ORIGINAL ARTICLE

Regular Allergen Exposure During Oral Immunotherapy Alters the Transcriptomic Innate Immune Response After Cellular Restimulation in Children With Egg Allergy

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Abstract

Background: Oral immunotherapy (OIT) is a promising treatment for food allergies. However, the molecular mechanisms leading to desensitization remain unknown.
Objective: To better understand the immunological mechanisms and transcriptional changes underlying desensitization to food allergens during OIT.
Methods: Our cohort consisted of 40 Finnish children with egg allergy who underwent OIT. Peripheral blood mononuclear cells (PBMCs) were collected at 0, 3, and 8 months of therapy and stimulated with an egg allergen extract. Differentially expressed genes (DEGs) were identified based on quantile-normalized and batch-corrected microarray data using a linear model. Gene enrichment and Pearson correlation analyses were conducted.
Results: After 8 months of therapy, 45% of patients were fully desensitized and 55% partially desensitized. Stimulation with egg yielded 49 DEGs at 0 months, 723 DEGs at 3 months, and 759 DEGs at 8 months in PBMCs after comparison with unstimulated controls. At 8 months of OIT, allergen stimulation led to down-regulation of proinflammatory pathways, as well as IL-4 and IL-13 signaling. At baseline, the immune response in the fully desensitized group was more reactive than in the partially desensitized group.
Conclusions: During OIT, general immune activity is increased, especially the number of down-regulated genes, suggesting active immune suppression. Transcriptomic profiles differ between fully and partially desensitized patients, with a notably more reactive immune response in the fully desensitized group at baseline. Innate immunity seems to play a significant role in the development of desensitization during OIT.

Key words: Food allergy. Oral immunotherapy. Microarray. Desensitization. Inflammation.

Resumen

Antecedentes: La inmunoterapia oral (ITO) es un tratamiento prometedor para las alergias alimentarias, pero aún no se han esclarecido los mecanismos moleculares que conducen a la desensibilización a los alérgenos.
Objetivo: Comprender mejor los mecanismos inmunológicos y transcripcionales que subyacen a la desensibilización a los alérgenos alimentarios durante la ITO.
Métodos: Nuestra cohorte consistió en 40 niños finlandeses con alergia al huevo que se sometieron a ITO. Se recogieron células mononucleares de sangre periférica (PBMC) a los 0, 3 y 8 meses de ITO y se estimularon con un extracto de huevo. Mediante un modelo lineal se identificaron genes expresados diferencialmente (DEG) a partir de datos de microarrays normalizados por cuantiles y corregidos por lotes. Se realizaron análisis de enriquecimiento de genes y correlaciones de Pearson.
Resultados: Tras 8 meses de tratamiento, el 45% de los pacientes estaban totalmente desensibilizados (total) y el 55% parcialmente desensibilizados (parcial). La estimulación con huevo produjo 49 DEGs en el mes 0, 723 DEGs en el mes 3, y 759 DEGs en el mes 8 en las PBMCs en comparación con controles no estimulados. A los 8 meses de ITO la estimulación con huevo condujo a la regulación a la baja de las vías proinflamatorias, así como de la señalización de IL-4 e IL-13. Al inicio del estudio, la respuesta inmunitaria del grupo total fue más reactiva que la del grupo parcial.
Conclusiones: Durante la ITO, aumenta la actividad inmunitaria general, especialmente el número de genes regulados a la baja, lo que sugiere la presencia de una inmunosupresión activa. Los perfiles transcriptómicos difieren entre pacientes con desensibilización completa y parcial, con una respuesta inmunitaria considerablemente más reactiva en el grupo con desensibilización completa al inicio. La inmunidad innata parece desempeñar un papel importante en el desarrollo del estado de desensibilización durante la ITO.

Palabras clave: Alergia alimentaria. Inmunoterapia oral. Microarray. Desensibilización. Inflamación.

Summary box

- **What do we know about this topic?**
  Oral immunotherapy (OIT) is a promising treatment for food allergies that modulates both the cellular and the humoral adaptive immune responses. However, the molecular mechanisms that lead to desensitization after repeated allergen exposure during OIT remain largely unknown.
- **How does this study impact our current understanding and/or clinical management of this topic?**
  In addition to changes in allergen-specific responses, our study highlights the importance of the responsiveness of innate immunity upon allergen exposure during OIT. Our results can help us to develop new tools to predict the outcome of OIT.

Introduction

Food allergies are a growing health concern, often developing early in life as part of the allergic march [1]. Oral immunotherapy (OIT) is a promising disease-modifying treatment for food allergies. It involves the gradual ingestion of increasing doses of the offending food, which raises the threshold for allergic reactions and results in desensitization to the culprit allergen [2]. Desensitization is considered a step toward achieving permanent tolerance, which develops over several years of regular reexposure to the allergen. OIT protocols have been established for egg, peanut, and milk, and success rates of up to 85% have been reported in some protocols [3,4]. The duration of OIT can vary greatly at the individual level. For some patients, OIT is accompanied by severe adverse effects, and success is not guaranteed for every patient [3]. Consequently, there is a need for a deeper understanding of the mechanisms underlying desensitization. Insights into these mechanisms could pave the way for more effective and reliable methods for the prevention and treatment of food allergies.

The molecular mechanisms that lead to desensitization to allergens during OIT remain unknown. However, administration of therapy is accompanied by a decrease in the concentration of IgE and the activity of T_H2 cells and an increase in the concentration of IgG4 [5]. In our previous study of 50 Finnish children with hen's egg allergy, we investigated transcriptomic changes in peripheral blood mononuclear cells (PBMCs) during OIT [6]. Our analyses revealed 467 differentially expressed genes (DEGs) at 3 and 8 months of treatment when compared to baseline (0 months). We observed a significant decline in inflammatory pathways and gene expression after 8 months of OIT.

