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ORIGINAL ARTICLE

RNA sequencing identifies global transcriptional changes in peripheral CD4$^+$ cells during active oesophagitis and following epicutaneous immunotherapy in eosinophilic oesophagitis

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Abstract

Objective. There are no disease-modifying therapies for the treatment of eosinophilic oesophagitis (EoE), which is driven by non-IgE-mediated allergic inflammation. A recent clinical trial of milk epicutaneous immunotherapy (EPIT) has shown initial promise, with 47% of treated EoE patients tolerating milk without recurrence of disease. Mechanisms of EPIT in EoE have not been studied in humans. Here, we identify transcriptional changes in the peripheral CD4$^+$ T-cell compartment during active EoE and following EPIT. **Methods.** RNA isolation, sequencing and integrative data analysis were performed on peripheral CD4$^+$ T cells isolated from 15 of 20 patients enrolled in a clinical trial of EPIT for EoE. Gene expression changes in peripheral CD4$^+$ T cells were examined during diet therapy and following trial of milk antigen EPIT. **Results.** We identify 244 differentially expressed genes in peripheral blood CD4$^+$ cells of EoE patients consuming versus those eliminating milk, and 129 DEGs in CD4$^+$ cells were isolated after EPIT versus after placebo (FDR $\leq$ 0.05). Gene set enrichment analysis identifies enrichment of hallmark interferon-α and interferon-γ response pathways in peripheral CD4$^+$ T cells from EoE patients during active disease on a milk-containing diet. We demonstrate overlap of this gene signature with the altered gene expression signature seen in EoE patient biopsy tissue. EPIT therapy response is associated with significant enrichment in pathways related to T-cell receptor signalling ($P = 1.16 \times 10^{-14}$), antigen presentation and costimulation, and cytokine signalling ($P = 1.11 \times 10^{-16}$), as well as upregulation of genes associated with regulatory T-cell function. **Conclusions.** EoE is associated with distinct global transcriptional changes in CD4$^+$ T cells, one feature of which is an IFN response signature. Clinically favorable response to EPIT is likely multifactorial but is associated with a distinct

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transcriptional profile in peripheral CD4+ cells supporting the hypothesis that EPIT alters peripheral CD4+ responses in EoE patients.

Keywords: CD4-positive lymphocytes, eosinophilic oesophagitis, food allergy, immunotherapy, interferon response, RNA sequencing

INTRODUCTION

Eosinophilic oesophagitis (EoE) is an inflammatory disorder of the oesophagus occurring in approximately 1 out of every 2000 people in the United States.[1,2] EoE is a major cause of GI morbidity and significant direct healthcare expenditures.[3] In contrast to immunoglobulin E (IgE)-mediated food allergies, disease resolution in EoE is rare and most patients require lifelong treatment.[4] The existing treatment options for EoE are limited and do not alter the underlying immunopathology. Therefore, when treatments are discontinued, inflammation recurs.

EoE is a multifactorial inflammatory disease of the oesophageal mucosa that is triggered by food allergens. EoE is thought to result from a combination of inherited risks and environmental exposures.[5–8] Over 90% of EoE patients will improve on a strict elemental diet, suggesting that antigen-specific adaptive immunity plays a central role in EoE pathogenisis.[9] Several lines of data implicate CD4+ helper T cells in the pathogenesis of EoE. Murine studies demonstrate that CD3+ T cells are critical for the establishment of experimental EoE.[10,11] Additionally, EoE patients have evidence of Th2 immune activation in the mucosa.[12] Th2 cytokines interleukin (IL)-4, IL-5 and IL-13 are expressed at high levels in EoE biopsy tissue, and single-cell RNA sequencing has demonstrated elevated numbers of CD3+ Th2-like GATA3+ cells in the oesophageal mucosa.[13,14] Further, peripheral CD4+CD154+ cells from EoE patients upregulate Th2 cytokine production of Th2 cytokines IL-5 and IL-13 when exposed to food antigens during *in vitro* antigen stimulation assays.[15,16] These data implicate aberrant antigen-specific T-cell responses as a key contributor to the pathogenesis of EoE.

Food allergen immunotherapy has shown considerable promise for the treatment of IgE-mediated food allergy. Although EoE is largely a food allergen-driven disease, the pathogenesis of EoE is non-IgE-mediated and therefore food allergy immunotherapy in EoE has not been utilised. Spergel *et al*.[17] have recently reported that epicutaneous delivery of milk antigen reduced oesophageal eosinophilia in paediatric EoE patients upon milk reintroduction, suggesting that epicutaneous immunotherapy (EPIT) may hold promise as a modality for food allergen immunotherapy in EoE. EPIT for milk may have broad applicability as cow's milk is the most frequent causative food antigen in EoE, and studies of EPIT in IgE-mediated food allergy patients thus far suggest an overall favorable safety profile.[18–20] Preclinical studies of EPIT in EoE suggest the CD4+ T-cell compartment is targeted by this therapy. Data from animal models demonstrate that EPIT induces epigenetic changes in milk antigen-specific CD4+ T cells, shifting the overall T helper cell balance away from a T helper 2 (Th2) phenotype.[21,22] Further, EPIT therapy induced regulatory T cells with unique trafficking properties which would permit access to the GI tract in addition to the skin.[23] In combination, it is hypothesised that these changes underlie the reduced mucosal inflammation seen with antigen rechallenge in these models. However, this has not been previously investigated in EoE patients, and it is unknown whether similar changes would occur in humans.

Here, we investigate transcriptional changes in the peripheral CD4+ T cells of paediatric EoE patients undergoing milk desensitisation with EPIT. Patients enrolled in the phase IIa study of milk EPIT for EoE were separately enrolled into this optional research investigation in order to test the hypothesis that alterations in human peripheral CD4+ T-cell responses underlie the mechanism of epicutaneous desensitisation in EoE patients.[17] RNA sequencing (RNA-seq) was used to compare transcriptome-wide changes in CD4+ gene expression in EoE patients at three time points: during active disease, during remission while excluding milk from the diet and again at the conclusion of the trial following EPIT therapy. Our analyses focused on several questions: (1) What are the transcriptional changes in the peripheral CD4+ T-cell population during active

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inflammation in EoE compared to an inactive state achieved by dietary therapy? (2) What are the transcriptional changes in the peripheral CD4$^+$ T-cell population that are associated with EPIT therapy? And (3) how do these changes relate to the tissue pathophysiology of EoE?

RESULTS

Patient cohort and response to EPIT

Fifteen out of 20 SMILEE study patients provided longitudinal blood samples during this research study. These patients had a median age of 11 years and were 68% male, and 86% had at least one comorbid atopic condition (Table 1, demographics). Patients underwent screening endoscopy to ensure milk-elicited oesophageal inflammation (Figure 1a). The patients enrolled in our research study did not have significantly different levels of mucosal inflammation prior to study randomisation that could explain differences in therapeutic response (Figure 1b). We collected a total of 11 peripheral blood samples from patients randomised to active EPIT treatment and three samples from patient randomised to placebo. The mean post-treatment placebo group biopsy was 80.3 $\pm$ 51.7 eos hpf^{-1}.

Table 1. Demographic characteristics

Age	Sex	Atopy	IgE-FA Trigger(s)	EoE trigger(s)	Enrolment biopsies (eos hpf^{-1})		Therapy type	Post-EPIT biopsy (eos hpf^{-1})	Study protocol deviations
					On-milk	Off-milk			
Placebo									
9	M	As		Milk	75	0	Placebo	140	Per-protocol
12	M	As, AR		Milk, soy	112	0	Placebo	50	Decreased milk intake at time of post-EPIT biopsy study
12	M	As	Egg, fish	Milk	30	0	Placebo	51	Per-protocol
EPIT, with therapeutic response after initial 11 months of therapy									
8	F	As, AR	Tree nut	Milk	30	5	Active	10	Per-protocol
8	M	As, AR, AD		Milk, soy	40	2	Active	8	Per-protocol
15	M	As, AR, AD		Milk, soy	50	10	Active	1	Per-protocol
14	M	AR		Milk	65	5	Active	0	Per-protocol
13	M		Peanut, tree nut, fish	Milk, peas, sweet potato	15	3	Active	11	Increased milk intake at time of post-EPIT biopsy
EPIT, no therapeutic response after initial 11 months of therapy									
11	M	AR		Milk	134	0–7	Active	128	Inconsistent PPI dosing
12	F			Milk	127	1	Active	65	Per-protocol
5	M	As, AR		Milk, pea, egg, beef	25	5	Active	20	Per-protocol
8	M	AD		Milk	30	6	Active	83	Timing on-milk confirmation endoscopy not per-protocol
7	M	As, AD		Milk	80	9	Active	75	Increased milk intake at time of post-EPIT biopsy; inconsistent PPI dosing
11	F			Milk	50	0	Active	110	Increased milk intake at time of post-EPIT biopsy; inconsistent PPI dosing
EPIT, withdrew									
15	F	AR		Milk	25	0	Active	–	N/A

[1]Fifteen of 20 patients randomised to therapy in the SMILEE trial provided additional informed consent for blood sample donation for this research study. One subject withdrew during the trial because of refusal to go back on milk-avoidance diet. All patients in this study were of white, non-Hispanic ancestry. Biopsy results are presented as eosinophils per high-powered field (eos hpf^{-1}). If patients had atopic comorbidities, these are listed (IgE-FA, IgE-mediated food allergy; As, asthma; AR, allergic rhinitis; AD, atopic dermatitis) and eliciting food are listed if patients had IgE-mediated food allergy. Active therapy was milk Viaskin EPIT patch, and placebo treatment was a placebo patch, and study protocol deviations are noted (PPI, proton pump inhibitor; EPIT, epicutaneous immunotherapy).

