream driver of these other significant inflammatory modules linked to higher TNSS and lower PNIF in response to allergen exposure. These modules contain several molecules known to mediate ER stress, which are discussed in the online repository. As such, the aspects of response to ER stress in these modules could represent master regulators of the mixed inflammatory pathways characterizing AR which would be useful molecules to further explore as potential therapeutic targets in order to potentiate the efficacy of AIT.

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The pathways decreased by SCIT and SLIT in PBMCs were narrower in scope than those in nasal brushings. PMBC module 4.B showed enrichment for cytokine mediating signaling molecules and appears to reflect specifically IL-4/IL-13 signaling, which we would anticipate being decreased by AIT. Module 11.B was enriched for molecules related to cellular stress response and cytokine stimulus. 11.B showed a significant causal mediation effect and as such likely contains regulators of the blood immune response in AR, which are further detailed in the online repository. Since we had pre- and post-NAC PBMC samples, we were able to confirm that allergen provocation increased the expression of 4.B and 11.B, which was then reduced by AIT at years 1 and 2.

AIT also increased the allergen-induced expression of 10 nasal gene modules, but no PBMC modules. Collectively, these nasal modules reflected AIT-induced expression of multiple epithelial functions, including notably a large set of genes related to epithelial barrier integrity and function as well as lipid metabolism. The importance of the epithelial barrier in AR was demonstrated by Ahuja et al, showing that individuals with house dust mite allergy had reduced expression of epithelial integrity-related genes following allergen challenge compared to individuals without house dust mite allergy[18]. We similarly observed epithelial integrity pathways that may be reduced in individuals with AR compared to healthy controls (Fig. E6). Our data further demonstrate that AIT increased gene expression associated with these pathways and that this effect may be related to the clinical effectiveness of AIT as both TNSS and PNIF strongly correlated with module expression. AIT-induced increases in one of these nasal modules, Module 2.N, had a significant causal mediation effect linking expression to the clinical efficacy of AIT, indicating that genes in this module may be regulators driving improvements in epithelial integrity pathways in response to AIT; these genes are further discussed in the online repository.

Additional research is needed to better understand how epithelial barrier dysfunction is associated with allergic disease, but it may be due to the enhanced ability of allergen to infiltrate the subepithelium when the epithelial barrier is disrupted, which may increase the interaction of an allergen with immune cells such as antigen presenting cells and/or mast cells promoting the allergic response[17]. Epithelial disruption can also lead to increased production of alarmins (IL-25, IL-33, TSLP) that promote type 2 inflammation[13]. Additionally, it is not clear how AIT may have led to the changes in epithelial barrier-related gene expression that we observed. It is possible that downregulation of the type 2 immune response, which occurred with active treatment in the GRASS study, played a role. A recent study showed that when IL-4, but not IFN-γ, was added to primary nasal epithelial cells in vitro, epithelial integrity was disrupted[28]. However, it is difficult to ascertain whether downregulation of type 2 immune responses was driving the increases in gene expression related to epithelial integrity in our study, or the reverse, since we observed changes to both by year 1 (the first post treatment sample collection time point). Perhaps, studies including repetitive evaluations at shorter intervals may reveal differences in the kinetics of these AIT effects leading to better understanding of their causal relation.

When we analyzed a cohort of healthy non-atopic controls, we observed that AIT altered the expression of most nasal modules of GRASS study participants to levels seen in HCs following the same experimental exposure to allergen, further validating our findings

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and supporting the notion that the gene expression changes we observed were clinically meaningful. Interestingly, two of the nasal modules shown to be causally mediating the clinical response to allergen (10.N and 2.N) had pre-treatment expression levels that were similar to those in the HCs, and then were expressed beyond the HC levels by active treatment. It is possible that successful AIT may be achieved by induction of changes in these causal gene pathways/transcription factors that are beyond “normal” or steady-state levels to result in downstream changes in the other identified pathways towards “normal” levels.

One goal of our work was to uncover differences in the molecular responses to SCIT and SLIT. However, we found that both therapies led to strikingly similar changes in the expression of gene modules in both the nasal epithelium and peripheral blood. A comparison of changes in module expression (SCIT vs SLIT) yielded no significant differences, indicating that SCIT and SLIT take advantage of similar mechanisms to improve clinical symptoms.

A potential shortcoming of our study was that we only evaluated transcriptional changes during AIT to a single allergen, so future studies will be necessary to determine whether our results are generalizable to AIT studies with other aeroallergens. GRASS participants received therapy for only 2 years which is insufficient for sustained clinical benefit, so it remains unclear whether there may be additional transcriptomic changes that would occur after a longer duration of AIT that would underlie sustained clinical response, i.e. tolerance. While we did not identify statistically significant transcriptomic differences between SCIT and SLIT, quantifiable differences between individual module expression in the two groups were noted, and our study may not have been powered adequately to capture potential differences. We did not perform any cell sorting so determining which cell type was driving the transcriptional changes we observed is imperfect. That said, using cellularly heterogeneous samples allowed us the opportunity to uncover a more complete picture of changes that were occurring in both immune and epithelial cells during AIT. Our sample collection methods did not allow for techniques such as single cell RNA sequencing to differentiate cell types, nor were we able to perform proteomic studies, which would be important to understand translation of expressed genes or to account for the impact of possible post-translational modifications. Future pursuits to validate the transcriptomic changes we have observed could also include *ex vivo* ALI studies from nasal samples collected throughout immunotherapy. The non-atopic healthy controls we studied were recruited in parallel with the GRASS study participants, tended to be slightly older, and included a higher percentage of females. However, adjustment for these variables did not change our results, suggesting our findings were not confounded by age or gender differences in gene expression and that the healthy controls were suitable as a comparator group despite these limitations.