In this study, we investigated changes in gene expression in PBMCs in response to stimulation with allergen during OIT. The study samples were from a subcohort of 40 patients from our previous study. We utilized genome-wide gene expression arrays to analyze the transcriptome at 3 time points: baseline (0 months), and after 3 and 8 months of OIT. We identified DEGs by comparing the gene expression profiles of PBMCs stimulated with egg allergen to those of the unstimulated control samples at each time point. Differences in gene expression of fully and partially sensitized patients were also determined. In addition, we examined how the DEGs correlated with concentrations of egg-specific antibodies and clinical parameters. Our aims were to study how the immune response to allergens changes during OIT and to identify which pathways are involved in the desensitization process.

Materials and Methods

Study Population

In our previous study, we evaluated 50 children and adolescents with egg allergy who received OIT (age, 6-17 years) [4]. The patients were diagnosed using double-blind placebo-controlled food challenge to heated egg. All relevant details, including inclusion and exclusion criteria, can be found in our previous study [4]. A subgroup of 40 patients was used for these analyses (Table 1). The study was approved by The Helsinki University Hospital of Children and Adolescents Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Additionally, each participant older than 6 years gave written informed consent.

OIT Protocol and Collection of Blood Samples

OIT was carried out according to the protocol outlined in our previous study [4]. Blood samples were collected from patients at baseline and at 3 and 8 months into the OIT. PBMCs were isolated from 8 mL of whole blood using a cell preparation tube (BD Vacutainer CPT, BD Biosciences). Plasma was separated and stored at –20°C, while PBMCs were extracted and frozen in cell-freezing medium (Gibco) and stored at –80°C. For the purposes of this study, a subset of 112 samples was used (40 collected at baseline, 34 at 3 months, and 38 at 8 months of OIT). Allergen-specific (Gal d 1-4) IgA, IgG4, and IgE concentrations were measured in our previous study using ImmunoCAP (Thermo Fisher Scientific) [4].

PBMC Stimulation and Microarrays

PBMCs were stimulated with ProEgg, a commercially available liquid egg white (Munax Oy). The liquid was diluted at 1:20 in phosphate-buffered saline (BioWhittaker), and endotoxins were removed using endotoxin removal columns (Pierce High-Capacity Endotoxin Removal Spin Column, Thermo Fisher Scientific) following the manufacturer's instructions. The liquid underwent testing for remaining endotoxins at the Finnish Institute for Occupational Health to ensure that it was safe for cell culture.

For stimulations, we randomized the order in which samples were thawed based on patient numbers and time points to mitigate day-specific batch effects. PBMCs were thawed as described here [7] and counted using the LUNA-FL Automated Fluorescence Cell Counter (Logos Biosystems). An amount of 1 000 000 cells was resuspended in RPMI 1640 medium (Gibco) containing 10% FBS (Gibco), 1% L-glutamine (BioWhittaker Lonza), 1% PEST (BioWhittaker Lonza), 1 mM sodium pyruvate (BioWhittaker Lonza), 1 × MEM NEAA (BioWhittaker Lonza), and 10 mM HEPES (MP Biomedicals). The egg allergen was added at a final concentration of 10 µg/mL. For the control samples, only medium was used. Cells were incubated at 37°C with 5% CO_2 for 6 hours. After incubation, cells were lyzed using lysis buffer (RL-buffer including 1% mercaptoethanol, Norgen Biotek Corp.) and frozen at –80°C. RNA was extracted from the samples using the Total RNA Purification Plus Kit (Norgen Biotek Corp.) following the manufacturer's instructions.

RNA concentration and quality were determined using Qubit and TapeStation 4200 at the Functional Genomics Unit at the University of Helsinki. Gene expression patterns in PBMCs were investigated using Agilent SurePrint G3 Human Gene Expression v3 arrays as described in our previous study [6].

Table 1. Patient Demographics.[a]

Clinical characteristics	All (N=40)	Full dose (n=18)	Partial dose (n=22)
DBPCFC severe reaction	8 (20%)	4 (22%)	4 (18%)
DBPCFC moderate reaction	32 (80%)	14 (78%)	18 (82%)
Asthma	23 (58%)	11(61%)	12 (55%)
Atopic dermatitis	28 (70%)	15 (83%)	13 (59%)
Allergic rhinitis	30 (75%)	16 (89%)	14 (64%)
Other food allergy	25 (63%)	12 (67%)	13 (59%)
All (n=40)	**Median**	**Mean**	**Range**
Age, y	10.6	11.3	6-17
Female (n=25, 62.5%)	12.5	11.9	6-17
Male (n=15, 37.5%)	9	10.2	6-17
Baseline DBPCFC maximum tolerated egg protein dose, mg	27.5	75.8	0-500
Tolerated egg protein dose, mg, at 8 mo OIT	700	640.5	20-1000
Baseline s-egg white IgE, kU_A/L	17.4	122.5	1-200
Baseline s-Gal d 1 IgE, kU_A/L	12.4	51.9	0.05-418.0
Baseline s-Gal d 2 IgE, kU_A/L	9.0	76.6	0.3-854.0
Full dose (n=18)	**Median**	**Mean**	**Range**
Age, y	9.8	10.7	6-17
DBPCFC maximum tolerated egg protein dose, mg	50	140	5-500
Tolerated egg protein dose, mg, at 8 mo OIT	1000	1000	1000
Baseline s-egg white IgE, kU_A/L	9.8	21.4	1.0-161.0
Baseline s-Gal d 1 IgE, kU_A/L	7.4	22.1	0.1-225.0
Baseline s-Gal d 2 IgE, kU_A/L	4.3	8.8	0.3-49.8
Partial dose (n=22)	**Median**	**Mean**	**Range**
Age, y	11.5	11.7	7-17
DBPCFC maximum tolerated egg protein dose, mg	5	23.2	0-200
Tolerated egg protein dose, mg, at 8 mo OIT	250	346	20-700
Baseline s-egg white IgE, kU_A/L	57.0	205.2	2.6-1200.0
Baseline s-Gal d 1 IgE, kU_A/L	42.4	76.3	3.4-418.0
Baseline s-Gal d 2 IgE, kU_A/L	39.4	132.0	0.3-854.0

Abbreviations: DBPCFC, double-blind, placebo-controlled food challenge; OIT, oral immunotherapy; s-, specific.

