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SUPPORTING INFORMATION

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Gastrointestinal $\gamma\delta$ T cells reveal differentially expressed transcripts and enriched pathways during peanut oral immunotherapy

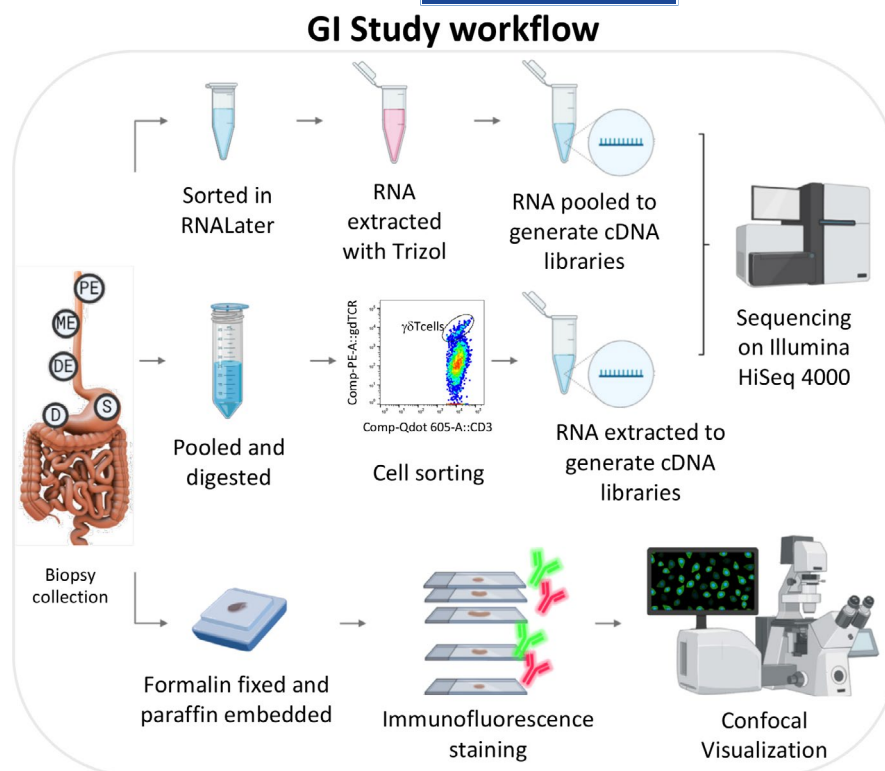
To the Editor,

Oral immunotherapy (OIT) has been successful in desensitizing patients to offending food allergens,¹ although the identification of tissue-resident T-cell subsets and cognate pathways leading to desensitization has been challenging. The $\gamma\delta$ T cells are a major T-cell subset of mucosal intraepithelial lymphocytes (IELs) and play a significant role in tissue homeostasis and repair.² In addition to aiding mucosal barrier function, $\gamma\delta$ T cells have also been recently discovered to be pivotal to cellular adaptations in response to nutrient sensing.³ In the broader context of atopy, $\gamma\delta$ T cells have been implicated both in IgE- and Th2-enhancing and IgE-suppressive effects.^{4,5} However, specifically with regard to peanut allergy, $\gamma\delta$ T cells were shown to be IgE-suppressive and thus protective in a study employing mouse models.⁶ In a recent study, peripheral $\gamma\delta$ Treg cells from

patients analyzed over 24 weeks of peanut OIT were shown to undergo dynamic changes in expression profiles, implicating pathways involved in immune homeostasis.⁷ To the best of our knowledge, the role of $\gamma\delta$ T cells in the intestinal mucosa of food allergic patients during immunotherapy has not been examined. To this end, we investigated whether $\gamma\delta$ T cells in the gastrointestinal (GI) tract exhibited changes during peanut OIT. We hypothesized that GI-resident $\gamma\delta$ T cells in peanut allergic patients would increase during the course of peanut OIT and might reveal transcripts and pathways relevant to the mechanisms of peanut desensitization.