In summary, our study identified nasal and PBMC gene expression pathways altered by AIT, which were associated with improvement in clinical responses induced by nasal allergen challenges. The specific gene pathways altered by AIT, and highlighted in our manuscript, can be tested as biomarkers to identify responder patients. Furthermore, the modified gene pathways have identified potential novel targets that could aid efforts to develop

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combination strategies with allergen immunotherapy for greater efficacy and sustained tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GRASS Study Contributors: *Imperial College Nursing Staff:* Andrea Goldstone, RN, Ms; Fotini Rozakeas, Rachel Yan, RN, Ms; *Imperial College Study management and administration:* Natalia Klimowska-Nassar, Mimi Poon. *Imperial College Laboratory projects:* Delica Kit Cheung, Constance Ito, Janice Layhadi, Elisabeth Lemm, Ellen Macfarlane, Orla MacMahon, Tomokasu Matsuoka, Rebecca Parkin, Amy Switzer. *ITN Staff:* Adam Asare, PhD (past); Eduard Chani, PhD; Judith Evind; Deborah Phippard PhD (past); Peter Sayre MD, PhD; Maureen Sharkey, MA (past); Don Whitehouse, MS. *DAIT-NIAID Staff:* Steven Adah, PhD (past); Theresa Allio, PhD; Christine Czarniecki, PhD; Jui Shah, PhD (past). *Rho Federal Systems Staff:* Travis Mason, Ann Nguyen, Shayala Gibbs, Spencer Childress. We especially thank the patients for their participation in this study.

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The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Data and Materials Availability:

The raw RNA-sequencing FASTQ data and Minimum Information about a high-throughput nucleotide SEQuencing Experiment (MINSEQE) have been deposited to the National Center for Biotechnology Information Gene Expression Omnibus (GEO) with accession number GSE206152. Data sets for these analyses are accessible through TrialShare, a public Web site managed by the Immune Tolerance Network (https://www.itntrialshare.org/GRASS_transcriptomics.url).

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Abbreviations used:

AR	allergic rhinitis
AIT	allergen immunotherapy
TGP	timothy grass pollen
SCIT	subcutaneous immunotherapy
SLIT	sublingual immunotherapy
GRASS	Gauging Response in Allergic Rhinitis to Sublingual and Subcutaneous Immunotherapy
NAC	nasal allergen challenge
PBMC	peripheral blood mononuclear cells
TNSS AUC	total nasal symptom score area under the curve
PNIF AUC	peak nasal inspiratory flow area under the curve
HCs	healthy controls
SPT	skin prick test

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Key Messages:

- Nasal epithelial transcriptomics identified multiple gene expression pathways directly associated with the clinical efficacy of grass allergen immunotherapy, defining mechanisms of effect and individual level responsiveness to therapy.
- Notably grass immunotherapy increased expression of multiple epithelial functions including those related to epithelial barrier integrity and lipid metabolism, while also decreasing components of type 2 inflammation and mucus secretion.

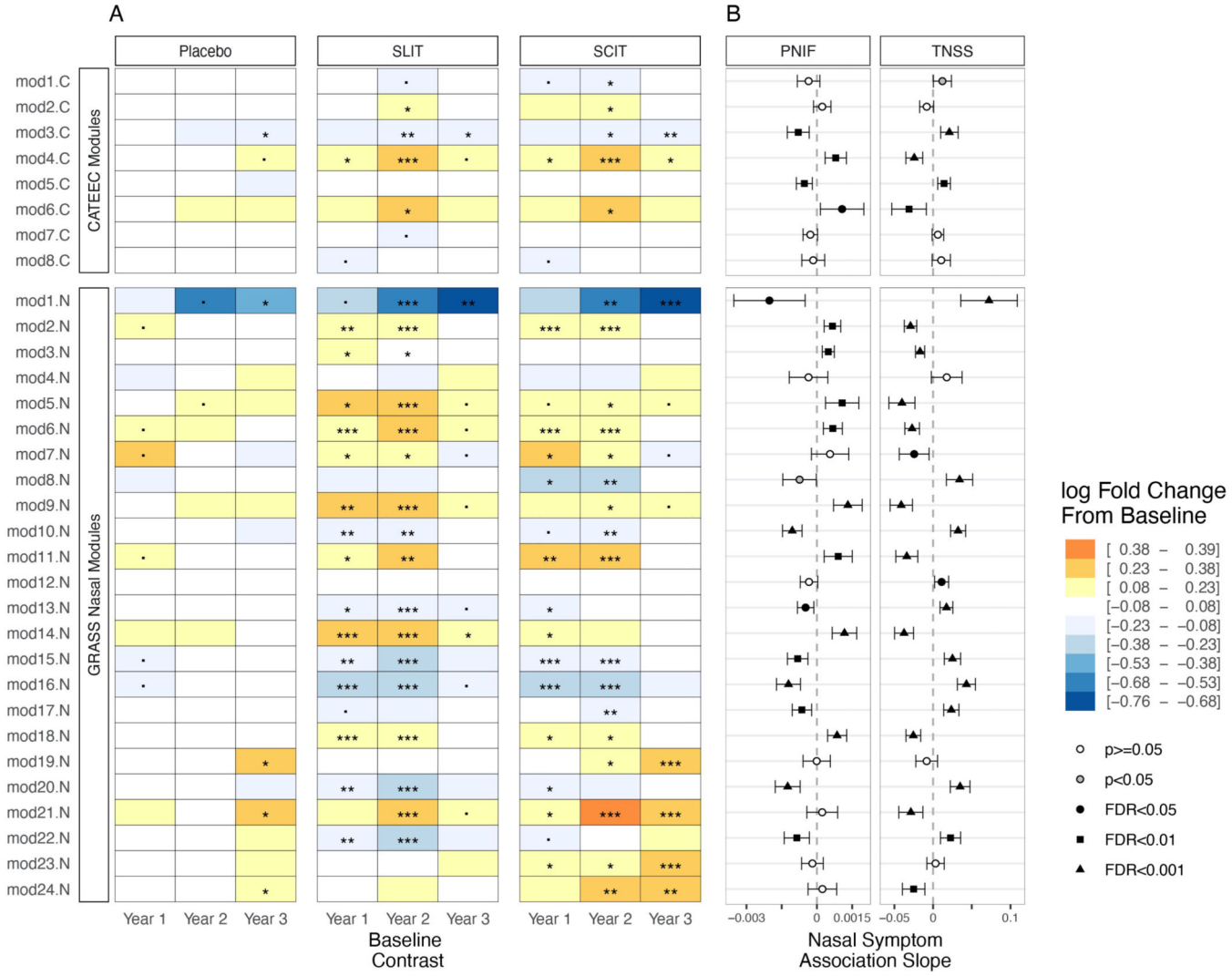

Figure 1.