[a]Clinical characteristics of 40 children undergoing egg oral immunotherapy. The 18 fully desensitized patients were able to consume 1000 mg of egg white protein (about 1/3 of an egg white), and the 22 partially desensitized patients were able to consume 20 mg (1/50 of an egg white) to 700 mg (about 1/5 of an egg white) daily without symptoms after 8 months.

ELISA

To validate our results, we measured heterodimer of S100A8/S100A9 and proinflammatory IL-1ß using ELISA kits (Proteintech™ Cat. KE00177 and KE00021). The analysis was performed according to the manual.

Cell Deconvolution Analysis

The CIBERSORT analysis tool (cibersort.stanford.edu) was used to estimate the leukocyte subset proportions of individual immune cells based on the validated gene signature matrices LM22 (for activated NK cells, monocytes, and mast cells) and DerM22 (for activated neutrophils) [8,9]. The estimations are based on 1000 permutations. No significance filter was applied to the estimated cell fractions to include all samples for further analysis.

Data Analysis

Microarray data were analyzed using eUtopia [10]. The median intensities were log2-transformed and quantile-normalized using the limma package (Bioconductor) [11]. A linear model was fitted to the dye and array batch-corrected data using sex as a covariate, and pairwise comparisons were performed using the empirical Bayes method [12]. Samples stimulated with egg allergen were compared to controls at each time point. Genes with a linear fold change larger than |0.33| and a Benjamini–Hochberg–adjusted P value ≤.05 were considered significantly differentially expressed.

Heatmaps were created using the Perseus data analysis platform [11]. The data were then z-score–normalized, and the Euclidean method and the k-means algorithm were used for clustering. Pearson correlation analyses were conducted.

For the identification of the most relevant pathways, we used the EnrichR [13] gene enrichment tool and ingenuity pathway analysis (QIAGEN IPA).

The distribution of DEGs between the different time points was determined using Venn diagram analysis (VENNY 2.0.2 [14]).

Results

Desensitization

At 8 months of OIT, 55% (22/40) patients were partially desensitized to egg and 45% (18/40) were fully desensitized. Full desensitization was defined as the ability to consume 1000 mg of egg white protein without symptoms. Partial desensitization was defined as the ability to tolerate varying smaller amounts below 1000 mg of egg protein. The results of the desensitization protocol are explained in more detail in our previous study [4].

Stimulation With Egg Allergens Changes Transcriptomics in PBMCs During OIT

At baseline, a total of 49 DEGs were identified between PBMCs stimulated with egg allergen and the controls (Table 2).

Table 2. Number of Differentially Expressed Genes (DEGs) and Percentage of the Down-regulated DEGs Between Egg-Stimulated Peripheral Blood Mononuclear Cells (PBMCs) and Unstimulated Control Samples at Baseline (0 Months), 3 Months, and 8 Months of Oral Immunotherapy.

Comparison	Total	Down	Up	% of down
Egg vs Ctrl 0 m	49	13	36	26.5
Egg vs Ctrl 3 m	723	427	296	59.1
Egg vs Ctrl 8 m	759	561	198	73.9

After 3 and 8 months of therapy, 723 DEGs and 759 DEGs, respectively, were detected (Table 2). Over time, the percentage of down-regulated genes increased from 26.5% to 73.9% (Table 2).

To visualize the changes in gene expression, we generated heatmaps using 49-50 of the most significant genes from each time point (Figure S1). At all timepoints, the genes clustered into 2 distinct groups, unstimulated and stimulated (Figure S1A-C). Genes up-regulated in PBMCs stimulated with egg allergen were enriched primarily in cell surface interactions with vascular and neuronal cells (Figure S1D) at baseline, in the RHO GTPase cycle and RHOGDI signaling at 3 months (Figure S1E), and in aryl hydrocarbon receptor signaling at 8 months (Figure S1F).

With the help of partial least-squares discriminant analysis (data not shown), we confirmed similar changes in protein expression level for the dimer S100A8/A9 (Figure S2A) and IL-1ß using ELISA (Figure S2B).

Distinct and Shared DEGs at Different Time Points of OIT

Of all the DEGs identified between the egg-stimulated and control groups at each time point, only 2 genes (*lnc-C14orf166-1* and *KLRB1*) were consistently shared across all time points (Figure 1A). In gene enrichment analysis, the 20 DEGs specific to baseline were significantly enriched in the "Adenine and Adenosine salvage" and "RNA degradation" pathways (Figure 1B). At 3 months, genes were primarily enriched in the "regulation of rRNA expression" and "DNA