Participants were recruited from a randomized, double-blind, placebo-controlled, phase II clinical trial of peanut OIT (POISED; NCT02103270).¹ Informed consent was obtained from all participants. Following dosage build-up over ~52 weeks, peanut-allergic

FIGURE 1 Mechanistic analyses of GI-resident immune cells. Illustration of the 5 sites of collection of GI tract biopsies, and downstream processing toward (i) bulk biopsy RNA-Seq, (ii) immune cell subset sorting from digested biopsies followed by RNA-Seq, and (iii) confocal microscopic evaluation of FFPE sections. Detailed methods appear in this article's Online Supplement



participants in the active arm ($n = 95$) were maintained on 4000 mg peanut protein until week 104. In an IRB approved sub-study involving esophagogastroduodenoscopies (EGDs), biopsies from 6 active arm participants (Table S1) were taken at baseline, Week 52, and Week 104.

We investigated peanut-OIT-induced transcriptional and frequency changes in GI-resident $\gamma\delta$ T cells at baseline vs. Week 52 and Week 104 (Figure 1). Transcriptomic analysis of sorted $\gamma\delta$ T cells from the GI tissue samples of 3 participants showed 1418 differentially expressed (DE) transcripts ($q < 0.3$) (Figure 2A, B, File S1). Transcripts of immunological interest among those DE genes along with respective fold change have been listed in Table S2. Notably, the DE transcripts included those encoding key regulatory molecules such as IL-10, IL2RA (i.e., CD25), elements crucial for B-cell signaling namely BTK, CD70, and CD72, and small intestinal-homing receptor-ligand pair CCR9-CCL25 (Table S2). Pathway enrichment analysis based on DE transcripts revealed the Notch signaling pathway (hsa04330; Figure S1A; DE transcripts: HES1, DLL1, NOTCH1), gap junction pathway (hsa04540; Figure S1B; DE transcripts: TUBA1A, TUBB2A, TUBB4A, TUBB8), B-cell receptor signaling pathway (hsa04662; Figure S1C), and TGF- β signaling pathway (hsa04350; Figure S1D; DE transcript: ACVR1B) (File S2 @ data link). Notch signaling pathway and gap junction proteins are instrumental in tissue homeostasis and maintaining barrier integrity, respectively, and impairment in each has been associated with atopy.^{8,9} We have also reported a transient increase in TGF- β -expressing peanut-specific CD4⁺ T cells among PBMCs from successfully desensitized POISED participants.¹⁰ Thus, this preliminary evidence of GI-resident $\gamma\delta$ T

cells being at the intersection of peanut-OIT-induced barrier function and immune regulation is indeed interesting and warrants further investigation.

Immunofluorescence staining of duodenum tissues for $\gamma\delta$ T cells revealed that in 4 of 6 patients, the total number of CD3⁺ TCR δ ⁺ T cells increased at Week 104 compared with baseline by an average of 3.8 cells per high-power field in the sections examined (Figure 2C, D).

To understand OIT-induced transcription changes in GI-resident cell subsets, we performed transcriptional analysis on RNA pooled from site-specific GI biopsies (Figure 1). Analysis of bulk RNA-Seq comparing baseline to Week 52 and Week 104 on 6 active participants yielded merely 33 DE transcripts (Figure S2A, S2B, File S3, @ data link) implying minimal detectable changes in mucosal epithelial tissue at the transcriptional level. No enriched pathways could be reported based on bulk RNA-Seq data given the low number of DE transcripts.

To the best of our knowledge, this is the first demonstration of DE transcripts and pathways from GI-resident $\gamma\delta$ T cells pointing to enrichment of specific signaling pathways during OIT-induced peanut desensitization. Although the transcriptomic investigation of sorted $\gamma\delta$ T cells from GI biopsies yielded a high number of DE transcripts, due to the low number of autologous samples used for comparisons, the statistical model fitting may not be robust and require more sampling for appropriate comparisons. However, our findings align with those showing changes in gene expression profiles of peripheral $\gamma\delta$ T cells in patients undergoing OIT,⁷ suggesting an important role of $\gamma\delta$ T cells in immune homeostasis in food allergy.