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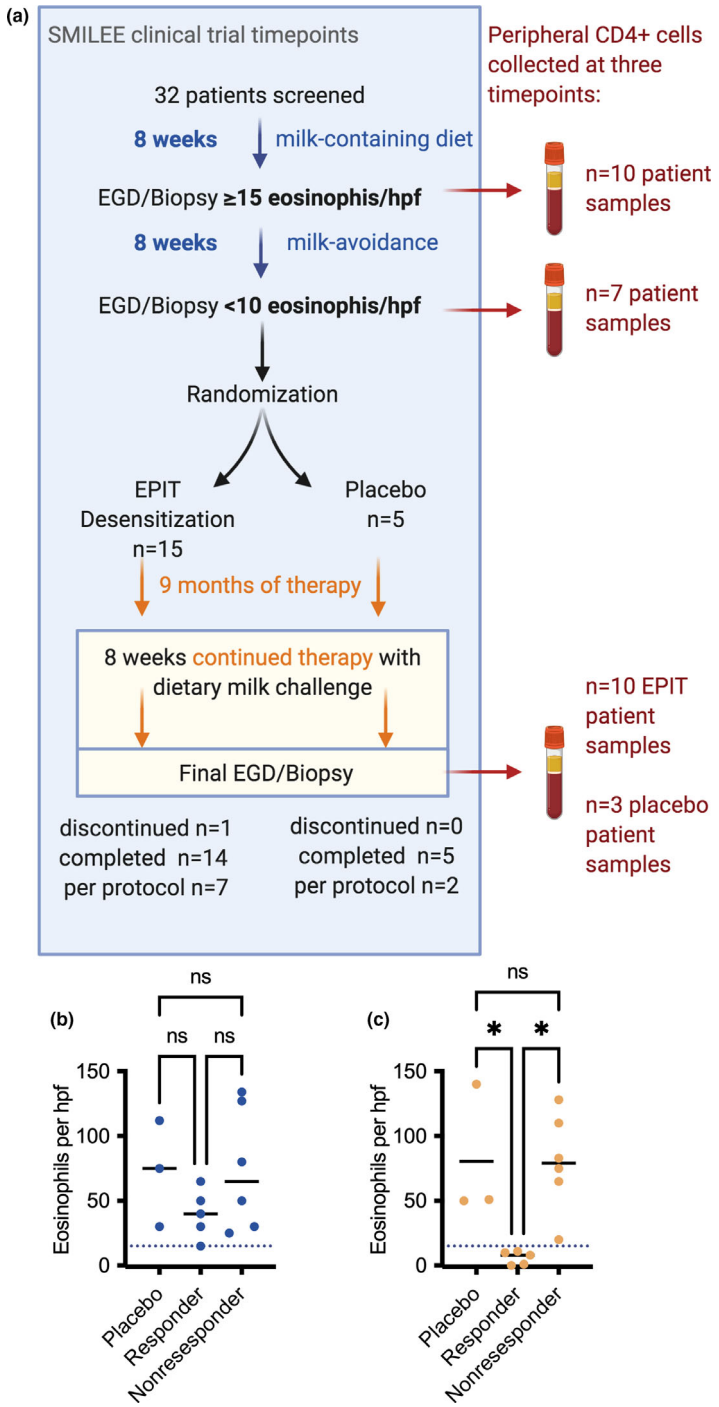


Figure 1. Overview of Study of Efficacy and Safety of Viaskin Milk for milk-induced EoE (SMILEE) trial time points and patient endoscopy results relevant to this study. **(a)** SMILEE time points and enrolment numbers. The numbers of peripheral CD4+ T-cell samples collected are shown at each of three visit time points: during the screening phase while on a milk-containing diet (n = 10 patient samples), during the screening phase while avoiding milk (n = 7 patient samples) and following 11 months of EPIT therapy (n = 3 placebo and n = 10 EPIT patient samples). **(b)** Results from the 'on-milk' pretreatment screening biopsy (eosinophils per high-powered field show no difference in biopsy results between patients randomised to placebo or active therapy). **(c)** Post-treatment biopsy results for the subset of placebo (n = 3) and active EPIT therapy patients in this research study. EPIT responder group had post-EPIT biopsy < 15 eos hpf^{-1} (n = 5) and nonresponder (n = 6) had post-EPIT biopsy ≥ 15 eos hpf^{-1}. Blue dotted line = 15 eos hpf^{-1} cut-off; black lines represent group mean. ANOVA F = 7.78 P = 0.0078, * denotes $P \leq 0.05$, Tukey HSD test.

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Within the subset of patients participating in this study, five patients randomised to EPIT had a favorable clinical response following 11 months of EPIT, with resolution of mucosal inflammation during the milk challenge (Figure 1c, classified as responder group; mean eos hpf^{-1} 6.0 $\pm$ 5.1). In contrast, six patients continued to have active mucosal inflammation at this final milk challenge despite receiving active EPIT therapy (Figure 1c, classified as nonresponder group, mean $\pm$ SD, 80.2 $\pm$ 37.6).

CD4$^+$ gene expression signature in EoE

We set out to test the hypothesis that CD4$^+$ T-cell responses are critical in the mechanism of EPIT in EoE patients. Peripheral CD4$^+$ T cells were isolated from paediatric EoE patients at the three study time points (Figure 1a), and total RNA was isolated for sequencing.

We first compared the gene expression of CD4$^+$ T cells from patients consuming cow's milk versus those on milk-avoidance diet to assess peripheral CD4$^+$ gene expression changes associated with active inflammation on cow's milk-containing diet in EoE patients. Within this set of samples, six sets of paired patient samples were analysed in addition to five additional patient samples from whom paired sample collection was not feasible (Supplementary table 1). A total of 839 transcripts were differentially expressed at a threshold cut-off of $P \leq 0.01$ (Figure 2a), with 345 genes upregulated and 494 downregulated in the on- compared to off-milk comparison. The majority of these had modest fold change difference, with 36.9% of transcripts over 1.5-fold change difference between groups. As shown in Figure 2b, we observe a relatively consistent gene expression pattern in CD4$^+$ cells of EoE patients on milk-containing diet and when not consuming milk. Similarly, we observe 647 differentially expressed transcripts ($P \leq 0.01$ cut-off) in CD4$^+$ from patients on EPIT therapy compared to those who were on placebo (Figure 2c). Of these, 445 were upregulated in EPIT-treated patients (EPIT > placebo) and 202 were downregulated in EPIT-treated patients (placebo > EPIT), with increased interpatient heterogeneity in gene expression (Figure 2d).

We examined the degree of expression overlap between the EPIT vs. placebo and on- vs. off-milk comparisons, hypothesising that patients in the placebo group have CD4$^+$ gene expression signatures comparable to those seen from the on-milk, pre-EPIT EoE patient group . In these comparisons, there is significant overlap of DEGs in the on-milk > off-milk and placebo > EPIT comparison (odds ratio 8.52; 95% CI [5.49, 12.84] P = 6.9e-17, Figure 2e). Further, we observe that the converse comparison of off- > on-milk genes to EPIT > placebo is also highly significant (OR 46.5; 95% CI [37.14, 58.22], P = 7.6e-206, Figure 2f). We also examined alternative comparisons, but there is no significant overlap between the off-milk < on-milk and placebo > EPIT comparison (P = 0.3008), and the off > on and placebo > EPIT DEG comparison has 26 overlapping genes with a lower odds ratio (OR = 2.94; 95% CI = [1.87, 4.46], P = 4.9e-06, Figure 2g). In total, this suggests there is a set of common differentially expressed genes (DEGs) in the peripheral CD4$^+$ T-cell compartment that characterises both the uninflamed and inflamed state in EoE patients. In our dataset, this is composed of 208 DEGs.

We selected the cut-off of $P \leq 0.01$ for differential gene expression analysis because the primary goal of our analyses was to identify potential pathways or mechanisms of interest in the peripheral CD4$^+$ T-cell response in EoE and to EPIT in particular. Alternatively, if using the Benjamini–Hochberg method with an FDR cut-off of 0.05, we identify 244 DEGs altered during milk-associated active EoE disease. Of these, 97 transcripts were upregulated and 147 were downregulated at the time of the active milk diet endoscopy (Supplementary table 2). Using the same FDR cut-off of 0.05, we observe a total of 129 DEGs, with 106 upregulated and 23 downregulated transcripts in the comparison of CD4$^+$ cells from EPIT versus placebo treated patients (Supplementary table 3).

Gene set enrichment analysis points to alterations in CD4$^+$ immune function following EPIT

We next performed gene set enrichment analysis (GSEA) to examine differences between these conditions as differentially expressed pathways using the MSigDB Hallmark gene set collection. In EoE patients before and after milk elimination diet (on- > off-milk), we observe enrichment of interferon (IFN)-α and IFN-γ response pathways (Figure 3a, Supplementary figure 1). We have previously reported that paediatric and adult EoE

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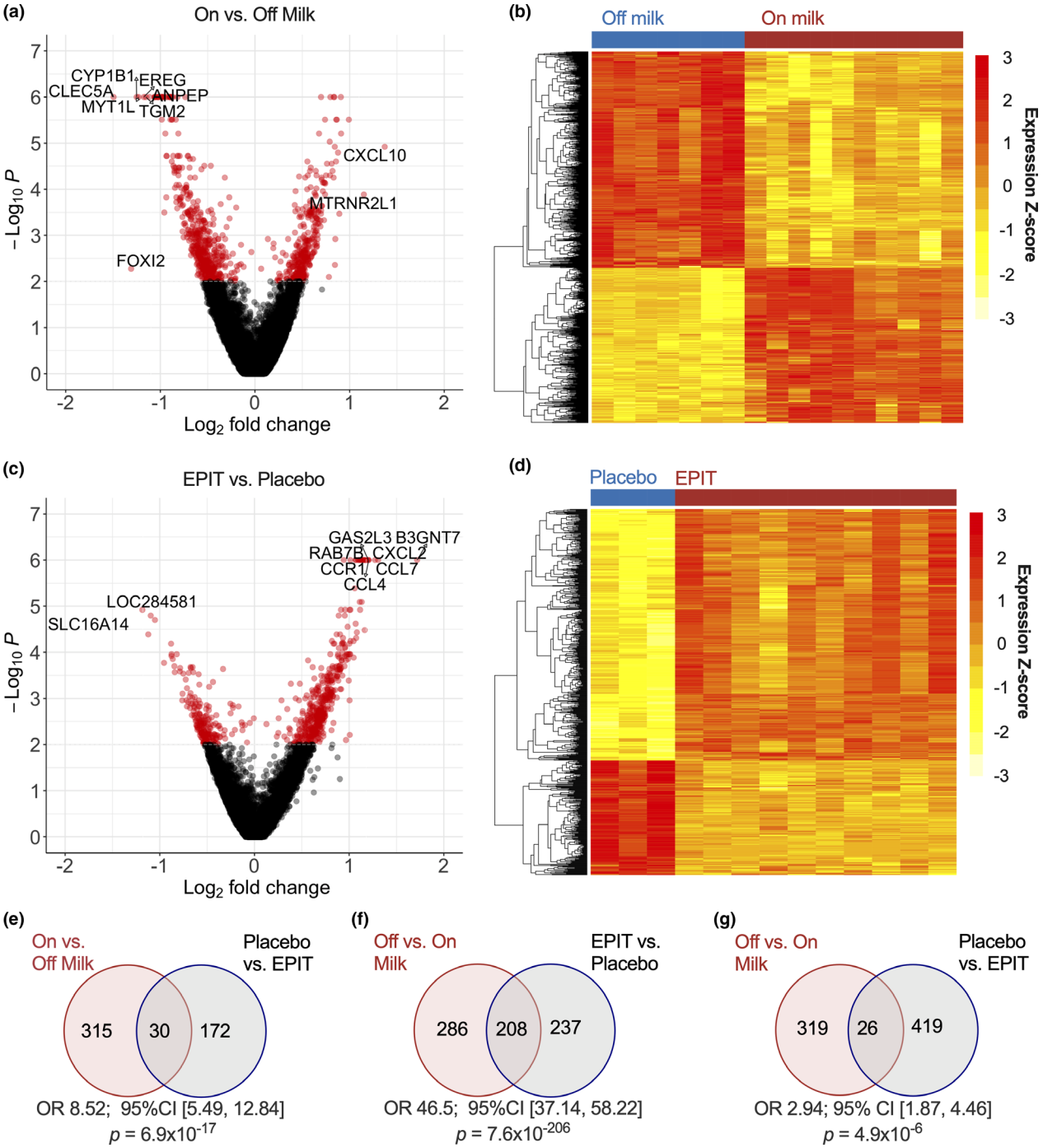


Figure 2. Differential gene expression in peripheral CD4+ cells identified by RNA sequencing. Differentially expressed genes (DEGs) from the peripheral CD4+ T cells of EoE patients collected while consuming versus abstaining from a cow's milk-containing diet ('on- > off-milk', n = 10 on-milk and n = 7 off-milk patient samples) represented in **(a)** volcano plot with P-value (Log_{10}) versus fold change expression (Log_2). Red data points denote $P \leq 0.01$. **(b)** Heatmap of DEGs at from on- versus off-milk comparison at $P \leq 0.01$. The legend displays mapping to row-wise Z-scores. **(c)** Volcano plot of DEGs from peripheral CD4+ T cells of EoE patients in the active EPIT versus placebo treatment groups ('EPIT > placebo', n = 3 placebo and n = 10 EPIT patient samples). Red data points denote $P \leq 0.01$. **(d)** Heatmap of DEGs from active EPIT versus placebo treatment comparison at $P \leq 0.01$. The legend displays mapping to row-wise Z-scores. **(e)** Venn diagrams of shared DEGs at $P \leq 0.01$ between on- > off-milk and placebo > EPIT, **(f)** off- > on-milk and EPIT > placebo, **(g)** off- > on-milk and placebo > EPIT with significance of gene overlapping determined using Fisher's exact test. The off- < on-milk and placebo > EPIT comparison is nonsignificant (P = 0.3).