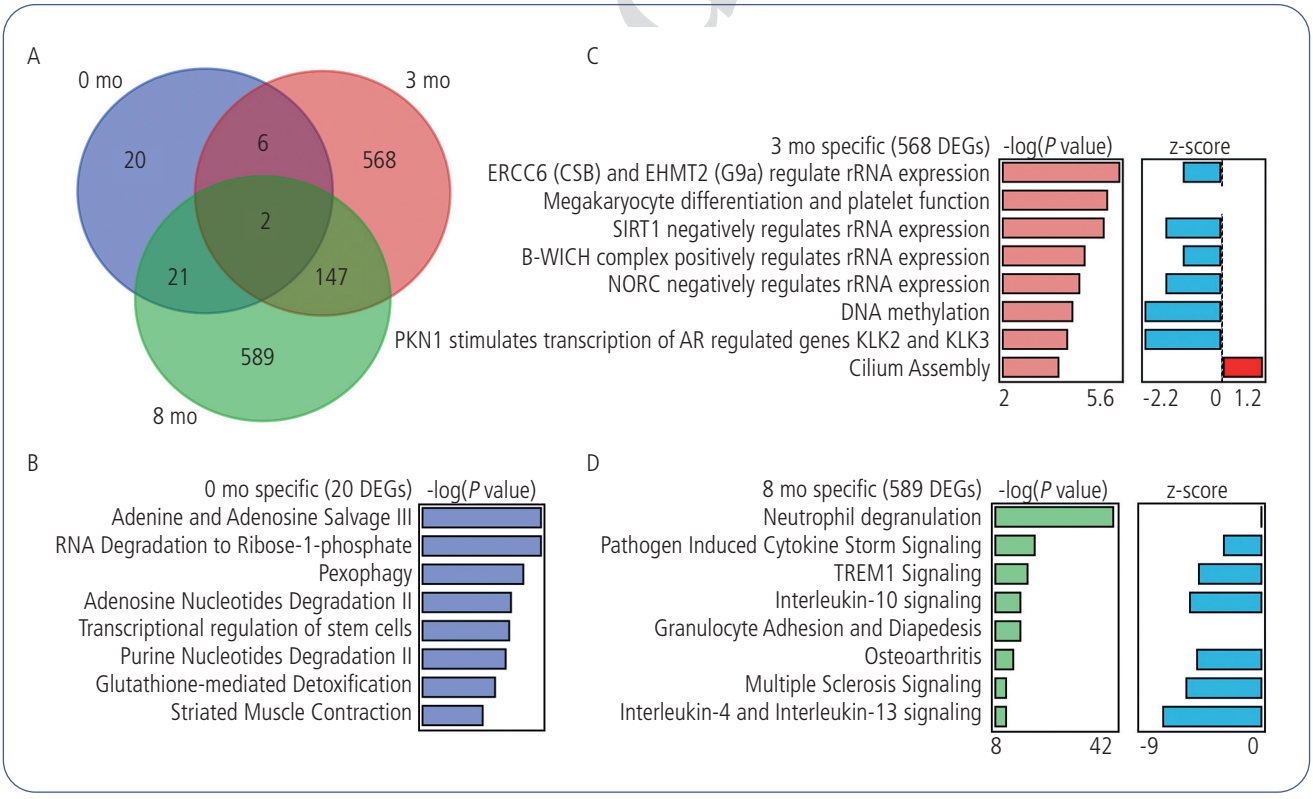

Figure 1. Specific changes in gene expression in egg extract–stimulated PMBCs at 0, 3, and 8 months. A, Venn diagram of DEGs between egg-extract stimulated PBMCs and controls at baseline (0 months), 3 months, and 8 months of OIT. Canonical pathways of specific DEGs at (B) 0 months, (C) 3 months, and (D) 8 months are shown using ingenuity pathway analysis. The negative logarithm of the *P* value (Fisher exact) and z-scores are shown.

methylation" pathways, with most being down-regulated (Figure 1C). By 8 months, DEGs were significantly enriched (*P* value 10^{-42}) in the "Neutrophil degranulation" pathway, with significant down-regulation also observed in other pathways associated with innate immunity and inflammation (Figure 1D).

The 8 DEGs shared between 0 and 3 months were most significantly enriched in the "Natural killer cell signaling" and "Neutrophil degranulation" pathways (Figure S3A). The 149 DEGs shared between 3 and 8 months were the most significantly enriched in pathways related to *IL17A* signaling and serotonin and melatonin biosynthesis (Figure S3B). The