Differential module expression from post NAC nasal brush samples. Indicated are **a)** fold change values of log2 normalized module expression (logFC) in each treatment group contrasting years 1, 2, and 3 to baseline values; and **b)** association (model βs; 95% CI) of two clinical endpoints to module expression, PNIF and TNSS. Annotations in panel A indicate significance of log FC as follows: ˙ p < 0.05, * FDR<0.05, **FDR<0.01, ***FDR<0.001. Corresponding significance of β-coefficients in panel B is indicated by point color and shape legend included in the figure.

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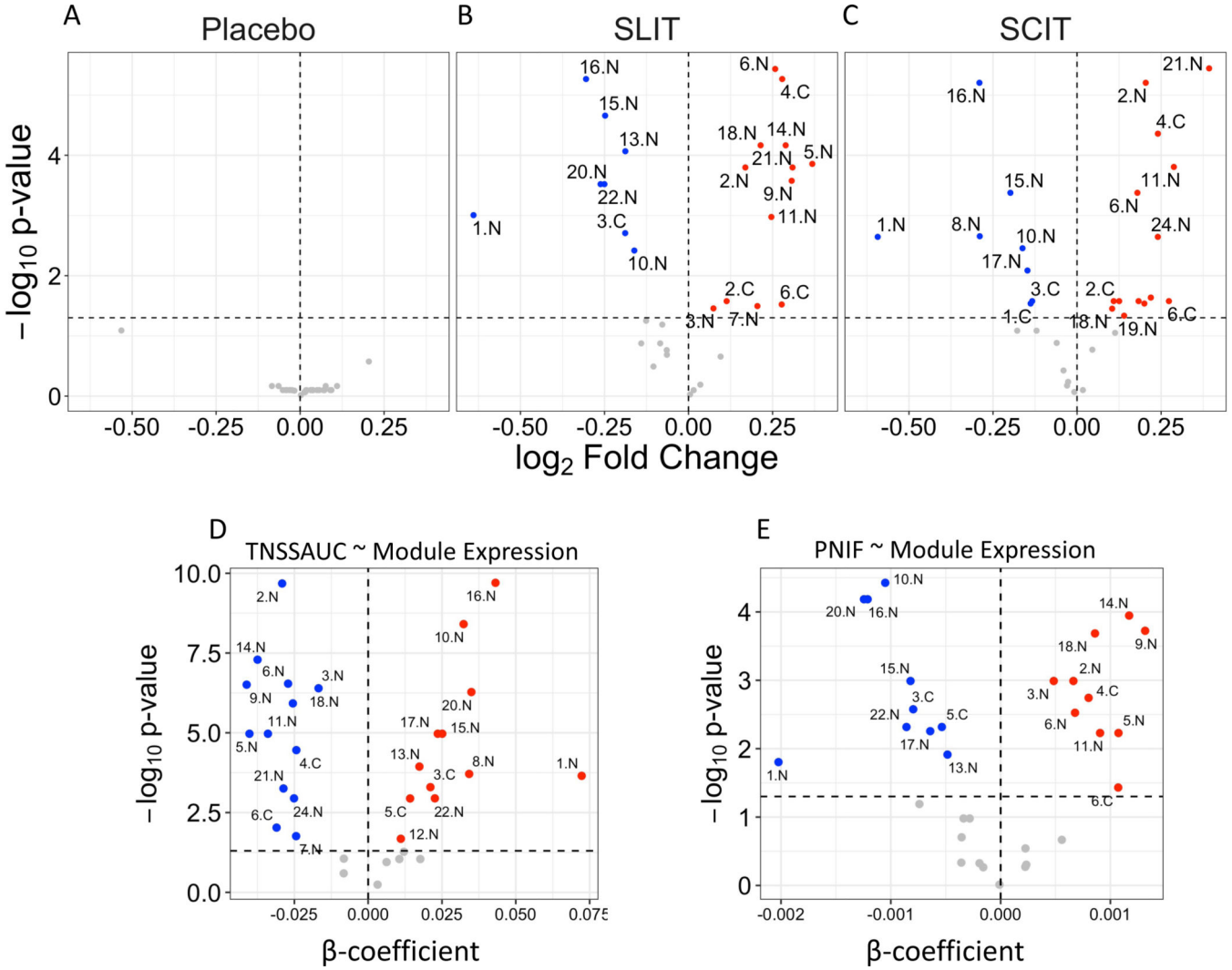

Figure 2.

Differentially expressed modules from nasal brush samples collected post NAC in each treatment group at year 2. Volcano plots of modules differentially expressed at year 2 in **(A)** placebo, **(B)** SLIT, **(C)** SCIT with a negative change (blue) or positive change (red) seen in log-2 fold change versus -log10 FDR-value (top row). Volcano plots of modular expression with association to the regression slope coefficient compared to TNSS **(D)** and PNIF **(E)** seen with negative association (blue) or positive association (red) (bottom row). 5/32 modules that showed negative change in expression in both SLIT and SCIT were found to be positively associated with TNSS while being negatively associated with PNIF. Conversely, 10/32 modules showed a positive change in differential expression in both SLIT and SCIT and were found to be negatively associated with TNSS while being positively associated with PNIF.