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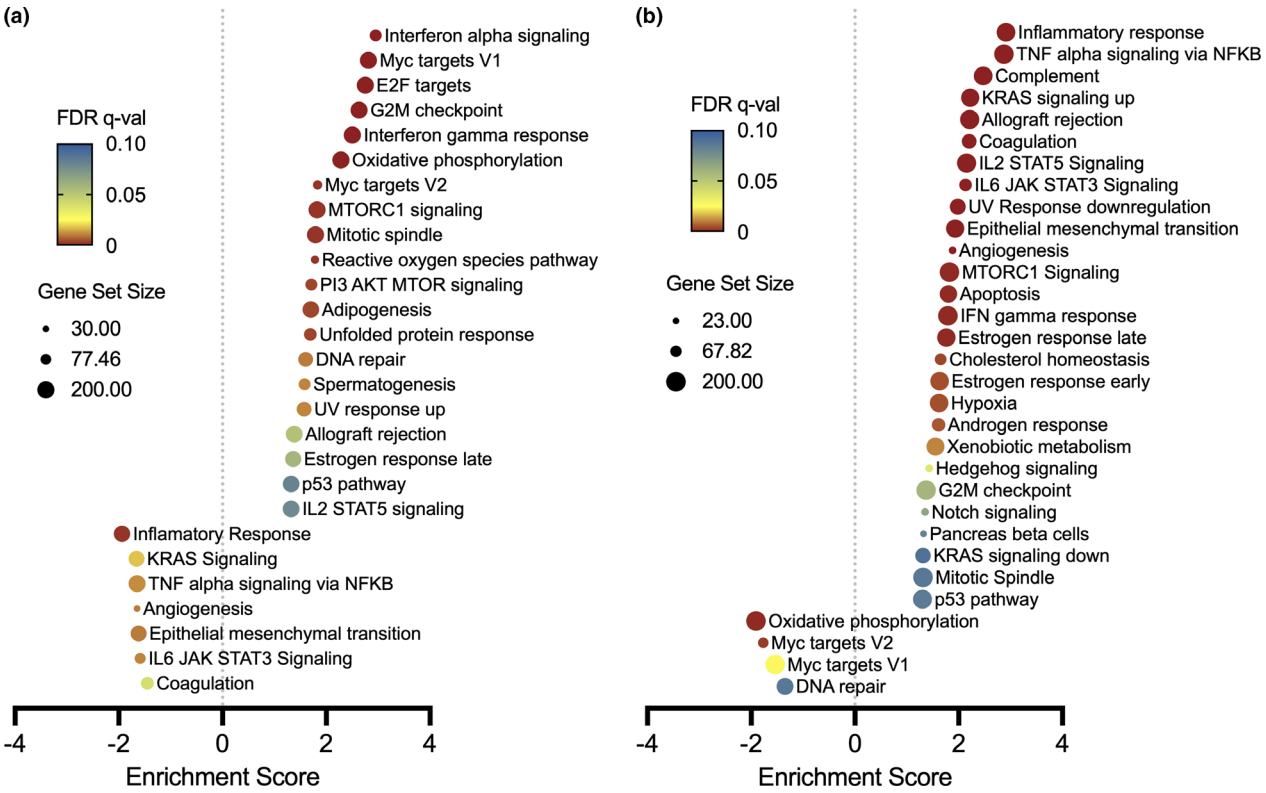


Figure 3. Functional enrichment analysis identifies potential peripheral CD4+ pathways associated with EPIT therapy. Hallmark GSEA from RNA-Seq experiments performed on CD4+ from EoE patients **(a)** consuming versus abstaining from a cow's milk-containing diet (on- > off-milk) and **(b)** in the active EPIT versus placebo treatment groups (EPIT > placebo). Pathway results with FDR *q*-val < 0.1 are plotted by normalised enrichment score (NES). Dot size represents the number of genes in the pathway, and colour represents the GSEA FDR as represented in the legend.

patients have a conserved IFN signature in biopsy tissue,[24] but in this study, we did not observe an IFN signature in whole blood samples from EoE patients compared to non-EoE controls. However, here, our transcriptional studies demonstrate that IFN pathway genes are enriched in the isolated CD4+ T cells of EoE patients.

We next performed GSEA in the EPIT > placebo sample comparison (Figure 3b). Because all patients were consuming milk at the time of the final study biopsy, we expect that pathway enrichment observed in the EPIT versus placebo comparison would relate to the effects of therapy. IFN-γ response is enriched; however, we do not see a significant enrichment in IFN-α response pathways. In the comparison of the patients on EPIT versus placebo, we observe positive enrichment in the 'TNFα signalling via NF-κB' pathway and 'Epithelial Mesenchymal Transition' pathways (Figure 3b, Supplementary figure 2). These are driven by modest upregulation in the expression of several key cytokines including *INHBA, TGFBR3, IL6, CXCL1, CXCL2 and CCL4*. As a feature in these pathways, we observe modest upregulation in *FOSB* (fold change 1.68, FDR = 0.042), suggesting that altered expression of the AP-1 transcription factor in peripheral T cells may be a potential mechanism of action of EPIT therapy in EoE patients.

Gene signature associated with favorable EPIT response

Within our patient cohort, five patients that were randomised to EPIT had final biopsy results with resolution of mucosal inflammation during the milk challenge (Figure 1c, responder group; mean eos hpf^{-1} 6.0 ± 5.1), whereas six patients continued to have active mucosal inflammation despite receiving active EPIT therapy (Figure 1c, nonresponder group, mean eos hpf^{-1} ± SD, 80.2 ± 37.6). Using cut-off of $P \leq 0.01$, we find

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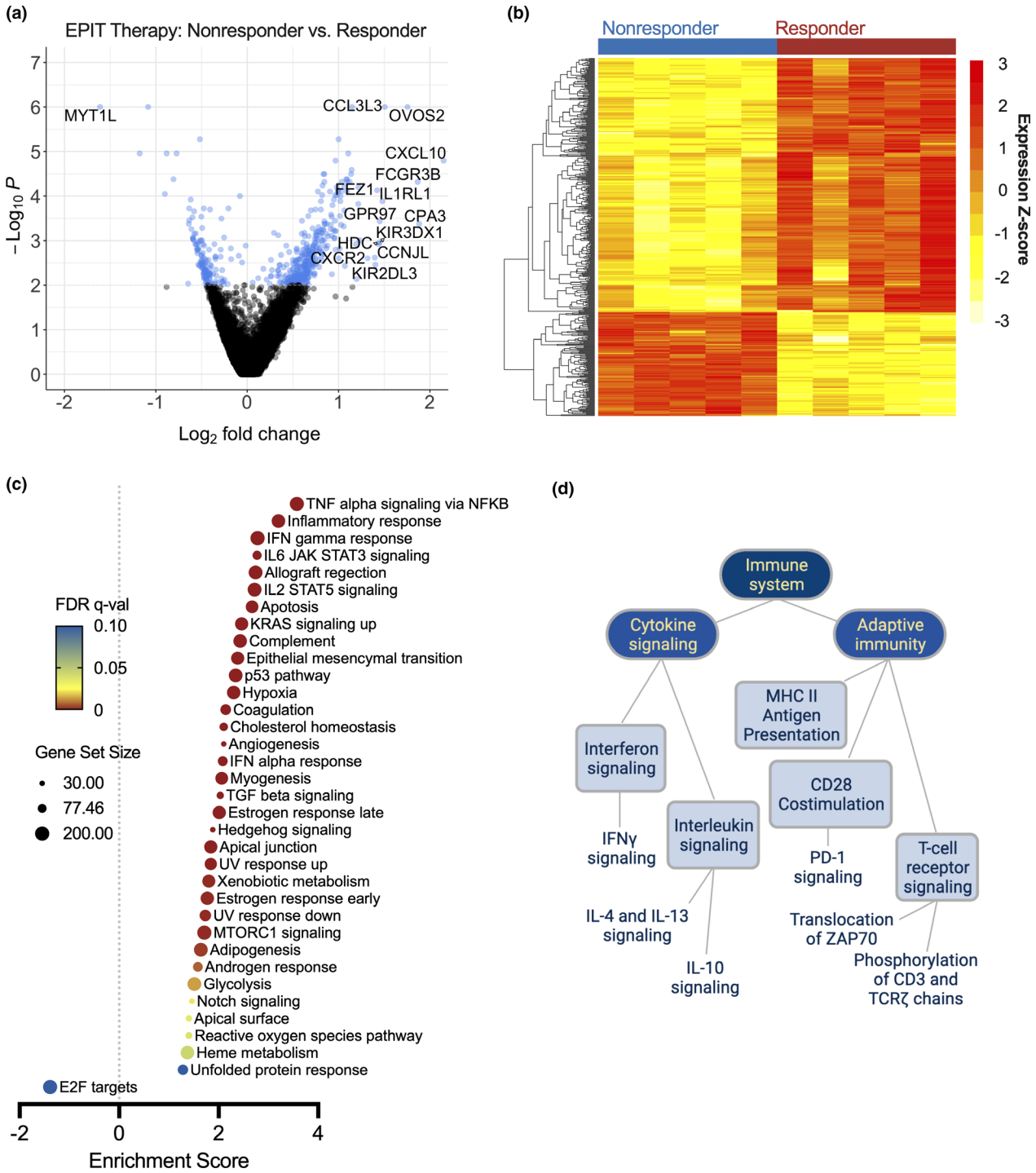


Figure 4. Peripheral CD4+ differential gene expression suggests several mechanisms may play a role in CD4+-mediated EPIT responses. Differentially expressed genes (DEGs) from peripheral CD4+ T cells of EoE patients with favorable (responders, $n = 5$, post-EPIT biopsy < 15 eos hpf^{-1}) vs unfavorable (nonresponders, $n = 6$, post-EPIT biopsy ≥ 15 eos hpf^{-1}) clinical response to EPIT represented as: **(a)** volcano plot showing *P*-value (Log_{10}) versus fold change expression (Log_2) with points $P \leq 0.01$ graphed in blue; **(b)** heatmap of DEGs at $P \leq 0.01$, with row-wise *Z*-score mapping as shown in the legend. **(c)** Hallmark GSEA from RNA-Seq experiments from responder vs. nonresponder DEGs. Results with FDR *q*-val < 0.1 are plotted by normalised enrichment score (NES), dot size represents the number of genes in the pathway, and colour represents the GSEA FDR as represented in the legend. **(d)** REACTOME pathway analysis of DEGs (FDR ≤ 0.1) from the comparison of EPIT responder to nonresponder groups. Pathways with FDR < 0.1 are shown.