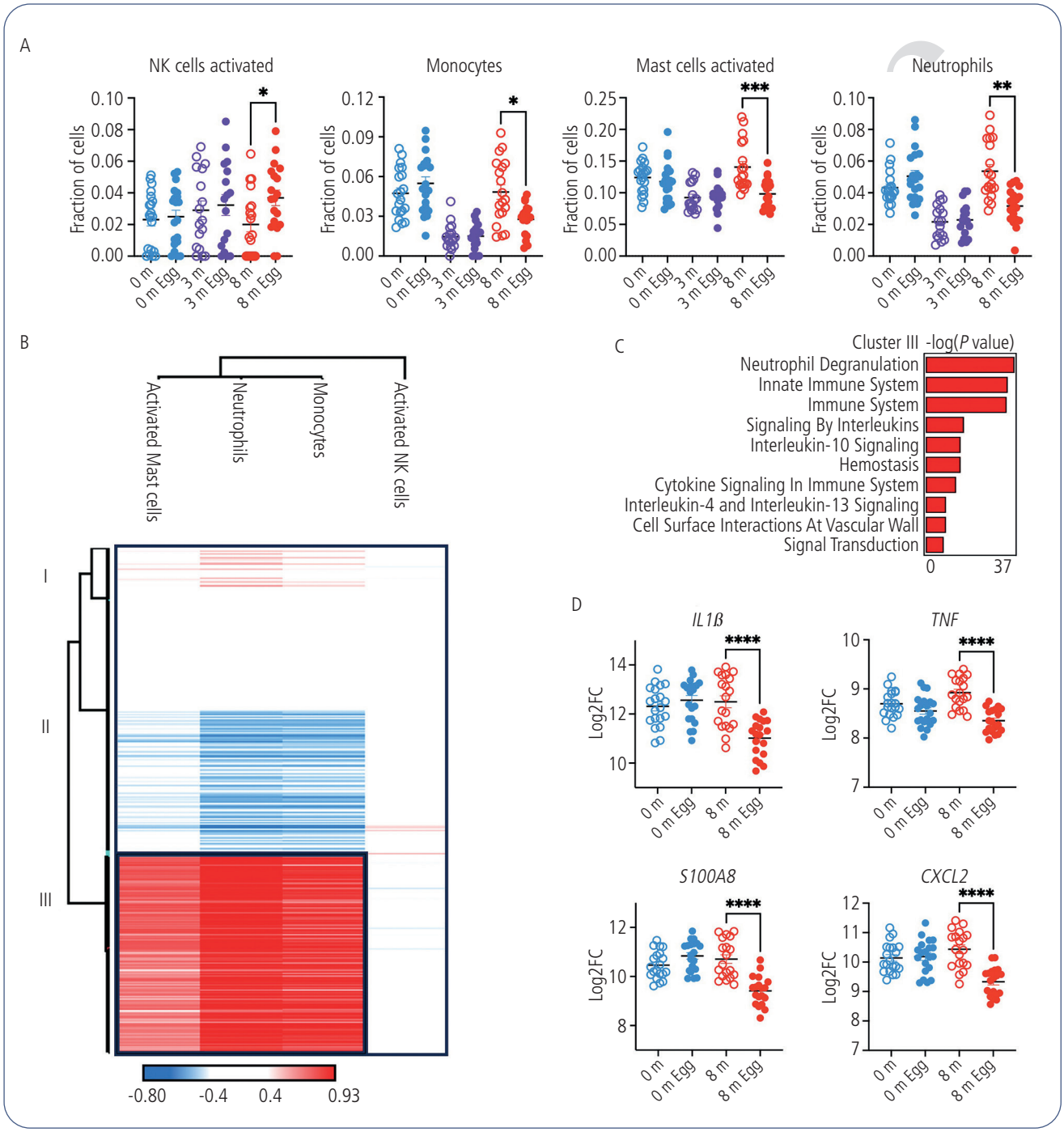

Figure 2. Cellular changes associated with differentially expressed genes (DEGs). A, Fractions of activated NK cells, monocytes, activated mast cells, and activated neutrophils from PBMC contents were deconvoluted at 0, 3, and 8 months with and without egg-stimulation using CiberSort. B, Pearson correlation of deconvoluted cell fractions and DEGs revealed a positive correlating cluster (black square), whose functions were studied in (C), a gene enrichment analysis (Reactome database). The negative *P* values (Fisher exact test) are shown as bars. D, Expression of selected target genes, including *IL1ß*, *TNF*, *S100A8*, and *CXCL2* from this cluster. ****, *P*<.0001

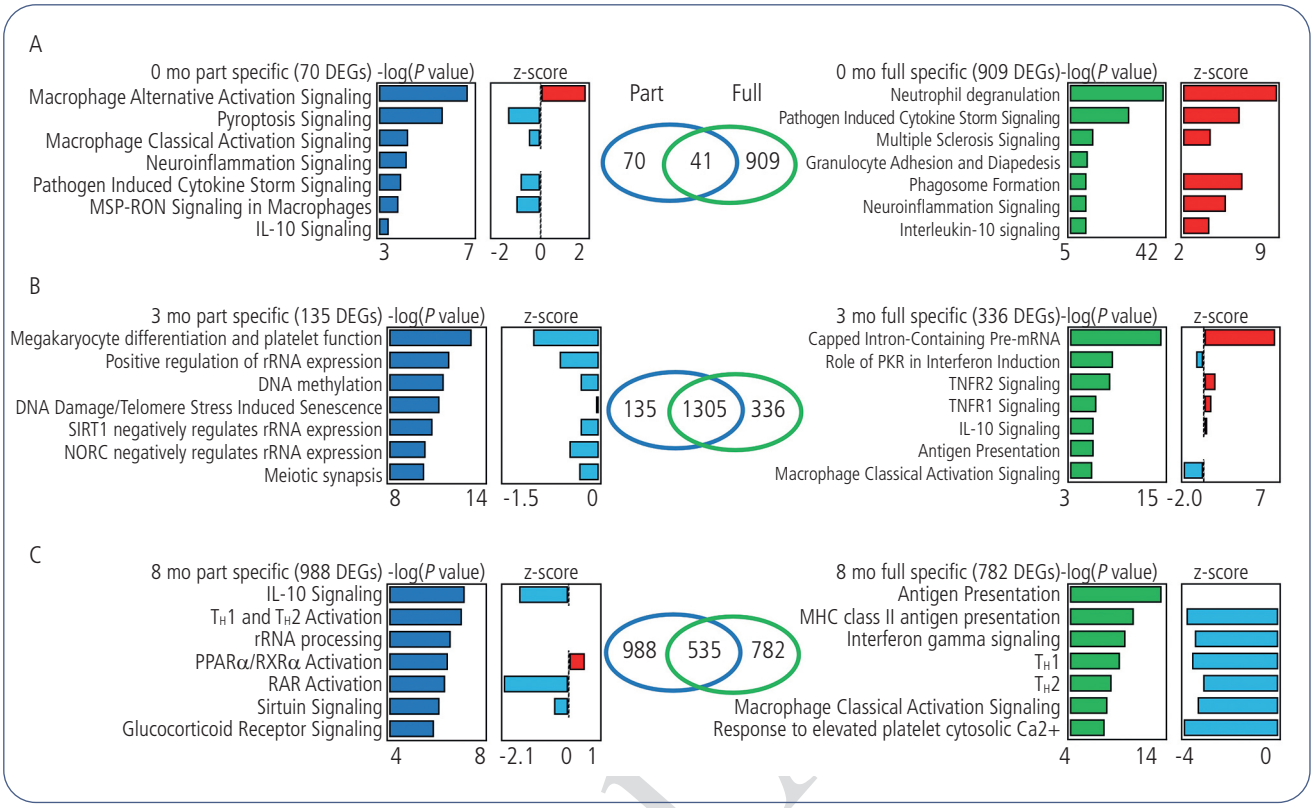

Figure 3. Peripheral blood mononuclear cells from partially and fully desensitized patients respond differentially to stimulation with egg extract. Canonical pathway analysis of specific DEGs is shown between partially (part) and fully (full) desensitized patients at baseline (A), at 3 months (B), and and 8 months (C). The Venn diagram in the middle of each section shows the number of specific and shared DEGs. The negative *P* values (Fisher exact test) are shown as bars; pathway directionality is shown as the z-score (ingenuity pathway analysis). DEG indicates differentially expressed gene.

23 DEGs shared between baseline and 8 months were notably enriched in the pathways "Phagosome formation", "Cell surface interactions at the vascular wall", and "Neutrophil degranulation". Up-regulation was detected at baseline in all of these pathways; down-regulation was deteted at 8 months of therapy (Figure S3C).