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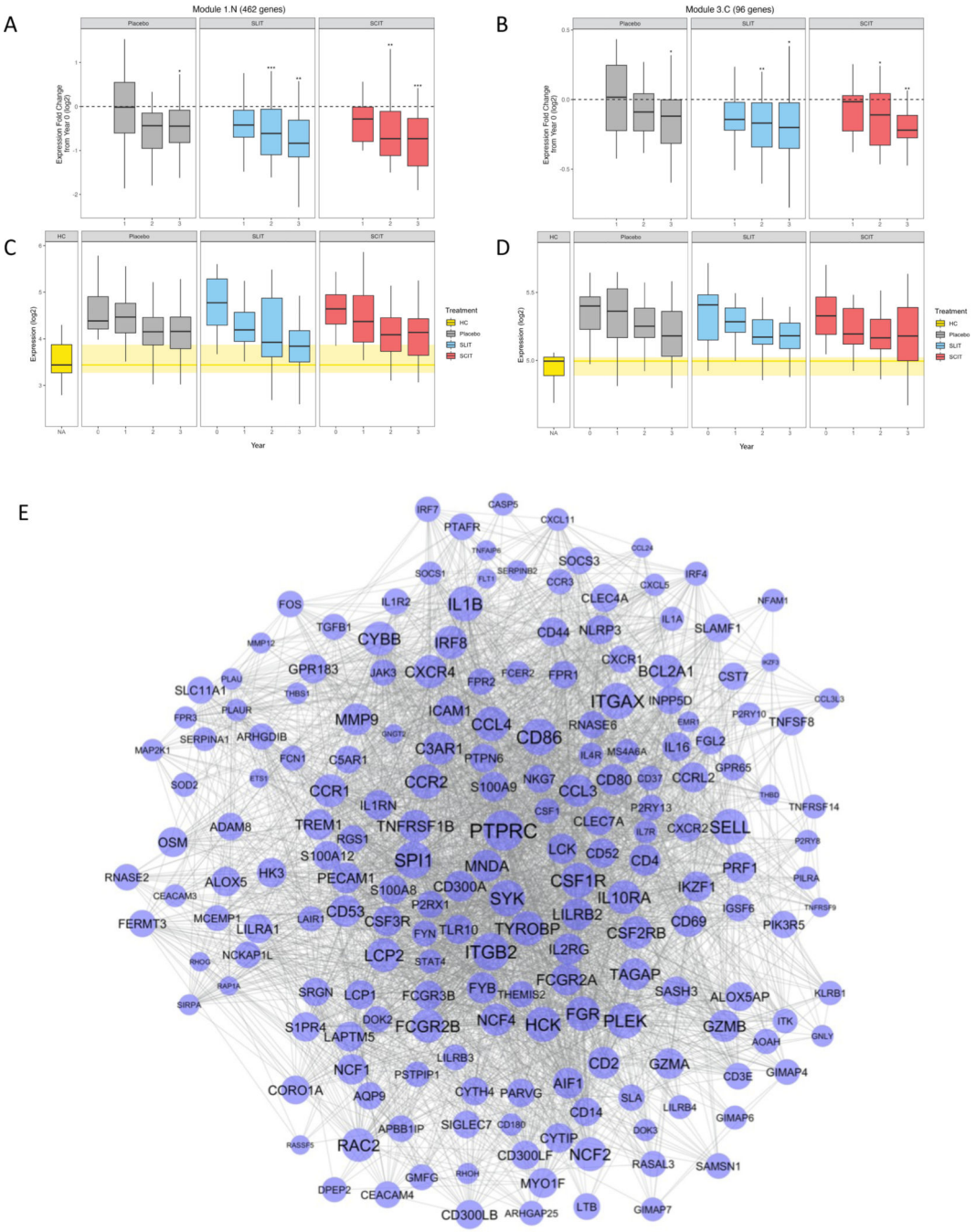

Figure 3.

Changes in modular gene expression from post NAC nasal brush samples over the duration of the GRASS study. Boxplot with log2 fold change at year 1, year 2, and year 3 relative to year 0 post NAC challenge in the placebo (grey), SLIT (blue) and SCIT (red) study groups. Modules with significantly decreased expression over time as compared to placebo are represented by upper row, **(A)** module 1.N (462 genes) and **(B)** module 3.C (96 genes). Gene expression in healthy controls (yellow) following nasal allergen challenge with grass pollen is also depicted for **(C)** module 1.N and **(D)** module 3.C. (**E)** Gene network plot

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of module 1.N from nasal brush samples depicting all known molecular interactions in STRING and significantly enriched for “T-cell immunity”. Genes are represented as circular nodes, and known gene–gene interactions from STRING are shown as connecting edges. The size of each node is proportional to the number of interactions. The networks are drawn as force-directed graphs, meaning that genes toward the center have the greatest centrality within the network. This module was seen to be decreased by immunotherapy. Significant changes within each group at a given timepoint relative to year 0 are noted with: *p-adj<0.05, **p-adj<0.01, ***p-adj<0.001.