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470 DEGs in the comparison of EPIT responder to nonresponder patient samples (Figure 4a), with 334 upregulated transcripts and 136 downregulated transcripts (Figure 4b, heatmap). We next performed ranked GSEA using MSigDB Hallmark gene set collection to examine enriched pathways in the DEGs in the comparison of patients with favorable clinical response to EPIT versus those on EPIT therapy without clinical response (Figure 4c, GSEA 'responders' vs 'nonresponders'). In this analysis, we observe enrichment of 'TNFα signalling via NF-κB', IFN-γ and IFN-α response pathways, and 'Epithelial mesenchymal transition', and 'IL6 JAK STAT3 signalling' pathways, similarly to that seen in the on- vs off-milk and EPIT vs placebo comparisons. Here, we observe negative enrichment in the 'E2F targets pathway', whereas downregulation of Myc targets was represented in two pathways in EPIT versus placebo comparison (Figure 3b).

We performed additional pathway analysis comparing therapy responders to nonresponders to more thoroughly examine the gene signature associated with a favorable response to therapy. We used the REACTOME database to identify possible pathways implicated in CD4$^+$ response to EPIT and explore how these pathways may interconnect mechanistically (Figure 4d, Supplementary table 5). Broadly, these pathways relate to several categories of immune function: T-cell receptor signalling ($P = 1.16 \times 10^{-14}$), antigen presentation and costimulation, and cytokine signalling ($P = 1.11 \times 10^{-16}$), including interferon gamma signalling ($P = 1.11 \times 10^{-16}$). Interleukin (IL)-4 and IL-13 signalling pathways were also identified as distinguishing EPIT responders from nonresponders, which corresponds to our previous data demonstrating that systemic antigen-specific Th2 cells could be detected in the peripheral circulation of EoE patients during active EoE disease.[16]

We next set out to examine the potential contributions of specific T-cell subsets to the peripheral CD4$^+$ EPIT response. To do so, we leveraged data from purified human T-cell populations[25–29] to generate gene expression signatures unique to CD4$^+$ helper T-cell and regulatory T-cell populations. We identified a set of unique DEGs for Th1, Th2 and Treg cell types, then compared our sample's average differential expression compared to these CD4$^+$ subset-specific gene groups. Overall, our analysis demonstrates a higher average expression of Th2-specific subset genes in the 'on- > off-milk' comparison when compared to the 'EPIT > placebo' comparison (Supplementary figure 3a). Post-EPIT, however, we observe higher expression corresponding with Th1-specific genes. This suggests that within our unsorted CD4$^+$ T-cell population there may be shifts in gene expression or cell number favoring Th1 type cells post-EPIT. Similarly, we applied this analysis using a signature from a purified human CD4$^+$CD25^{hi}CD127^{low} regulatory T-cell population as a comparator (Supplementary figure 3b). Peripheral CD4$^+$ from EPIT-treated patients have a higher per cent average expression of genes specific for regulatory T cells.

Correlation of peripheral T-cell gene expression to EoE transcriptome

The mucosal tissue in EoE is characterised by an eosinophil-rich, dense inflammatory infiltrate and reactive epithelial changes. Biopsy tissue from patients with EoE has reproducible alterations in gene expression which are collectively known as the EoE transcriptome.[30,31] Rochman and colleagues have previously demonstrated that 35–40% of these transcripts arise uniquely from the oesophageal epithelium; however, many of the dysregulated transcripts within EoE biopsies can likely be attributed to hematopoietic cell types.

Using EoE transcriptome data from Sherrill *et al.*,[30] we compared genes uniquely expressed in oesophageal tissue during active EoE to our on- versus off-milk CD4$^+$ DEGs. Our goal was to determine which alterations in peripheral CD4$^+$ signalling may have the most physiologic relevance to the mucosal disease seen in EoE patients. These data sets had 14 281 shared transcripts available for analysis, from which we analysed the overlap of the top 250 genes with $P \leq 0.01$ and highest fold change (Figure 5a). Within this subset of overlapping genes, the magnitude of the fold change in expression is higher between EoE and control biopsy tissue than it is in the on- versus off-milk CD4$^+$ samples (Figure 5b). Among the DEGs, there is a significant correlation between transcripts upregulated in inflamed EoE biopsies (EoE > control) and in the on- versus off-milk EoE patient samples (Figure 5c; OR 5.51 95% CI [3.29, 8.93], $P = 3.1e\text{-}09$). There also significant overlap between the off- > on-milk and control > EoE comparison (Figure 5c; OR 2.67 95% CI [1.29,

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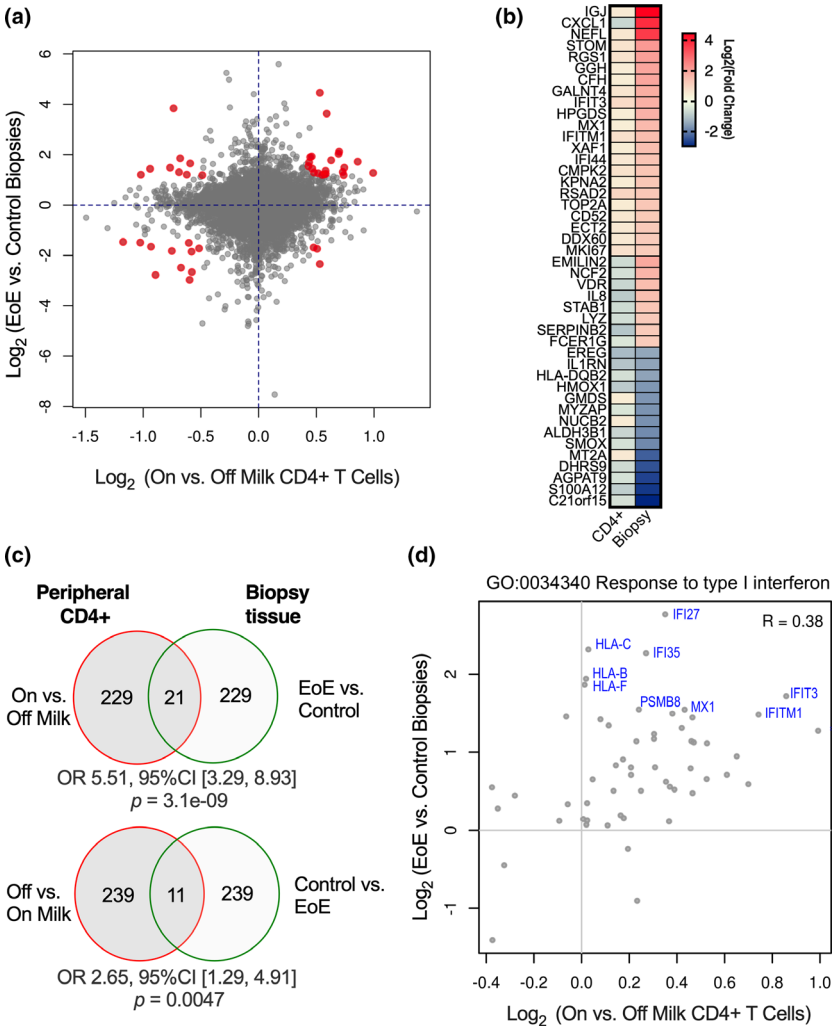


Figure 5. Differentially expressed genes from peripheral CD4+ T cells and from EoE-specific oesophageal biopsy transcriptome. **(a)** Fold change overlap of differentially expressed genes from EoE patient peripheral CD4+ samples (on and off of milk-containing diet) compared to RNA-seq dataset from Sherrill *et al* (EoE vs. control biopsy).[30] The top 250 genes with the highest fold change and $P \leq 0.01$ were analysed to determine degree of overlap and are shown in red. **(b)** Heatmap display of Log_2(fold change) of 44 overlapping genes from on- vs. off-milk peripheral CD4+ samples and EoE vs control biopsies. **(c)** Venn diagram of overlapping differential gene expression (at $P \leq 0.01$) between on- > off-milk and EoE > control biopsy and off- > on-milk and control > EoE biopsy. Significance determined using Fisher's exact test. Additional comparisons were not significant. **(d)** Fold change graph of the transcripts in the 'Gene Ontology: resource type I interferon response' pathway, annotated to display transcripts enriched in both biopsy tissue as well as CD4+ samples.

4.29], $P = 0.0047$). Other comparisons were not significant.

We hypothesised that gene expression changes in the peripheral CD4+ T-cell compartment of EoE patients on milk-containing diets (i.e. on- > off-milk DEGs) may correlate with EoE disease activity through T-cell-mediated mucosal mechanisms. To examine this hypothesis, we performed over-representation analysis using the overlapping set of expressed genes to determine possible mechanistic pathways. Results with *P*-values ≤ 0.01 in both the EoE versus control biopsy and on-milk versus off-milk SMILEE peripheral CD4+ samples demonstrated several overlapping pathways between the on- > off-CD4+ T cells and EoE biopsy transcriptome (Supplementary table 6). Of particular interest, we observe genes in the IFN response signature pathway are upregulated in both the EoE biopsy tissue as well as the bulk CD4+ RNA-seq from EoE patients during active disease (GO:0030198, Figure 5d). Specifically, this includes upregulation of IFN-inducible genes and

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HLA-class I molecules in both samples. In contrast, other overlapping pathways (Supplementary figure 4) involving expression of other cytokine and histone genes are not as clearly correlated between the peripheral blood CD4$^+$ and mucosa during active disease.

DISCUSSION

In this study, we examined the gene expression profiles of peripheral CD4$^+$ cells in eosinophilic oesophagitis patients. We identify specific gene expression changes that occur during active EoE in patients on a milk-containing diet, as well as those who have completed therapy with milk antigen EPIT. In this small cohort of patients, we observe relative homogeneity in the peripheral CD4$^+$ gene expression profile of active EoE patients (on-milk) compared to inactive patients (off-milk).

We identify IFN-α and IFN-γ response signatures as prominent pathways represented in GSEA from CD4$^+$ DEGs of active EoE patients on milk-containing diet when compared to nonmilk containing, inactive state. We have previously identified that EoE patients have milk antigen-specific circulating T cells.[16,24] These antigen-specific T-cell populations are not present in healthy control patients, and although the numbers of these cells are reduced during periods of inactive EoE, they remain detectable using *in vitro* assays.[16] We have recently shown that an IFN-α and IFN-γ gene response signature is consistently seen in both paediatric and adult EoE biopsy tissue.[24] In this current analysis, we identify upregulated IFN responses as a distinguishing feature in unstimulated peripheral CD4$^+$ cells from patients with EoE patients. Our prior work has additionally shown that EoE patients have a population of milk antigen responsive peripheral CD4$^+$IFNγ T cells. Although EoE is known to have a reproducible pattern of mucosal Th2 cytokine elevation,[14,32] our results suggest altered IFN responses are present both in the mucosa and in the circulating CD4$^+$ T cells of EoE patients and may be another important feature of the inflammatory milieu in these patients.