Correlation of DEGs with Clinical Parameters

We conducted a Pearson correlation analysis to explore potential correlations between clinical parameters and DEGs (Figure S4). The analysis yielded 3 distinct, positive clusters (Figure S4A). In cluster I, development of tolerance was correlated with genes enriched in signaling of IL-17, IL-4, and IL-13 (Figure S4A-B). Symptom severity during the food challenge correlated with the regulation of rRNA expression and DNA methylation in cluster II (Figure S4C), whereas in cluster III, the tolerated dose in the food challenge (FC_tolerated_dose) was enriched in the "Neutrophil degranulation" and "TREM1 signaling" pathways (Figure S4D). Notably, cluster II included numerous long noncoding RNAs (lncRNAs), the functions of which remain largely unknown.

In the graph shown in Figure S4A, the terms "Tolerance_8_m" and "OIT_months" correspond to the ability to consume 1000 mg of egg proteins and the duration of therapy, respectively. The severity of symptoms during the food challenge is expressed as a numerical value ("FC_symptom_Score") or is verbally described by the physician from mild to moderate and severe ("FC_Severity").

Egg Stimulation Decreases the Fractions of Monocytes, Mast Cells, and Neutrophils at 8 Months

We applied transcriptome-based cellular deconvolution to examine changes in cell composition during OIT (Figure 2). A statistically significant increase in activated NK cells and a decrease in monocytes, activated mast cells, and neutrophils were observed at 8 months of therapy between PBMCs stimulated with egg and controls, while at other time points, egg-stimulation did not yield statistically significant changes (Figure 2A). Next, we investigated the correlation between DEGs in which the deconvoluted cell fractions showed a highly positive cluster of activated mast cells, neutrophils, and monocytes correlating with genes enriched in neutrophil degranulation and in the innate immune system (Figure 2B-C). Gene-specific changes in the highly positive correlating cluster (cluster III) were further identified based on the significant decrease in the expression levels of IL-1β, TNF, S100AB, and CXCL2 at 8 months (Figure 2D).

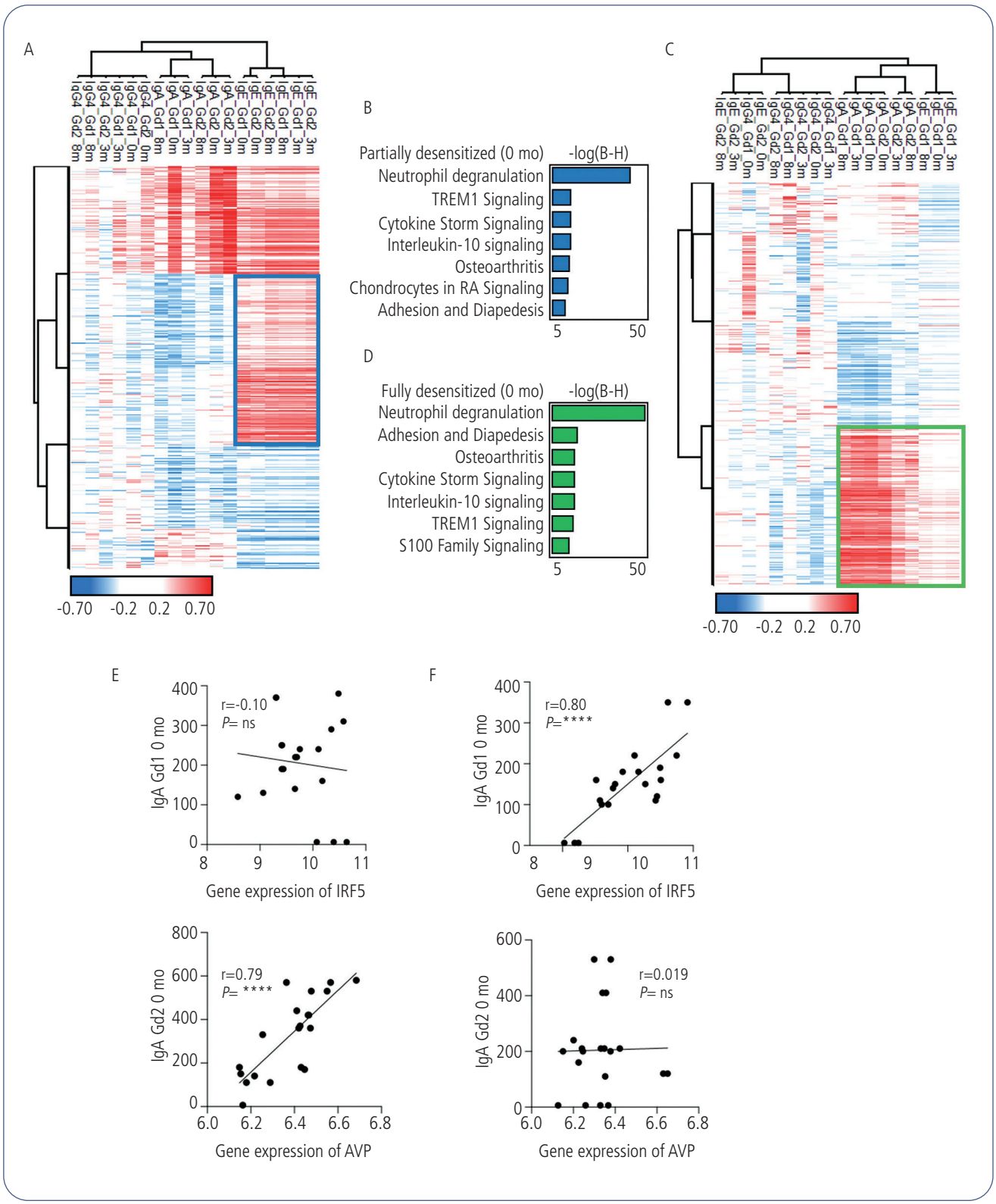

Figure 4. DEGs from partially and fully desensitized patients associate differentially with egg-specific antibodies at initiation of OIT. Pearson correlations of DEGs and ovomucoid-specific (Gd1) and ovalbumin-specific (Gd2) IgA, IgG4, and IgE antibodies in partially desensitized (A) and fully desensitized patients (C) at 0 months of OIT. The gene cluster highlighted in the blue box in (A) represents canonical pathways (ingenuity pathway analysis) shown in (B), and the gene clusters highlighted in the green box in (C) represent canonical pathways in (D). Pearson correlation of IgA Gd1 at 0 months with expression of the *IRF5* gene and IgA Gd2 at 0 months with the *AVP* gene in partially desensitized patients (E) and fully desensitized patients (F). DEG indicates differentially expressed gene; OIT, oral immunotherapy; AVP, arginine vasopressin.