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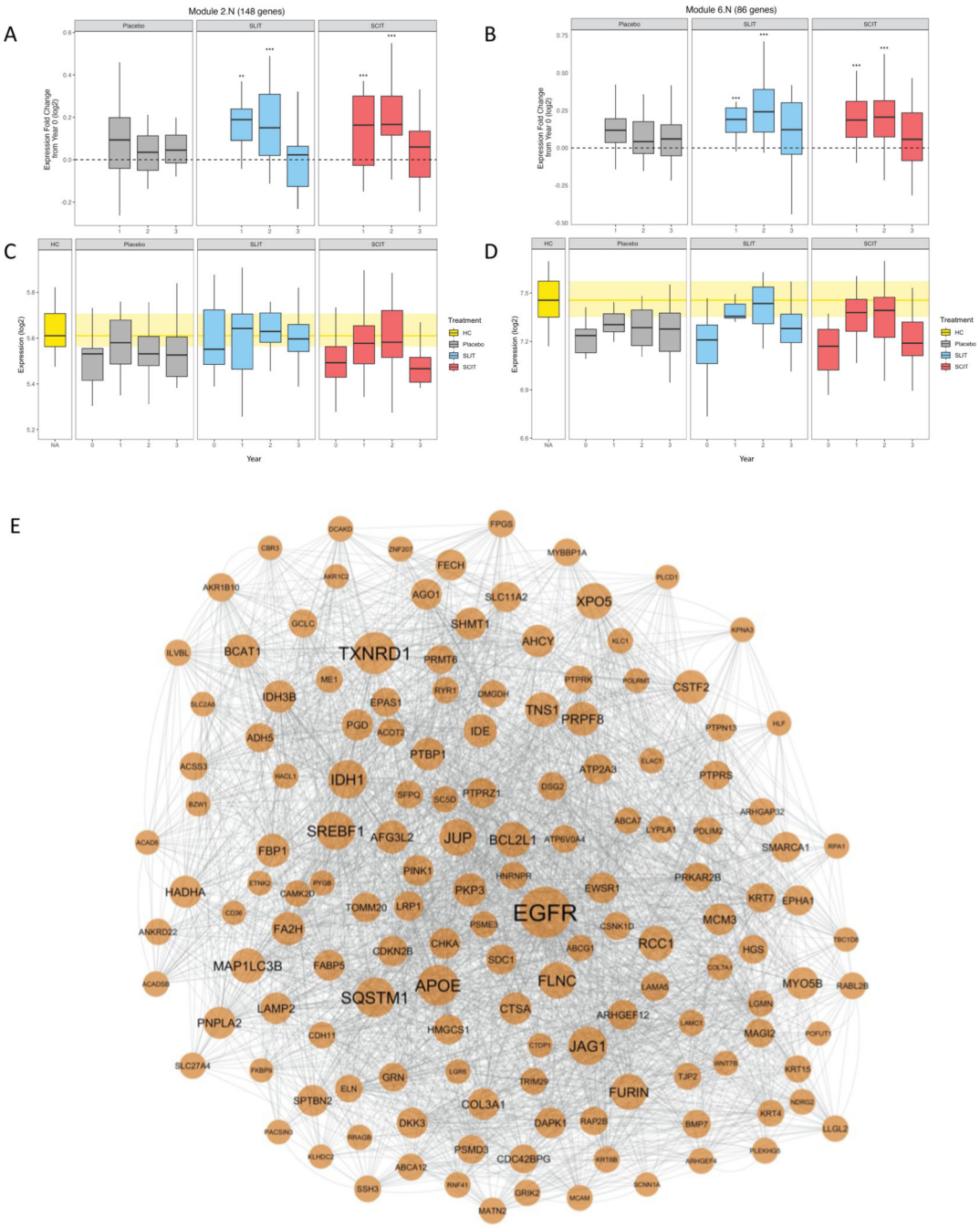

Figure 4.

Changes in modular gene expression from post NAC nasal brush samples over the duration of the GRASS study. Boxplot with log2 fold change at year 1, year 2, and year 3 relative to year 0 post NAC challenge in patient groups of placebo (grey), SLIT (blue) and SCIT (red). Modules with significantly increased expression noted in lower row by **(A)** module 2.N (148 genes), and **(B)** module 6.N (86 genes). Gene expression in healthy controls (yellow) following nasal allergen challenge with grass pollen is also depicted for **(C)** module 2.N and **(D)** module 6.N. **(E)** Gene network plot from nasal brush samples of modules 5.N, 6.N,

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7.N, 9.N, 11.N, 18.N, 21.N depicting all known molecular interactions from STRING and significantly enriched for pathways related to both lipid metabolism and epithelial barrier integrity, which were seen to be increased by immunotherapy. Genes are represented as circular nodes, and known gene–gene interactions from STRING are shown as connecting edges. The size of each node is proportional to the number of interactions. The networks are drawn as force-directed graphs, meaning that genes toward the center have the greatest centrality within the network. Significant changes within each group at a given timepoint relative to year 0 are noted with: *p-adj<0.05, **p-adj<0.01, ***p-adj<0.00.