We selected the cut-off of $P \leq 0.01$ for our differential gene expression analysis because our primary goal was to perform ranked gene set enrichment analyses. This strategy would allow us to identify potential pathways or mechanisms of interest in peripheral CD4$^+$ T-cell responses before and after EPIT therapy in EoE patients. From these pathway analyses, we chose an FDR cut-off of 0.1 for downstream pathway analyses to balance the risk of false-positive results given the sample size and heterogeneity. One strength of our current study is that in the on- and off-milk comparison we had a high proportion of paired samples (Supplementary table 1), reducing the impact of interindividual variability present in cross-sectional studies. The determination of enriched pathways in the CD4$^+$ cells isolated from these patient groups allowed us to find gene sets that may help identify genes/proteins with important biological relevance to the pathogenesis of EoE as well as therapeutic response to EPIT. Focusing on single genes alone would have missed such pathways and subnetworks.

In the pathway analysis from on- vs off-milk, placebo vs EPIT, and responder vs nonresponder conditions, we observe enrichment in sex steroid response pathways. This cohort is 68% male, which is consistent with US cohorts and reflects the male predilection described in EoE. However, because our cohort was small and the number of patients of each sex was unevenly distributed, we did not consider Y chromosome genes in our analysis. Therefore, these findings require validation in a larger cohort where more detailed analyses stratified by sex could be performed.

We performed exploratory analyses to determine whether transcriptional changes following EPIT could be correlated with gene expression signatures from purified T-cell subsets (Supplementary figure 3). Following EPIT, there is a decrease in the average expression of Th2-associated transcripts, while the average expression of regulatory T-cell-associated transcripts increases. An imbalance of Th2 and Th1 T-cell responses has been shown to play a critical role in allergic disease, including but not limited to EoE.[13,16,33] Altering the balance between these Th2 and Th1 responses is one of several important mechanisms invoked by other successful immunotherapy strategies.[34] We observed an increase in the average gene expression of transcripts specific to CD4$^+$CD25^{hi}CD127^{low} T_{reg} cells (Supplementary figure 3b). These findings are consistent with our REACTOME pathway analyses (Figure 4d) identifying aspects of Th2 signalling (i.e. IL-4 signalling, IL-13 signalling), regulatory T-cell pathways (IL-10, TGFβ and activin/follistatin), in addition to TCR and costimulatory pathway signalling, as gene expression pathways that differ

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in patients with a favorable clinical response to EPIT versus those without.[35] In preclinical studies, increased numbers of gastrointestinal-homing LAP$^+$FoxP3$^-$ T_{reg} cells were observed following EPIT.[21,36] However, because our experiments examine RNA-seq signatures from the polyclonal peripheral CD4$^+$ population, we cannot establish whether the observed changes are attributable to altered numbers of T-cell subsets or because of significant transcriptional changes in a relatively constant proportion of T-cell subsets.

The SMILEE trial was a small phase IIa trial ($n = 20$ patients).[17] Patients following the predefined EPIT protocol had significant biopsy improvement (25.57 $\pm$ 31.19 eos hpf^{-1}) compared to the placebo (95 $\pm$ 63.64 eos hpf^{-1}).[17] Limitations to our study include protocol violations that occurred during the SMILEE study (Table 1). An additional caveat to our findings is that all patients within the SMILEE study had similar histories of atopic comorbidity but this was not specifically matched in patients and controls. It is possible that antigen-specific responses to EPIT therapy, as well as the transcriptional differences that we observe could be different in patients with a different disease phenotype. Recent studies illustrate differing biopsy transcriptional profiles are one feature of potential EoE endotypes,[32,37] and it is unclear whether peripheral lymphocyte gene expression could differ based on endotype as well. Because we assessed gene expression at the time of oesophageal biopsy, one strength of our study is that we were able to clearly correlate peripheral CD4$^+$ gene expression changes with EoE disease activity.

EoE has a unique non-IgE-mediated pathophysiology that has previously limited the use of food antigen immunotherapy strategies for its treatment. However, the SMILEE trial has been the first of its kind to demonstrate initial promise using an immunotherapy approach to treat patients with EoE. Following a total 22-month open-label extension of the SMILEE trial, 47% of patients tolerated a milk reintroduction challenge without any recrudescence of oesophageal eosinophilia. In conjunction with our prior work that demonstrates antigen-specific CD4$^+$ T cells are present in the peripheral blood of EoE patients,[16] we now show that active inflammation in EoE is associated with global alteration in CD4$^+$ gene expression. A distinct feature of CD4$^+$ gene expression in EoE patients is IFN-α and IFN-γ response signatures. This suggests that the T-cell population we have seen during *in vitro* milk antigen stimulation followed by flow cytometry[24] is present in patients and may play a role in EoE pathophysiology. Further, milk EPIT in EoE patients alters a subset of transcripts within the overall CD4$^+$ T-cell population, suggesting that further examination of peripheral CD4$^+$ T-cell responses in EoE is necessary to dissect the specific populations implicated in patients' response to EPIT and understand how to implement this therapy more effectively for EoE patients.

METHODS

Study subjects

This additional research investigation and the SMILEE (Study Efficacy and Safety of the Viaskin Milk for Treating Milk Induced Eosinophilic Esophagitis in Children) trial were approved by the Institutional Review Board of the Children's Hospital of Philadelphia. All procedures were in accordance with the ethical standards of the committee and the 1964 Declaration of Helsinki and its amendments. Written informed consent was obtained from all participants for participation in the SMILEE clinical trial, as well as for this separate investigational study as an independent consideration. Full results from the SMILEE double-blind, placebo-controlled, phase IIa safety and efficacy study of Viaskin Milk have been published elsewhere (DBV Technologies, Bagneux, France; ClinicalTrials.gov, NCT02579876).[17]

Peripheral blood samples were collected from the subset of SMILEE patients electing to participate in this additional, optional, research study (Table 1). Briefly, patients were eligible to enrol in the SMILEE trial if they were between the ages of 4 and 17 years with a history of confirmed EoE diagnosis defined as oesophageal symptoms consistent with EoE and history of prior biopsy with $>$ 15 eosinophils per high-powered field (eos hpf^{-1}) following a minimum 2-month period of high-dose proton pump inhibitor (PPI, i.e. 1–2 mg kg^{-1} omeprazole twice daily). To ensure that cow's milk was a relevant EoE antigen for each patient, screening endoscopies were performed prior to randomisation in the SMILEE study (Figure 1, schematic). Subjects underwent endoscopy while eating a cow's milk-containing diet (minimum of 240 mL milk per day) for 8 weeks minimum. A second endoscopy was performed following 8 weeks of a milk-free diet. If biopsies confirmed milk-responsive EoE ($>$ 15 eos hpf^{-1} on milk-containing diet and $<$ 10 eos hpf^{-1} on milk-free diet), patients were eligible for randomisation in the SMILEE trial with Viaskin Milk (500 μg of milk proteins) or placebo patch (Figure 1, DBV Technologies, Paris, France).

Following 9 months of EPIT while on a milk-free diet, EPIT was continued throughout a 2-month period of milk reintroduction (minimum 240 mL milk daily). Patients then underwent repeat endoscopy with biopsy to assess response to therapy. Patients randomised to the active (milk peptide Viaskin EPIT) treatment arm were further classified as treatment responders or nonresponders based on these post-EPIT biopsy results. Patients were classified as therapy

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'responders' if post-EPIT biopsy was < 15 eos hpf^{-1} and 'nonresponders' if post-EPIT biopsy continued to have significant inflammation with > 15 eos hpf^{-1}.

CD4$^+$ T-cell isolation

Peripheral blood samples were collected at each SMILEE study endoscopy ('on milk-containing diet' screening visit, 'off-milk-containing diet' screening visit and following EPIT therapy, Figure 1). Whole-blood samples were stored at 4°C for less than 4 h before PBMCs were separated by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Lifesciences, Marlborough, MA, USA) and cultured on a polystyrene flask for 1 h to remove remaining adherent cells. CD4$^+$ cells were isolated via positive selection using magnetic beads (Invitrogen Dynabeads Human CD4$^+$ Positive Isolation Kit, Carlsbad, CA, USA), which achieved a median purity of 95% CD4$^+$ T cells confirmed by flow cytometry analysis.

RNA isolation and sequencing

Total CD4$^+$ T-cell RNA was extracted (TRIzol reagent, Invitrogen, Carlsbad, CA, USA) and purified (RNeasy Mini Kit, Qiagen, Hilden, Germany). RNA preparations with an A260/280 range of 1.8–2.1 and RNA integrity number over 7 were used for further analysis. cDNA was generated and libraries were made with Ovation Ultralow Library System V2 (NuGen, Redwood City, CA, USA), samples were pooled, and paired-end reads were generated using an Illumina HiSeq 4000 (Illumina, San Diego, CA, USA) with a minimum of 50×10^6 confirmed reads per library.

Analysis of RNA-Seq data

Sequence reads were aligned to the human reference genome GRCh38 and transcriptome using STAR.[38,39] Gene-level read counts (in FPKM) were normalised using the Rlog method and $\log_2$-transformed.[40] Genes with total read counts < 6 were removed from analysis. As a result of unequal distribution of patients of different sexes between groups, Y chromosome genes were not included in the analysis. Reads mapping to both strands of transcripts were counted to account for double-stranded libraries. Differential gene expression between treatment groups was tested using the rank products method,[41] which is a robust nonparametric test suitable to highly variable clinical samples. The data from this publication have been deposited in NCBI's Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) at accession number GSE173895.

Gene set enrichment and pathway analysis

Genes were ranked by their *P*-values and direction of change and input into gene set enrichment analysis (GSEA) algorithms.[42,43] Hallmark gene sets from the molecular signatures database were used to identify pathways significantly enriched with differentially expressed genes (DEGs).[44] Pathways with an FDR < 0.1 were selected for comparison between on-milk, off-milk and post-therapy comparisons. Reactome pathway analysis was performed for genes in the responder vs nonresponder comparison using DEGs meeting FDR < 0.1 cut-off, and REACTOME pathways with FDR < 0.1 are reported.[45,46]

Comparative analyses

Data were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) to perform comparative analyses.[25–29,47] Purified, unstimulated, human T-cell subset gene expression data (GSE3982 and GSE107011) were used to determine difference in average gene expression from our patients' CD4$^+$ samples compared to Th1, Th2 and regulatory T-cell subsets. Published datasets were analysed to derive unique gene expression signatures for regulatory (GSE107011) and Th1/Th2 (GSE3982) CD4$^+$ T-cell subsets.[28,29,47] For each GEO data set, ANOVA was used to identify genes differentially expressed between all T-cell subtypes and Limma was used to identify unique differentially expressed genes for Th1, Th2 and Treg cell types. Then, each patient sample was compared to this T-cell subset data. We calculated per cent change of all genes in our samples across the specific comparisons (on- vs off-milk and EPIT vs placebo comparisons) for the sets of Th1, Th2 and regulatory CD4$^+$ T-cell genes identified.

EoE mucosal tissue is characterised by a reproducible set of transcriptional changes, as demonstrated by Sherril and colleagues using RNA sequencing of biopsy tissue from EoE patients and controls (GSE58640).[25,26,30] Sample CD4$^+$ data were compared to this dataset to examine potential overlapping signatures of biologic importance in EoE, using ANOVA to identify genes expressed between both datasets.