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Differences Between Partially and Fully Desensitized Patients

To investigate potential differences between partially and fully desensitized patients, we compared gene expression in cells stimulated with egg allergen from each group and in controls at various time points of OIT. All the group-specific DEGs and those shared between the 2 groups are listed in Table S1. At baseline, we identified 70 DEGs for partially desensitized patients; these were enriched in up-regulation of the "Macrophage alternative activation" pathway and down-regulation of signaling in the innate immunity pathway (Figure 3A). The 909 DEGs identified in the fully desensitized patients were enriched in the "Neutrophil degranulation" and "Pathogen-induced cytokine storm signaling" pathways and up-regulated (Figure 3A). At 3 months, there were 135 DEGs in partially desensitized patients enriched in the down-regulated "Megakaryocyte differentiation and platelet function", "Regulation of rRNA expression", and "DNA methylation" pathways, while the 336 DEGs in fully desensitized patients were enriched mostly in the "Capped intron-containing pre-mRNA" pathway (Figure 3B). At 8 months, 988 DEGs in partially desensitized patients were enriched in the "IL-10 signaling" and "T_H1, and T_H2 activation" pathways, while the 782 DEGs in fully desensitized patients were enriched in pathways related to antigen presentation and T_H1/T_H2 and IFN-γ signaling, which were all down-regulated (Figure 3C).

Correlation Between DEGs in Fully and Partially Desensitized Patients and Clinical Parameters and Antibodies

We correlated the 1531 DEGs with antibody concentrations at baseline in the partially and fully desensitized patient groups. We correlated the DEGs at baseline with ovomucoid-specific (Gal d 1) and ovalbumin-specific (Gal d 2) IgA, IgE, and IgG4 antibody concentrations within these 2 patient groups (Figure 4). In the partially desensitized group, Gal d 1– and Gal d 2–specific IgE antibodies correlated positively with the "Neutrophil degranulation" pathway (Figure 4A-B) (highlighted in blue), whereas in the fully desensitized group, Gal d 1– and Gal d 2–specific IgA correlated with the "Neutrophil degranulation" pathway (highlighted in green) (Figure 4C-D). A strong positive correlation was observed in the fully desensitized group between Gal d 1–specific IgA and the *IRF5* gene at baseline, although not in the partially desensitized group (Figure 4E-F). Conversely, there was a strong correlation between Gal d 1–specific IgA at baseline and the *AVP* gene in patients in the partly desensitized group but not in the fully desensitized group (Figure 4E-F).

In addition, we examined the correlation between the same DEGs at baseline in the partly and fully desensitized groups and clinical parameters (Figure S5). To do so, we used all the DEGs identified between the stimulated cells and controls at different time points during therapy. In the partially desensitized group, DEGs correlated positively with duration of therapy (OIT_m) and severity of symptoms in the food challenge (FC_severity), as well as with the food challenge symptom score (FC_symptom_score) (Figure S5A). This gene cluster, which included many lncRNAs, was enriched in canonical pathways related to ferroptosis and ESR-mediated signaling (Figure S5B). We further individually examined correlations between 2 cluster genes, *DIXDC1* and *lnc-EIF2SL.1-2*, and duration of therapy in the partly desensitized group, although we found no statistically significant results (Figure S5C).

Conversely, in the fully desensitized group, DEGs correlated statistically significantly with duration of therapy. In the canonical IPA, DEGs were enriched in pathways related to neutrophil degranulation (Figures S5D-E). In comparison to the partially desensitized patients, the fully desensitized group was characterized by a significant positive correlation with *DIXDC1*, a positive regulator of the Wnt signaling pathway. Furthermore, a significant negative correlation was found with *lnc-EIF2SL.1-2* in the fully desensitized patients (Figure S5F).

Discussion

OIT is an increasingly recognized treatment option for management of food allergy. In this study, we showed that the transcriptomic response to egg allergens in PBMCs changes during OIT when compared to the unstimulated control samples at baseline, 3 months, and 8 months.

We found that at baseline, ie, before the initiation of egg OIT, the difference in the number of DEGs between egg-stimulated and unstimulated control cells was small (49 DEGs) compared to findings at 3 and 8 months of therapy (723 and 759, respectively). At baseline, most DEGs were up-regulated, whereas at 8 months, most of the DEGs (73.9%) were down-regulated. At 8 months, proinflammatory pathways such as "cytokine storm signaling", "TREM-1", and "IL-4 and IL-13 signaling" were down-regulated, suggesting that allergen exposure following desensitization results in the down-regulation of these immune responses. Therefore, the increase in the number of DEGs (from 49 to 759) with the shift from gene up-regulation to down-regulation indicates that gene expression is actively modulated and increased as therapy progresses.

During the OIT, stimulation with egg allergen induced different kinds of changes in gene expression. Two of the genes (*KLRB1* and *lnc-C14orf166-1*) were differentially expressed at all timepoints when compared to unstimulated cells. *KLRB1*, also known as *CD161*, has been found to be expressed on a subtype of T_H2 cells that are associated with allergic disorders [15]. Especially at 3 months, genes were primarily enriched in the "regulation of rRNA expression" and "DNA methylation" pathways, with most being down-regulated. Many of these genes were identified as lncRNAs of unknown function. This aligns with our previous study, where we found that a significant number of the DEGs at 3 months of OIT were lncRNAs [6]. Apart from their suggested role in the regulation of transcription [16-18], the involvement of noncoding RNAs in allergic diseases and the modulation of immune responses has been investigated [19]. At 8 months of OIT, the DEGs were significantly enriched (*P* value 10^{-42}) in the "Neutrophil degranulation" pathway, with other pathways associated with innate immunity and inflammation showing significant down-regulation. This decrease in neutrophil degranulation was also observed in a study examining the whole blood gene

expression profile before and after 2-3 years of omalizumab-assisted peanut OIT [20]. Our findings suggest that lncRNAs significantly influenced modulation of the immune response at 3 months of therapy. Furthermore, the down-regulation of pathways associated with innate immunity at 8 months indicates the crucial role of modulation of the innate immune system in the desensitization process.