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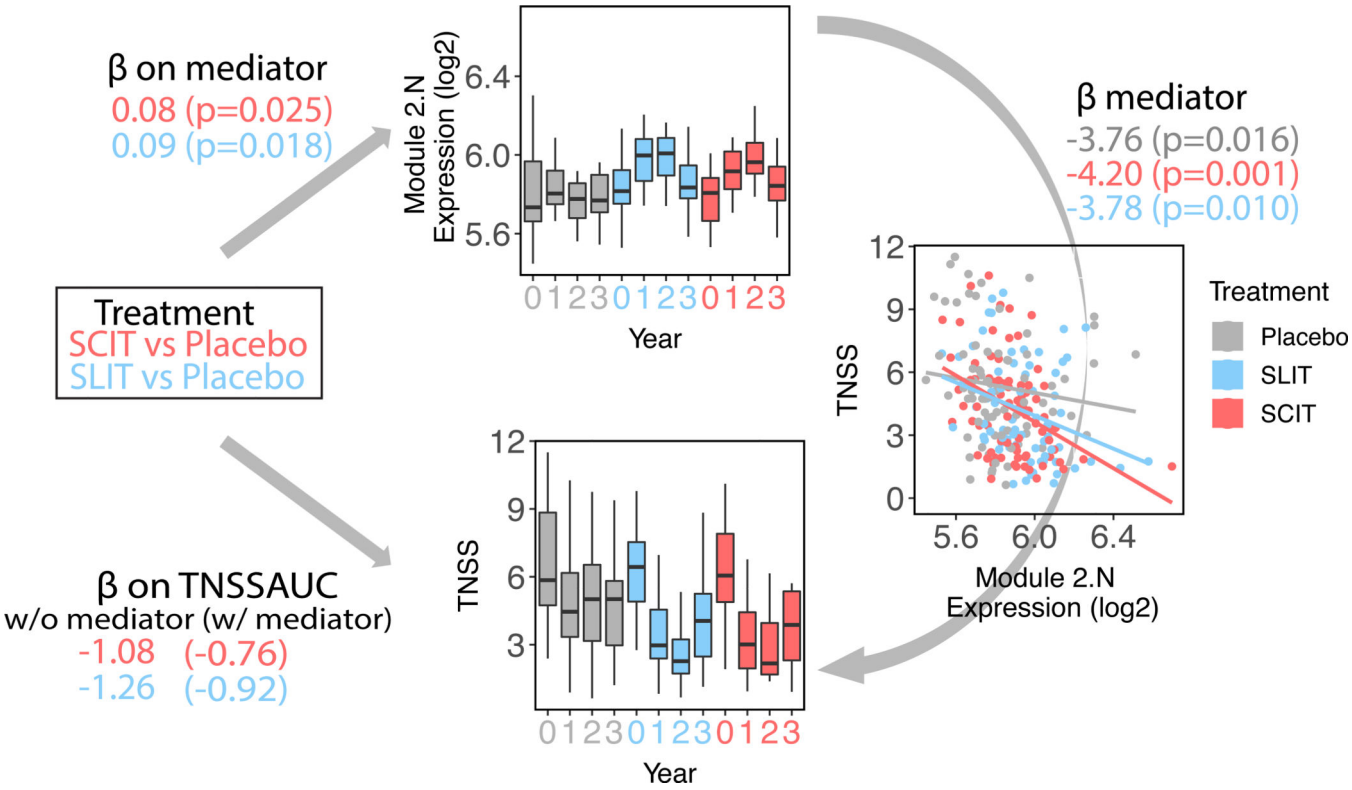

Figure 5.

Schematic showing causal mediation of treatment effects (SCIT and SLIT) on TNSS by module 2.N. in nasal brush samples. Lower panel shows observed direct effects of SCIT (red) and SLIT (blue) therapies on TNSS (outcome) relative to Placebo (grey) at each time point. Upper panel shows effects of SCIT and SLIT therapies on Module 2.N expression (mediator) relative to Placebo at each time point. Right panel shows the relationship of Module 2.N expression to TNSS within each group, by which the direct effect on TNSS may be mediated. Module 2.N demonstrated significant mediating effect of both SCIT and SLIT on TNSS across the duration of the GRASS study in a moderated causal mediation analysis, accounting for 30.0% and 27.0% of the effect in SCIT and SLIT, respectively ($p<0.05$). Statistical results of this analysis are presented in the figure; "β on mediator" indicates the β-coefficient and associated p-value of the model comparing Module 2.N expression by each treatment group contrasted to placebo while adjusting for visit; "β mediator" indicates the β-coefficient and associated p-value of module expression in relation to TNSS by treatment group adjusting for visit; "β on TNSSAUC w/o mediator (w/ mediator)" shows the change in the β-coefficient of the model comparing TNSS by each treatment group contrasted to placebo, adjusting for visit, either without or with adjusting for Module 2.N expression, showing had Module 2.N expression partially accounts for the treatment effect on TNSS.

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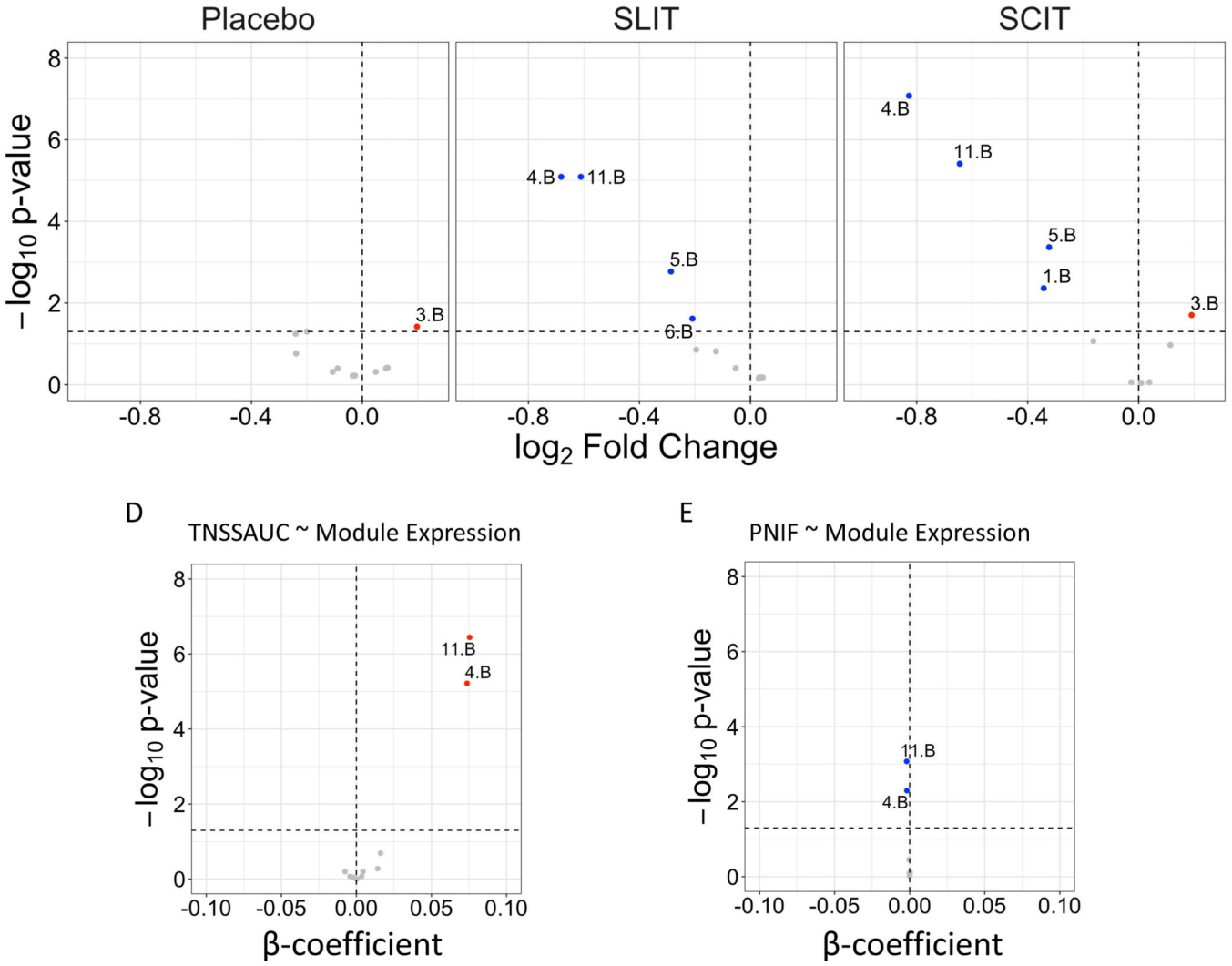