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CONFLICT OF INTEREST

Drs Spergel and Cianferoni have consulting agreements and clinical trial grants with DBV Technologies. Dr Spergel has stock equity with DBV Technologies. DBV Technologies provided the Viaskin Patch used in the SMILEE clinical trial and had no role in the collection, analysis or interpretation of the data presented in the current study. The remaining authors declare no competing financial interests or relationships pertinent to the work reported in this paper.

AUTHOR CONTRIBUTIONS

Melanie A Ruffner: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. **Zhe Zhang:** Data curation; Formal analysis; Methodology; Visualization; Writing-review & editing. **Kelly Maurer:** Investigation; Methodology; Project administration. **Amanda B Muir:** Methodology; Project administration; Resources. **Antonella Cianferoni:** Project administration; Resources; Writing-review & editing. **Kathleen E Sullivan:** Conceptualization; Formal analysis; Methodology; Resources; Supervision; Validation; Visualization; Writing-review & editing. **Jonathan M Spergel:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Visualization; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Table 1: Information regarding patient sample pairing for enrollment endoscopy samples. Age, sex and biopsy results from each EoE patient before and after milk-containing diet are shown. Dashed lines indicate that there was no sample available for analysis at that timepoint.

Age	Sex	"Off milk" Biopsy eos/hpf	"On Milk" Biopsy eos/hpf
11	M	0-7	134
9	M	0	75
13	M	--	15
12	M	--	112
8	F	5	30
12	F	1	127
5	M	5	25
6	M	--	20
12	M	--	30
15	F	3	25
8	M	6	--

Supplementary Table 2: Differentially expressed genes, EoE patients on milk containing diet (n=10 patient samples) versus off milk (n=7 patient samples) containing diet. Using the Benjamini-Hochberg method with an FDR cutoff of 0.05, we identify 244 differentially expressed genes (DEGs) altered during milk-associated active EoE disease. Of these, 97 transcripts were upregulated and 147 were downregulated at the time of the active milk diet endoscopy. Genes with absolute fold change over 1.75 are shown in the table.

Gene Symbol	Log_2 (Fold Change)	FDR
CXCL10	1.3711	0.0045
MTRNR2L1	1.1504	0.02
RSAD2	0.9921	0.0013
HBB	0.9099	0.0024
PPIB	0.9074	$< 10e^{-4}$
LOC101929643	0.8912	0.034
CD69	0.8773	0.0055
LMOD2	0.865	0.0013
IFIT3	0.8583	0.0013
LAMP3	0.8562	0.0071
ASB15	0.8434	0.0045
LOC284581	0.8383	$< 10e^{-4}$
RPS14	0.832	$< 10e^{-4}$
KLRD1	0.831	0.011
C1orf95	0.8194	0.009
GBP1	0.8099	0.0078
TMEM52B	-0.8085	0.01
ITGAX	-0.8099	0.0058
VCAN	-0.8103	0.0024
THBS1	-0.8196	$< 10e^{-4}$
RHOB	-0.8207	0.0058
LOC339874	-0.8219	0.031
C5AR1	-0.8269	0.009
MRAS	-0.8302	0.014
RNASE2	-0.8304	0.022
CD300LF	-0.8316	0.025
AQP9	-0.8366	0.0013
PPARG	-0.8373	0.0083
PILRA	-0.846	0.016
ADORA2B	-0.8504	0.009
ALDH1A1	-0.8507	0.0096
JDP2	-0.8534	0.0083
LOC729737	-0.8627	0.0083
TREM1	-0.8645	0.0058
OLR1	-0.8675	$< 10e^{-4}$
CCDC183-AS1	-0.8754	0.0013
DOK3	-0.8786	0.0013
CLEC7A	-0.8794	0.0071
CREB5	-0.8801	$< 10e^{-4}$
CCL3	-0.8901	$< 10e^{-4}$
S100A12	-0.8931	0.0013
SLC7A11-AS1	-0.8945	$< 10e^{-4}$
CD163	-0.895	$< 10e^{-4}$
BMS1P2	-0.8982	$< 10e^{-4}$
MMP19	-0.9031	$< 10e^{-4}$
BASP1	-0.9249	$< 10e^{-4}$
HMOX1	-0.9321	0.0058

Supplementary Table 2, continued

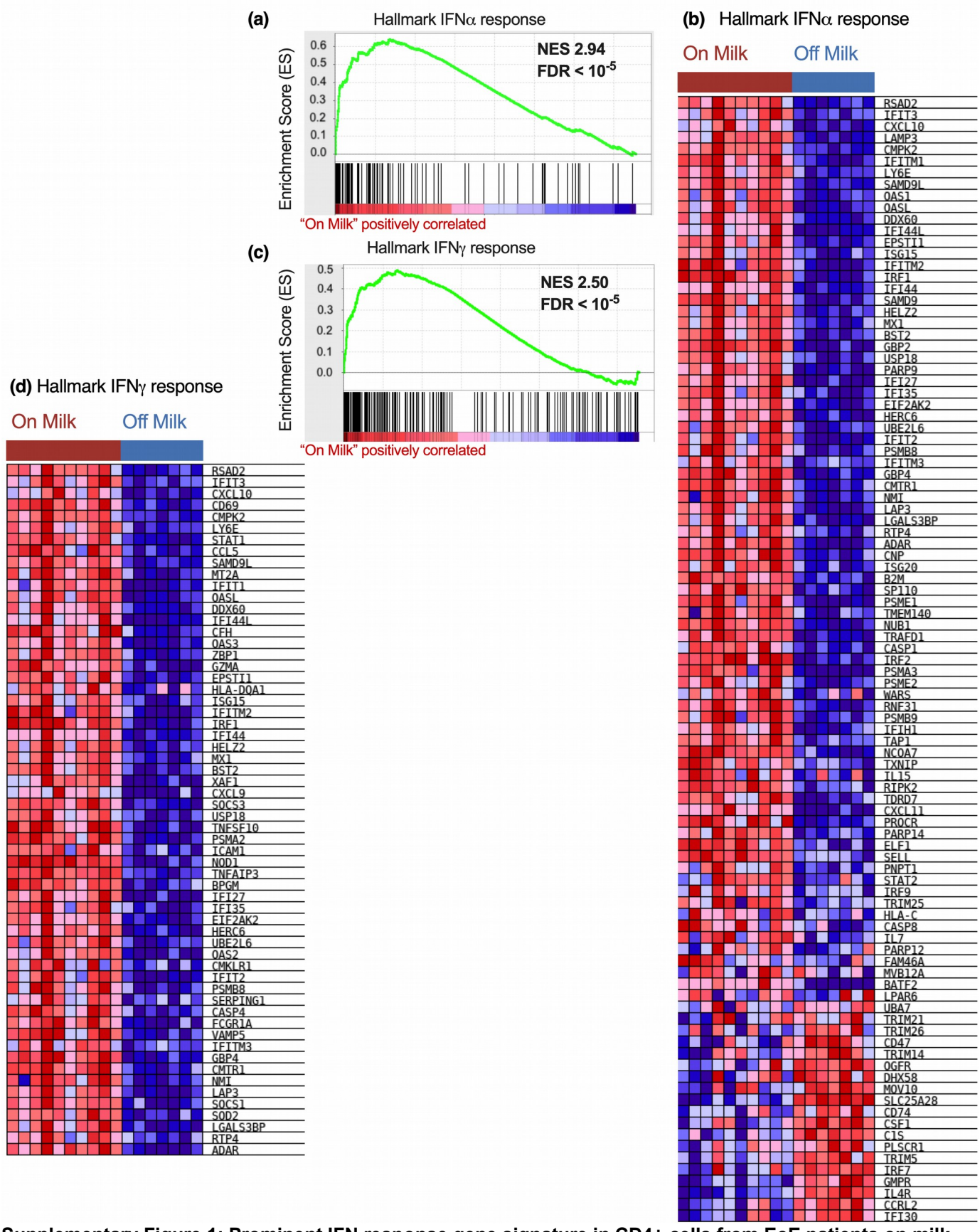
Gene Symbol	**Log_2 (Fold Change)**	**FDR**
PTX3	-0.9366	0.0058
KCNMB1	-0.9373	0.022
IL8	-0.9399	$< 10e^{-4}$
LOC145474	-0.9474	0.0024
GPNMB	-0.9582	0.033
SLC37A2	-0.9627	$< 10e^{-4}$
SGMS2	-0.9733	$< 10e^{-4}$
RPH3A	-0.979	$< 10e^{-4}$
LIPN	-0.9833	$< 10e^{-4}$
KYNU	-0.9839	$<10e^{-4}$
TUBB6	-0.9851	0.0013
CXCL2	-0.9903	$< 10e^{-4}$
SERPINB2	-1.0198	$< 10e^{-4}$
FCAR	-1.0203	$< 10e^{-4}$
IL1RN	-1.0253	$< 10e^{-4}$
THBD	-1.0344	$< 10e^{-4}$
FGD4	-1.0453	$< 10e^{-4}$
GAS2L3	-1.0596	$< 10e^{-4}$
MAFB	-1.0606	$< 10e^{-4}$
UGDH-AS1	-1.0607	$< 10e^{-4}$
ANPEP	-1.1138	$< 10e^{-4}$
TGM2	-1.1527	$< 10e^{-4}$
EREG	-1.1738	$< 10e^{-4}$
MYT1L	-1.2308	$< 10e^{-4}$
CYP1B1	-1.2505	$< 10e^{-4}$
CLEC5A	-1.4939	$< 10e^{-4}$

Supplementary Table 3: Differentially expressed genes, EPIT therapy versus placebo therapy patients. Using the Benjamini-Hochberg method with an FDR cutoff of 0.05, we observe a total of 129 DEGs, with 106 upregulated and 23 downregulated transcripts in CD4+ samples collected from EoE patients on EPIT (n = 7 patients) compared to those on placebo (n=3 patients). Genes with absolute fold change over 1.75 are shown in the table.