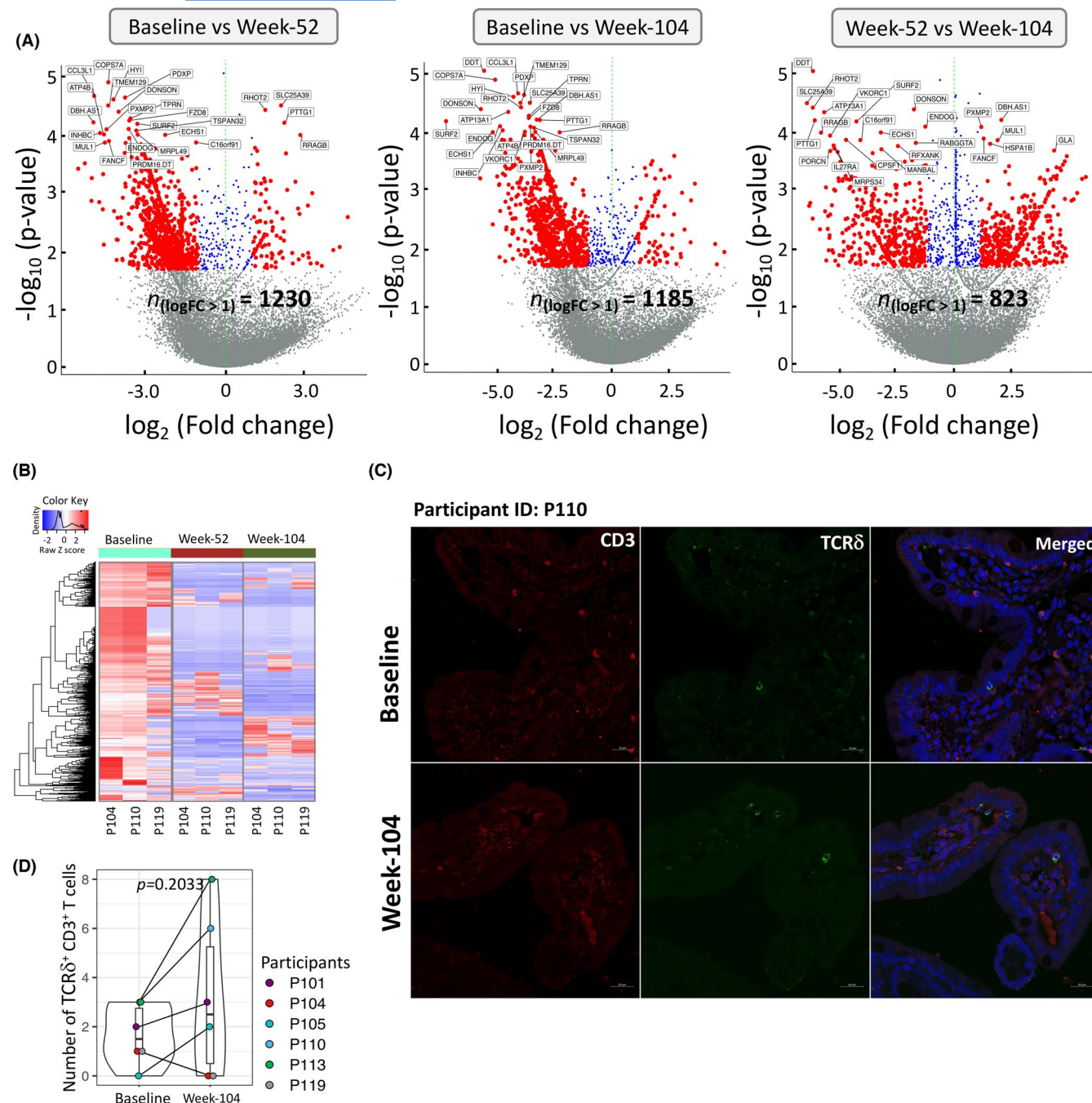


FIGURE 2 OIT-induced transcriptional and frequency changes in GI-resident $\gamma\delta$ T cells. (A) Volcano plots representing DE transcripts in sorted $\gamma\delta$ T cells from autologous samples ($n = 3$) assayed at indicated time points using RNA-Seq. Red = significant DE transcripts with absolute $\log_2(\text{Fold Change}) > 1$, and $q < 0.3$; Blue = transcripts with absolute $\log_2(\text{Fold Change}) < 1$, but $q < 0.3$; Gray = transcripts with no significant change in expression. (B) Heatmap depicting 1418 DE transcripts across indicated time points in RNA-Seq of $\gamma\delta$ T cells sorted from autologous samples (clustering method = linkage). (C) Representative immunofluorescence images of duodenal biopsy section showing CD3, TCR δ , and TCR δ^+ merged with CD3 stained cells. (D) Total cell counts for CD3⁺ TCR δ^+ cells in duodenal biopsy sections from 6 participants at baseline vs. Week 104. p value was calculated using Wilcoxon signed-rank test

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CONFLICT OF INTEREST

Dr. Nadeau reports grants from National Institute of Allergy and Infectious Diseases (NIAID), National Heart, Lung, and Blood Institute (NHLBI), National Institute of Environmental Health Sciences (NIEHS), and Food Allergy Research & Education (FARE); stock options from IgGenix, Seed Health, ClostraBio, and ImmunelD; is Director of World Allergy Organization (WAO), Advisor at Cour Pharma, Consultant for Excellergy, Red tree ventures, Eli Lilly, and Phylaxis, Co-founder of Before Brands, Alladapt, Latitude, and IgGenix; and National Scientific Committee member at Immune Tolerance Network (ITN), and National Institutes of Health (NIH) clinical research centers, outside the submitted work; patents include, "Mixed allergen composition and methods for using the same", "Granulocyte-based methods for detecting and monitoring immune system disorders", "Methods and Assays for Detecting and