Figure 6.

Differentially expressed modules from post NAC PBMC samples in each treatment group at year 2. Volcano plots of modules differentially expressed at year 2 in placebo **(A)**, SLIT **(B)**, SCIT **(C)** with a negative change (blue) or positive change (red) seen in log 2 fold change versus -log10 p-value (top row). Volcano plots of modular expression with association of **(D)** TNSS and **(E)** PNIF seen with negative association (blue) or positive association (red) in Bcoefficient versus -log10 p-value (bottom row).

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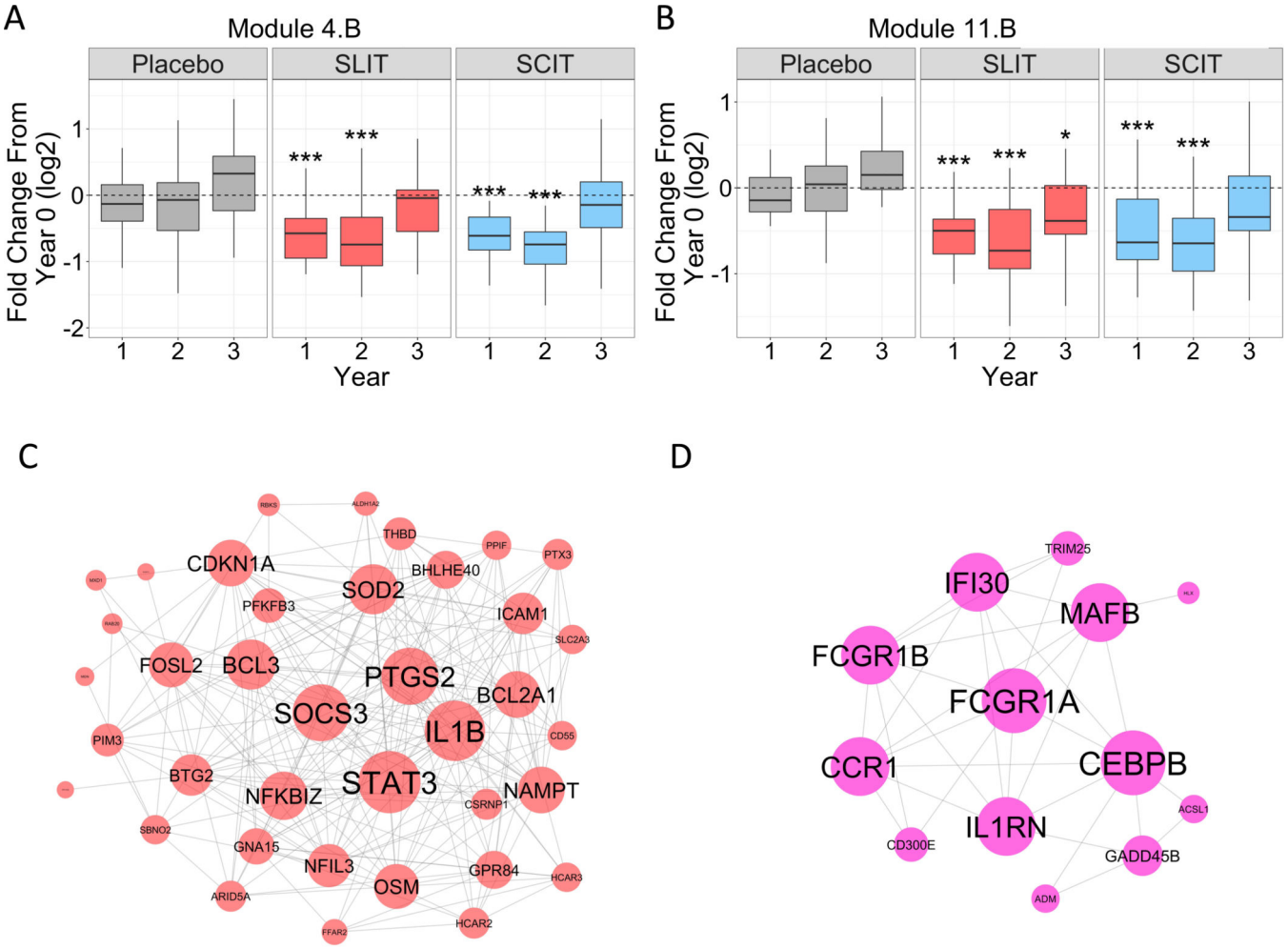

Figure 7.