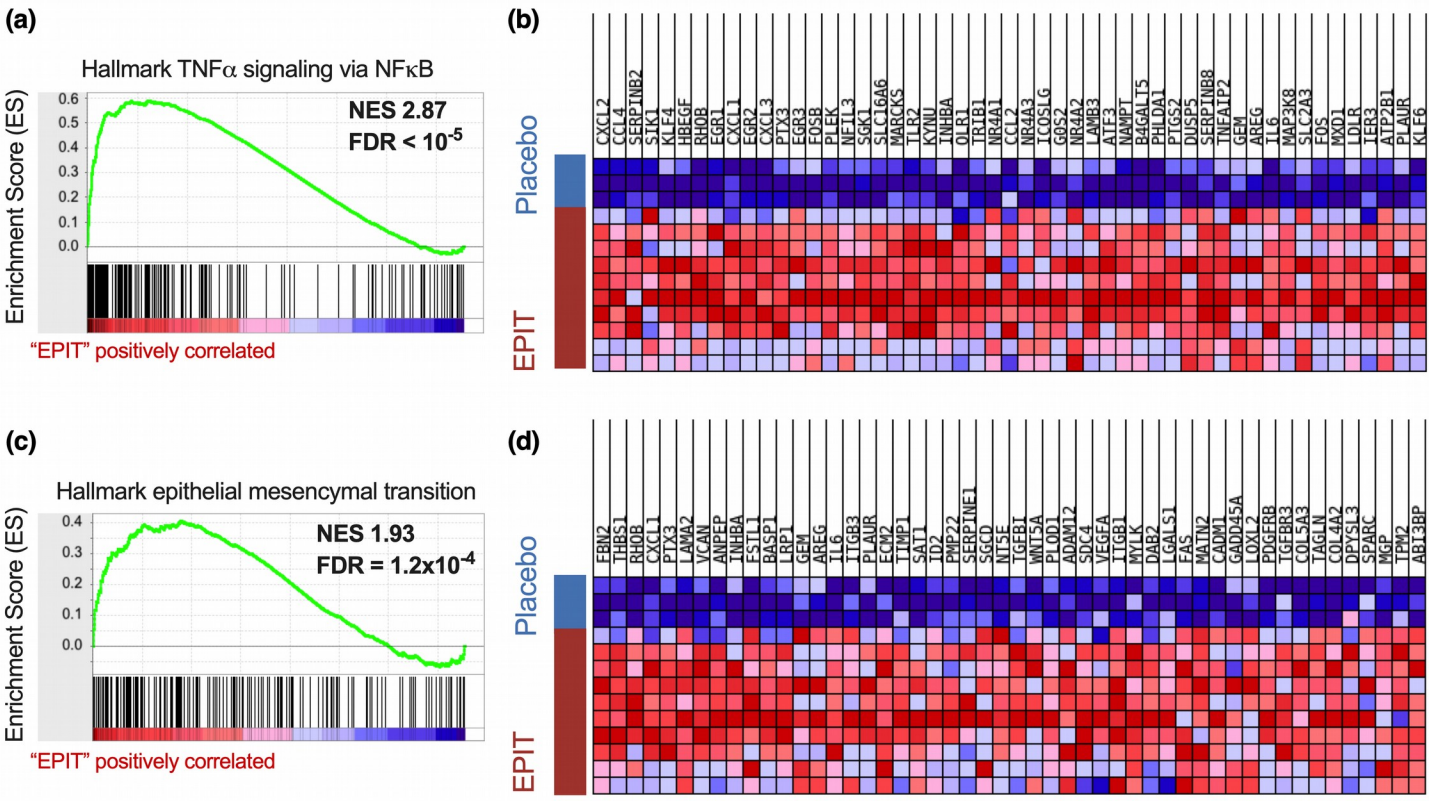
Gene Symbol	Log_2 (Fold Change)	FDR
B3GNT7	1.7174	$< 10e^{-4}$
CCL7	1.3058	$< 10e^{-4}$
CXCL2	1.273	$< 10e^{-4}$
CCL4	1.2067	$< 10e^{-4}$
GAS2L3	1.2036	$< 10e^{-4}$
CCR1	1.1948	$< 10e^{-4}$
RAB7B	1.1838	$< 10e^{-4}$
SGMS2	1.1721	$< 10e^{-4}$
MSR1	1.1697	$< 10e^{-4}$
CX3CR1	1.1637	0.015
EMR3	1.1603	$< 10e^{-4}$
SIGLEC10	1.1527	$< 10e^{-4}$
TNFSF15	1.1491	$< 10e^{-4}$
LILRA1	1.1369	$< 10e^{-4}$
FBN2	1.1353	$< 10e^{-4}$
LOC102724696	1.1326	0.0059
THBD	1.1223	$< 10e^{-4}$
THBS1	1.1209	$< 10e^{-4}$
GPRC5A	1.1158	0.0072
SLC9A7P1	1.112	0.0059
MAFB	1.1102	$< 10e^{-4}$
EMP1	1.0987	$< 10e^{-4}$
SLC2A14	1.0959	$< 10e^{-4}$
RHOB	1.0815	0.017
NID1	1.0738	0.02
SERPINB2	1.07	$< 10e^{-4}$
MNDA	1.0698	$< 10e^{-4}$
EREG	1.0686	0.012
SIK1	1.0588	0.0032
RP11-277P12.20	1.0479	0.033
FCRL1	1.0463	0.022
FGD4	1.0133	0.022
CD300E	1.0132	$< 10e^{-4}$
SIRPB1	1.0129	0.0072
MS4A7	1.0101	0.03
EGR2	1.0081	0.03
FCAR	1.0049	0.0072
EGR1	1.0021	0.022
SLC8A1	0.9953	0.031
MYOM2	0.9886	0.034
CTSW	0.9843	0.03
HBEGF	0.9838	0.014
CLEC7A	0.9744	0.03
FCGR3A	0.9722	0.031
DOCK5	0.966	0.035
LOC729737	0.9582	0.0072
MAP7D2	0.9573	0.025
FCGR2A	0.9556	0.026
JDP2	0.9556	0.031
KLF4	0.9538	0.0088
LOC102723797	0.9506	0.041
SLC44A5	0.9428	$< 10e^{-4}$
MET	0.9413	0.022
PPARG	0.935	0.02

Supplementary Table 3, continued

Gene Symbol	Log_2 (Fold Change)	FDR
RP11-362F19.1	0.9326	0.022
FCN1	0.9297	0.022
IGSF6	0.9291	0.03
LYN	0.9286	0.049
SH2D1B	0.9239	0.033
MN1	0.922	0.035
DUSP6	0.9217	0.022
FPR1	0.9209	0.035
C5AR1	0.9154	0.042
FCER1A	0.9139	0.0072
IRAK3	0.9069	0.042
AGPAT9	0.9056	0.041
CSF1R	0.9049	0.042
CD163	0.9042	0.017
EMR2	0.9015	0.043
SGK1	0.9007	0.045
TLR4	0.8971	0.041
PTX3	0.8958	0.033
CSF2RA	0.8946	0.042
GAS7	0.8942	0.041
SLC7A11-AS1	0.8912	0.026
LRRK2	0.8896	0.0088
PLEK	0.8836	0.042
CXCL3	0.8812	0.031
AQP9	0.8802	0.031
ZEB2	0.8774	0.049
NFIL3	0.8721	0.042
PYGL	0.8718	0.041
FPR2	0.8708	0.049
CHAC1	0.8681	0.049
ANKRD20A11P	0.8677	0.017
RET	0.8519	0.042
P2RY1	0.8473	0.049
SLC7A11	0.8445	0.022
MGAM	0.8405	0.045
CXCL1	0.8344	0.022
LAMA2	0.8255	0.041
EGR3	0.8217	0.042
CREB5	0.8183	0.022
COL18A1	-0.8213	0.031
S1PR4	-0.842	0.045
LOC101929796	-0.8462	0.041
LIMS2	-0.863	0.034
TMIGD2	-0.8762	0.031
DBH-AS1	-0.8766	0.031
SRGAP2D	-0.8809	0.022
RPPH1	-0.9554	0.038
LINC00997	-1.0514	0.011
ADARB2	-1.0957	0.0088
CMSS1	-1.1218	0.017
LOC284581	-1.1842	0.0072
SLC16A14	-2.2291	0.012



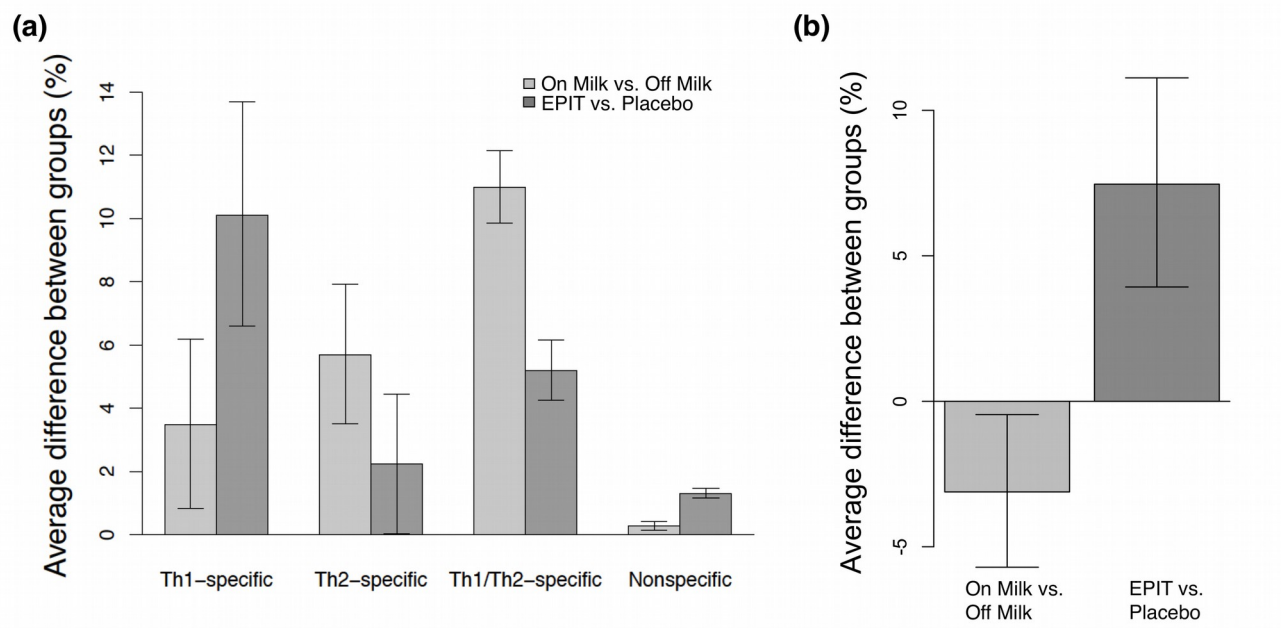
Supplementary Figure 1: Prominent IFN response gene signature in CD4+ cells from EoE patients on milk-containing diet. Gene set enrichment analysis (GSEA) was performed with the canonical pathway gene sets in GSEA Molecular Signatures Database using On versus Off milk EoE patient samples (n=10 and n=7). (A) GSEA Enrichment plot (score curves) for Hallmark IFN alpha pathway and (B) the heatmap panel visualizes all genes contributing to the enriched pathway. (C) GSEA Enrichment plot (score curves for Hallmark IFN gamma pathway and (D) the heatmap visualizes the top 60 genes contributing to the enriched pathway.



Supplementary Figure 2: Gene set enrichment analysis (GSEA) was performed with the canonical pathway gene sets in GSEA Molecular Signatures Database using differentially expressed genes of CD4+ cells from patients on milk EPIT (n=10) versus placebo therapy (n=3). **(a)** GSEA Enrichment plot (score curves) **(b)** the heatmap of top 50 contributing enriched genes in the TNFα signaling via NFκB signaling pathway. **(c)** GSEA Enrichment plot (score curves) **(d)** the heatmap of top 50 contributing enriched genes in the Epithelial Mesenchymal transition pathway.