Quantifying Pure Subpopulations of White Blood Cells in Immune System Disorders," and "Methods of isolating allergen-specific antibodies from humans and uses thereof"; Dr. Chinthrajah reports grants from NIAID, CoFAR, Aimmune, DBV Technologies, Astellas, Regeneron, Stanford Maternal and Child Health Research Institute (MCHRI), and FARE. She is an Advisory Board Member at Alladapt Therapeutics, Novartis, Genentech, Sanofi, Allergenics, and Nutricia; Dr. Galli reports grants from the NIH; Dr. Boyd has consulted for Regeneron, Sanofi and Novartis on topics unrelated to this study, owns shares in AbCellera, has patents awarded or submitted related to immunoglobulin gene and protein analysis, and reports grants from the NIH; Dr. Andorf reports grants from the NIH. Drs. Zhang, Dhondalay, Hoh, Liu, Kaushik, DeKruyff, Kambham, Fernandez-Becker, Manohar, Kwok, and Desai indicate no conflict of interest.

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IL-33 prevents the enhancement of AP-N, DPP4, and ACE2 expression induced by rhinovirus HRV16 in the human lung endothelium—potential implications for coronaviral airway infections

To the Editor,

The lung vascular endothelium is a semi-permeable barrier that regulates the flow of nutrients, ions, cytokines, and immune cells between blood and lung tissues. Being a source of inflammatory mediators, it plays a crucial role in maintaining pulmonary homeostasis and in driving immune responses.¹ The main target for rhinoviruses (HRV) is the airway epithelium. However, we have recently shown that HRV16 may also infect the lung vascular endothelium via ICAM-1.¹ As the vascular endothelium may express coronaviral entry receptors, including aminopeptidase N (AP-N), dipeptidyl peptidase 4 (DPP4), and angiotensin-converting enzyme 2 (ACE2), it may possibly be infected by human coronavirus 229E (HCoV229E) and highly pathogenic MERS-CoV and SARS-CoV-2.² The latter one is responsible for currently ongoing COVID-19 pandemics.³