Changes in post NAC PBMC modular gene expression over time. Boxplot showing Log2 fold changes in the allergen-provoked expression of **(A)** module 4.B and **(B)** module 11.B from year 0 to year 1, year 2, and year 3 in placebo (grey), SLIT (red) and SCIT (blue) participants. **(C)** Gene network plot of genes from 4.B containing molecules significantly enriched for IL-4/−13 signaling, and decreased by immunotherapy. **(D)** Gene network plot of 11.B, containing molecules significantly enriched as cytokine signaling, and decreased by immunotherapy. Significant changes within each group at a given timepoint relative to year 0 are noted with: *p-adj<0.05, **p-adj<0.01, ***p-adj<0.001

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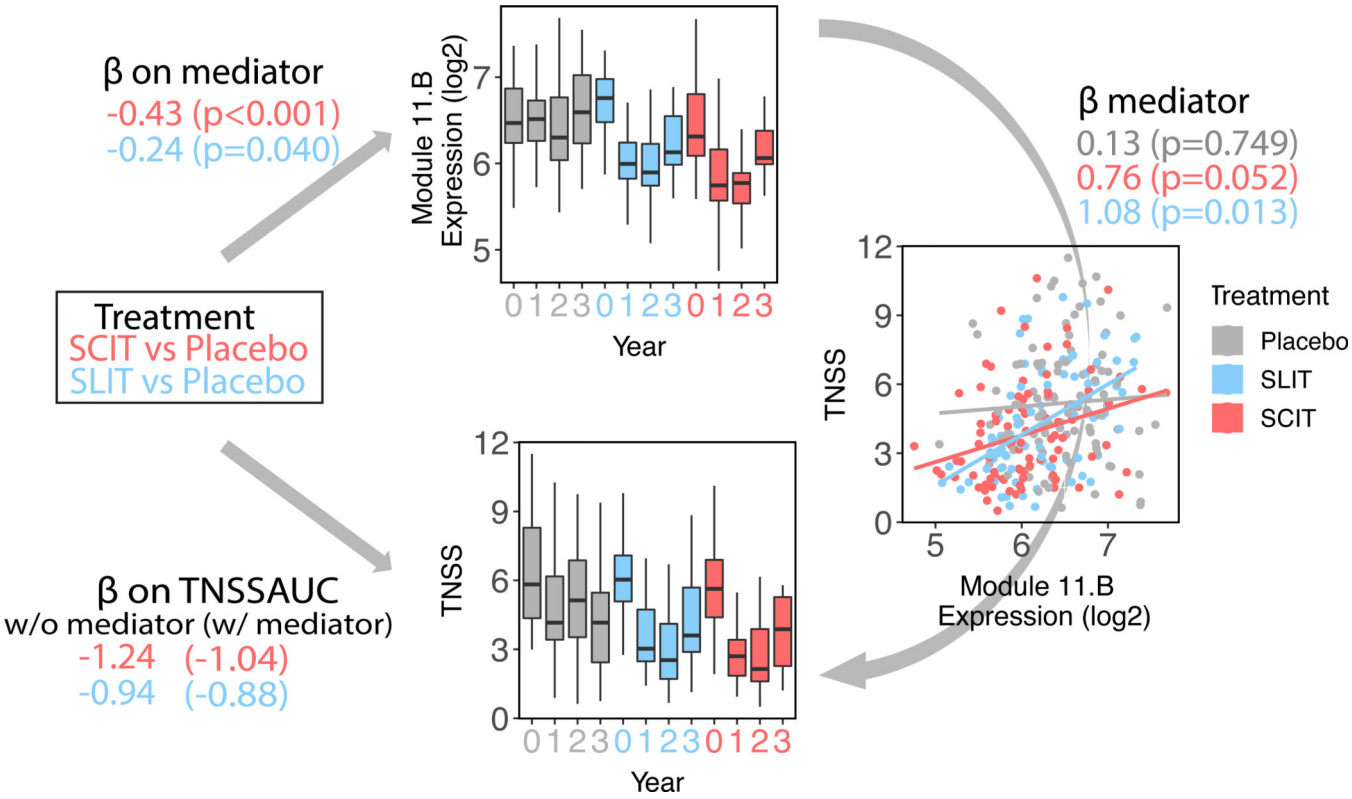

Figure 8.

Schematic showing causal mediation of treatment effects (SCIT and SLIT) on TNSS by module 11.B in PBMC samples. Lower panel shows observed direct effects of SCIT (red) and SLIT (blue) therapies on TNSS (outcome) relative to Placebo (grey) at each time point. Upper panel shows effects of SCIT and SLIT therapies on Module 11.B expression (mediator) relative to Placebo at each time point. Right panel shows the relationship of Module 11.B expression to TNSS within each group, by which the direct effect on TNSS may be mediated. Module 11.B demonstrated significant mediating effect of both SCIT and SLIT on TNSS across the duration of the GRASS study in a moderated causal mediation analysis, accounting for 21.4% and 15.1% of the effect in SCIT and SLIT, respectively ($p<0.05$). Statistical results of this analysis are presented in the figure; "β on mediator" indicates the β-coefficient and associated p-value of the model comparing Module 11.B expression by each treatment group contrasted to placebo while adjusting for visit; "β mediator" indicates the β-coefficient and associated p-value of module expression in relation to TNSS by treatment group adjusting for visit; "β on TNSSAUC w/o mediator (w/ mediator)" shows the change in the β-coefficient of the model comparing TNSS by each treatment group contrasted to placebo, adjusting for visit, either without or with adjusting for Module 2.N expression, showing had Module 11.B expression partially accounts for the treatment effect on TNSS.