Using a transcriptome-based cell deconvolution method, we found that the fraction of activated NK cells was increased, while that of monocytes, activated mast cells, and neutrophils decreased in egg-stimulated PBMCs after 8 months of OIT. We did not observe any differences between egg-stimulated PBMCs and unstimulated control cells at 3 or 8 months, indicating, potentially, that after a certain degree of desensitization has been reached, allergen exposure results in deletion or suppression of these cell types. A previous study has shown that stimulation with peanut allergen led to the differentiation of 2 DC subsets: $CD209^+$ monocyte-derived dendritic cells and $CD23^+$ myeloid DCs in peanut-allergic patients but not in healthy controls. The differentiation was driven by $CD4^+$ T cells, and the $CD209^+$ cells in turn reinforced the production of T_H2 cytokines, thus building a positive feedback loop. The same study showed that OIT resulted in a decrease in the number of $CD209^+$ DCs [21]. The correlation of the deconvoluted fractions of activated mast cells, neutrophils, and monocytes correlated (r=–0.80 to +0.93) significantly with genes involved in neutrophil degranulation and the innate immune system, indicating that gene expression of this type involves regulation of immune cell populations and mechanisms influencing cell activation, proliferation, and function. Our results show very significant down-regulation of the proinflammatory cytokines IL-1ß and TNF, antimicrobial peptide S100A8, and chemokine CXCL2, which are usually produced by antigen-presenting cells. Similar patterns of changes were found for IL-1ß and S100A8/9 proteins in our ELISA analyses, suggesting that innate immune cells play an important role in the induction and continuation of OIT. Further, our results indicate that after 8 months of OIT, proinflammatory signals are suppressed in response to allergen exposure.

The present results show that the immune responses of PBMCs are more reactive to egg allergens in fully desensitized patients than in partially desensitized patients. At baseline, there was a significantly higher number of DEGs specific to the fully desensitized group (909 DEGs) than to the partially desensitized group (70 DEGs). In the partially desensitized group, many innate immunity pathways were down-regulated, whereas in the fully desensitized group, pathways such as "Neutrophil degranulation" and "Pathogen-induced cytokine storm signaling" were mostly up-regulated, suggesting an elevated response to allergens in the fully desensitized group. At 3 months, the number of specific DEGs in both groups decreased, while the number of shared DEGs increased significantly, indicating that the changes are very similar in both groups. Additionally, the 135 DEGs in the partially desensitized group were enriched in the down-regulated pathways "Megakaryocyte differentiation and platelet function" and "Regulation of rRNA expression", while the 336 DEGs in the fully desensitized group were enriched mostly in the "Capped intron-containing pre-mRNA" pathway. Platelets are known to play a role in allergic diseases [22]. Our results indicate that platelet function may also play a role in OIT. At 8 months, DEGs in partially desensitized patients were enriched in "IL-10 signaling", and "T_H1 and T_H2 activation", while DEGs in fully desensitized patients were enriched in the "Antigen presentation" and "IFN-gamma signaling" pathways, which were highly down-regulated. T_H1- and T_H2-related pathways were also down-regulated in the fully desensitized group. A study on peanut-allergic patients found that OIT specifically suppresses allergen-specific T_H2 and T_H1 effector cells and their phenotypes but not regulatory T-cell phenotypes [23]. Our findings further confirm that the suppression of these cell types plays an important role in desensitization to allergen. In summary, patients who are fully desensitized to the allergen at 8 months already have a more "reactive" immune system at baseline, and their responses show more powerful down-regulation than in the partially desensitized patients.

In the partially desensitized group, IgE antibodies correlated positively with neutrophil degranulation–associated genes. In fully desensitized patients, these genes also correlated positively with IgA concentrations, suggesting that the expression of neutrophils and innate immunity–related genes might impact the production of IgE and IgA. The increase in IgA has been linked to formation of natural tolerance [24], in both egg allergy [25] and milk allergy [26]. These same neutrophil-associated genes, which often play a role in various other innate immune cells, correlated positively with the duration of OIT in the fully desensitized group, indicating that expression of these genes is associated with the time taken to achieve desensitization. This trend was not observed in the partly desensitized group. Our studies highlight that patients with highly active innate immune systems respond more successfully to OIT and achieve desensitization to egg faster than those with less active immune responses.

The strengths of this study include a carefully selected, clinically relevant, and well-represented cohort of children with moderate-to-severe egg allergy. The cohort was moderately sized, and the participants demonstrated strong commitment and consistent adherence to the protocol guidelines. Processing all the samples after collection and randomizing the sample handling order minimized potential biases in sample collection, cell stimulation, RNA extraction, and transcription analysis. The study was also subject to limitations. The cohort could have been larger, and including younger children might have yielded more impactful results, given the greater effectiveness of OIT in younger age groups. Additionally, including a sample taken at a later time point could have provided additional insights into the long-term effects of OIT, such as the development of tolerance.

Conclusion

Our study revealed dynamic changes in the immune response to exposure to egg allergen over time. The transcriptomic profiles differ between fully and partially desensitized patients, and innate immunity seems to play a significant role in the development and success of

desensitization during OIT. Our results suggest that patients who have more reactive immune cells at initiation of OIT develop more active suppression of antigen-specific responses and reach tolerance quicker than patients with less responsive immune cells. Further studies are needed to identify the best biomarkers for this reactive phenotype, which could prove beneficial in the planning of personalized OIT protocols.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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