Seasonal peaks for HRV infections occur in early fall (September–November) and spring (March–May), whereas low pathogenic HCoVs which evoke mostly mild upper airway infections appear in winter and early spring (December–April). Both viruses overlap in March and April. HRV and HCoV co-infections or subsequent infections may lead to severe viral pneumonias. In such patients, the diffuse alveolar lung damage and thrombotic angiopathy in alveolar capillaries have been observed, which suggests an active involvement of the lung vascular endothelium.⁴ The effect of HRV on the expression of HCoV entry receptors on the lung vascular endothelium and its modulation by IL-33 widely distributed in asthmatic airways has not been elucidated.

Firstly, we confirmed the HRV16 infection (MOI3) of human lung microvascular endothelial cells (HMVEC-L) (Figure 1A) and we observed the increase of IFN- β , RANTES (Figure 1B,C) IL-6, and TNF- α protein release (Figure S1A,B,C). Then, we noticed that HRV16 may enhance the expression of AP-N, DPP4, and ACE2 in HMVEC-L (Figures 1 and 2). The pattern of HRV16-induced ACE2 mRNA expression varied from DPP4 and AP-N. AP-N and DPP4 mRNA expression began to grow at 24 h and further increased at 72 h upon

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incubation with HRV16 (Figure 1D,G). In contrast, the transient enhancement of ACE2 mRNA expression occurred already at 5 hours (Figure 2A). Regardless of these differences, HRV16 led to the significant increase of AP-N, DPP4, ACE2, and TMPRSS2 surface protein expression observed by flow cytometry (Figures 1E,H and 2B,D) and confocal microscope (Figures 1F,I and 2C,E) (isotype controls and gating strategy: Figures S7 and S8). We did not notice any significant effect of HRV16 in lower MOI (0.1 and 1) on the expression of any receptor (Figures S2A–C).

Recently, we have shown that IL-33 may enhance HRV-induced release of cytokines, chemokines, and growth factors by the lung vascular endothelium.⁵ In order to analyze the effect of IL-33, HMVEC-L were pre-incubated with IL-33 (10 ng/ml) for 24 h and subsequently exposed to HRV16 (MOI 0.1; 1; 3). These results confirmed our previous findings showing that IL-33 increased the capture of HRV16 by cells (Figure 1A). Despite IL-33 prevented the induction of AP-N, DPP4, and ACE2 mRNA expression by HRV16 (Figures 1D,G and 2A). At the same time, AP-N, DPP4, ACE2, and TMPRSS2 surface expressions were significantly lower, when HMVEC-L were infected with HRV16 upon the pre-treatment with IL-33 (Figures 1E,F,H,I and 2B–E). This effect of IL-33 was partially diminished by the blockade of ST2 receptor with anti-ST2 antibodies (3 μ g/ml) before the pre-treatment of cells with IL-33 (Figure S3A,B,C). To note, IL-33 alone affected neither AP-N nor ACE2 and TMPRSS2 expression, whereas it slightly decreased the surface density of DPP4 (Figure 1H). IL-33 did not significantly change the effect of HRV16 in lower MOI (0.1; 1) on the receptor expression (Figure S2A,B,C).

Our results led us to pose the question about the possible mechanism of the induction of AP-N, DPP4, and ACE2 with HRV16. First, heat-inactivated HRV16 did not exert any effect (Figure S4A,B,C,D), which indicates that the change in AP-N, DPP4, and ACE2 expression requires an active virus. Second, the pattern of ACE2 mRNA expression was similar to the expression of both HRV16 copy number and IFN- β mRNA, which suggests an activation of common cytoplasmic