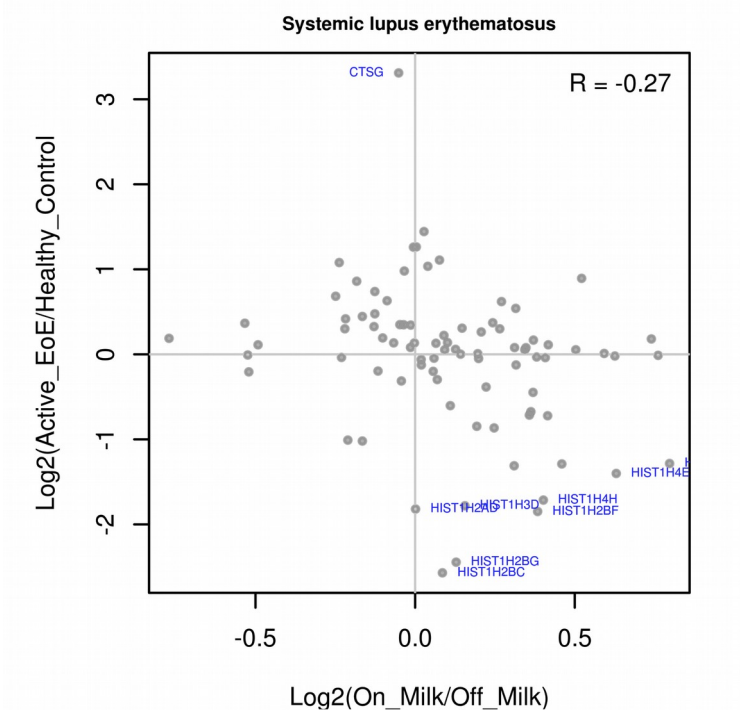
Supplementary Table 4: Differentially expressed genes, EPIT therapy responders versus nonresponders. Using the Benjamini-Hochberg method with an FDR cutoff of 0.05, we observe a total of 42 DEGs, with 33 upregulated and 9 downregulated transcripts in CD4+ samples collected from EoE patients with favorable (responders, n = 5, post-EPIT biopsy < 15 eos/hpf) vs unfavorable (nonresponders, n = 6, post-EPIT biopsy ≥ 15 eos/hpf) clinical response to EPIT.

Gene Symbol	Log2(Fold Change)	FDR
CXCL10	2.1496	0.023
FCGR3B	1.8649	0.036
OVOS2	1.7539	$< 10e^{-4}$
CCL3L3	1.5056	$< 10e^{-4}$
FEZ1	1.4216	0.042
NKG7	1.2168	0.041
CCL2	1.1478	$< 10e^{-4}$
CCL3	1.1431	0.035
STEAP4	1.1371	0.035
SIGLEC5	1.1211	0.036
APOBEC3A	1.1053	0.018
PRSS23	1.0951	0.036
TNFAIP6	1.0934	0.036
FCRL6	1.091	0.042
C19orf59	1.0834	0.036
FPR1	1.0605	0.041
HLA-DQB1	1.0348	0.036
CCL4L1	1.031	0.042
CCL5	1.0211	0.045
SIK1	1.0004	0.014
PTGS2	0.9948	0.042
HLA-DRB5	0.9915	0.042
IER3	0.9838	0.045
EMP1	0.9801	0.041
LOC101927861	0.9669	0.023
GJB2	0.9625	0.045
ZNF319	0.9053	0.042
GTF2H2B	0.8446	0.035
LOC441081	0.8441	0.036
LOC100996385	0.834	0.035
LONRF2	0.8242	0.041
NAPRT1	0.8197	0.045
OVOS	-0.8071	0.036
ZNF90	-0.882	0.018
RNF17	-0.9013	0.045
CDO1	-1.0847	$< 10e^{-4}$
FST	-1.1767	0.018
MYT1L	-1.611	$< 10e^{-4}$

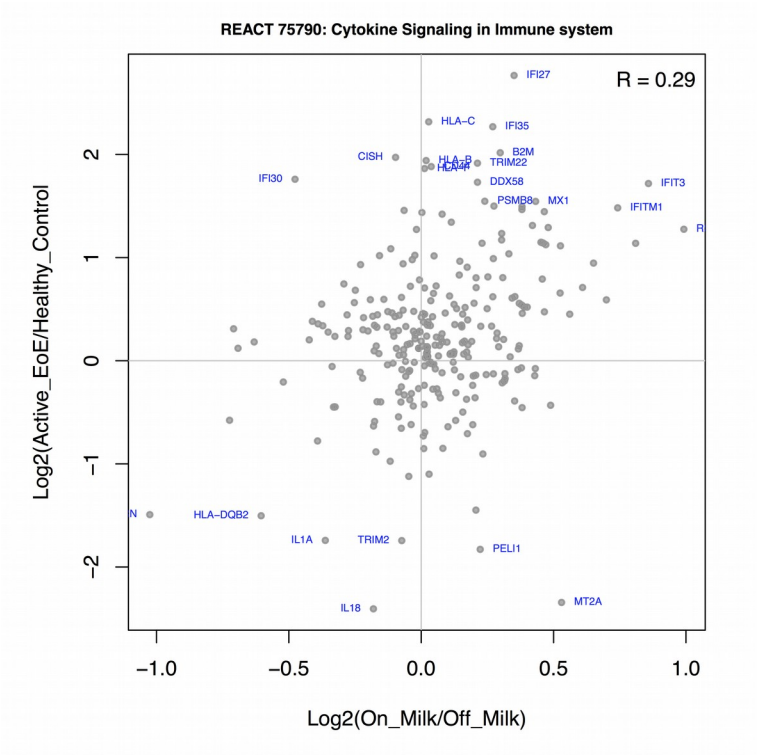


Supplementary Figure 3: Deconvolution analysis of T-cell subset gene signatures suggests EPIT alters balance of T cell subsets. Published datasets were analyzed to derive unique CD4+ T cell subset signatures for T_{helper} (GSE3982) and $T_{regulatory}$ (GSE107011) cell subsets.[34–36] For each GEO data set, ANOVA was used to identify genes differentially expressed between all T cell subtypes and Limma was used to identify unique differentially expressed genes for Th1, Th2 and Treg cell types. We calculated average percent change of all genes in our samples across the specific comparisons (On vs off milk and EPIT vs placebo comparisons) for the sets of Th1, Th2 and Treg genes identified (Average Change %). **(a)** Average change in expression of peripheral CD4+ RNA-seq genes following EPIT (blue) has a higher percent average expression of Th1 signature genes, whereas before therapy the percent average expression of Th2-type genes is higher. **(b)** Peripheral CD4+ from patients post-EPIT have a higher percent average expression of genes specific for regulatory T cells.

(a)



(b)



Supplementary Figure 4: Representation of gene expression patterns in biopsy tissue and circulating CD4+ cells in EoE patients during active disease. Fold change graphs of analysis results for the top 250 common highly expressed transcripts with $P \leq 0.01$ in the EoE patient peripheral CD4+ samples collected before EPIT, (while on and off of milk containing diet) and the EoE biopsy tissue RNA-seq dataset from Sherrill *et al.*[37] Results for **(a)** KEGG Systemic lupus erythematosus and **(b)** REACTOME Cytokine signaling in immune system are shown.

Supplementary Table 5: REACTOME pathway analysis results, EPIT responder vs. nonresponder groups. Reactome pathway analysis was performed using differentially expressed genes from the EPIT nonresponder vs responder comparison meeting FDR < 0.1 cutoff. REACTOME pathways with FDR < 0.1 are reported.[30]

Pathway Name	Number found	Total in Pathway	Ratio	*P*-value	FDR
Translocation of ZAP-70 to Immunological synapse	16	42	0.003	1.11E-16	2.44E-15
Phosphorylation of CD3 and TCR zeta chains	16	45	0.003	1.11E-16	2.44E-15
Interleukin-10 signaling	16	86	0.006	1.11E-16	2.44E-15
PD-1 signaling	16	45	0.003	1.11E-16	2.44E-15
Generation of second messenger molecules	16	59	0.004	1.11E-16	2.44E-15
Interferon gamma signaling	24	252	0.017	1.11E-16	2.44E-15
Costimulation by the CD28 family	16	97	0.007	1.11E-16	2.44E-15
Cytokine Signaling in Immune system	42	1,312	0.089	1.11E-16	2.44E-15
Interferon Signaling	24	396	0.027	6.66E-16	1.27E-14
Downstream TCR signaling	16	124	0.008	8.88E-16	1.51E-14
TCR signaling	16	147	0.01	1.19E-14	1.85E-13
MHC class II antigen presentation	16	148	0.01	1.32E-14	1.85E-13
Immune System	53	2,869	0.196	8.29E-13	1.08E-11
Signaling by Interleukins	18	639	0.044	5.75E-07	6.90E-06
Chemokine receptors bind chemokines	6	57	0.004	3.77E-06	4.14E-05
Adaptive Immune System	19	999	0.068	7.24E-05	7.96E-04
Peptide ligand-binding receptors	8	211	0.014	1.38E-04	1.38E-03
Neutrophil degranulation	12	480	0.033	1.59E-04	1.43E-03
Interleukin-33 signaling	2	4	0	3.91E-04	3.52E-03
Formyl peptide receptors bind formyl peptides and many other ligands	2	11	0.001	2.86E-03	2.29E-02
Interleukin-4 and Interleukin-13 signaling	6	211	0.014	4.05E-03	3.24E-02

Supplementary Table 6: Overrepresentation analysis comparison of biopsy and circulating CD4+ gene expression in active EoE. We examined overlap of differentially expressed genes from EoE patient peripheral CD4+ samples collected before EPIT, (while on and off of milk containing diet) and the EoE biopsy tissue RNA-seq dataset from Sherrill *et al.*[37] The top 250 genes from the two comparisons with $P \leq 0.01$ and highest fold change in common genes were analyzed, and results from overrepresentation analysis pathways with $P \leq 0.05$ are shown.

Collection	Name	Within (*N*)	Total (*N*)	Within (%)	Odds Ratio	*P*-Value	FDR	Direction of Change
KEGG_pathway	Cytokine-cytokine receptor interaction	8	127	3.2	3.69	2.3e-03	7.3e-01	Down; On vs Off Milk
KEGG_pathway	Cytokine-cytokine receptor interaction	11	188	4.4	4.28	1.1e-04	3.1e-02	Up; EoE vs Control
GO_BP	GO:0006954_inflammatory response	20	257	8.0	4.83	1.0e-07	2.5e-03	Down; On vs Off Milk
GO_BP	GO:0006954_inflammatory response	21	345	8.4	4.62	0.0e+00	9.8e-05	Up; EoE vs Control
GO_BP	GO:0030198_extracellular matrix organization	11	177	4.4	3.67	4.2e-04	3.8e-01	Down; On vs Off Milk
GO_BP	GO:0030198_extracellular matrix organization	11	284	4.4	2.76	3.5e-03	2.4e-01	Up; EoE vs Control
GO_BP	GO:0034340_response to type I interferon	9	54	3.6	11.08	5.0e-07	1.2e-03	Up; On vs Off Milk
GO_BP	GO:0034340_response to type I interferon	15	56	6.0	25.86	0.0e+00	0.0e+00	Up; EoE vs Control
GO_BP	GO:1901568_fatty acid derivative metabolic process	6	75	2.4	5.91	8.5e-04	1.5e-01	Up; EoE vs Control
GO_BP	GO:1901568_fatty acid derivative metabolic process	6	75	2.4	5.91	8.5e-04	6.7e-01	Down; EoE vs Control
KEGG_pathway	Systemic lupus erythematosus	11	66	4.4	11.17	0.0e+00	9.2e-05	Up; On vs Off

								Milk
KEGG_pathway	Systemic lupus erythematosus	6	88	2.4	4.97	1.9e-03	7.6e-01	Down; EoE vs Control