A molecular basis for milk allergen immune recognition in eosinophilic esophagitis

Key words: *Food allergy, eosinophilic esophagitis, T cell, TCR, antigen*

Abbreviations used
EoE: Eosinophilic esophagitis
peT_H2: Pathogenic effector T_H2
T_{conv}: $CD25^-CD4^+$ conventional T

To the Editor:

Since the early 1980s, eosinophilic esophagitis (EoE) has been managed as a clinical food allergy.[1] However, immunologic evidence of food antigen recognition by the immune system in patients with EoE has only recently become available.[2-4] Despite these advances, a food antigen–specific T cell has yet to be identified in a patient with EoE. To address this knowledge gap, we enrolled a subject with clinically established EoE milk allergy (EoE subject 1) in an institutional review board protocol (18-015524), collected the patient's peripheral blood, and stimulated the PBMCs with milk proteins as previously described.[3] We then performed single-cell RNA sequencing with linked, paired *TRA* and *TRB* sequencing to determine the activation states and T-cell receptor (TCR) clonotypes of $CD4^+CD45RO^+$ memory T_H cells that expanded in response to milk protein stimulation.

Clonal expansion of a subset of memory T_H cells and enrichment of unique TCRs were observed in response to milk stimulation (false discovery rate ≤ 0.05 [Fig 1, *A*]). Previous studies of esophageal biopsy samples from patients with EoE milk allergy identified clonally expanded pathogenic effector T_H2 (peT_H2) cells.[4] Consistently, we observed upregulation of a peT_H2 gene signature in several clonotypes among the 20 most milk-expanded TCR pairs (Fig 1, *B*). Of these, we chose eoeTCR-4, eoeTCR-6, and eoeTCR-7 for functional testing, as they were the most unique from one another among the peT_H2 cell–high clonotypes and therefore maximized the likelihood of identifying a milk-specific TCR.

TCRs were transduced into sort-purified $CD25^-CD4^+$ conventional T (T_{conv}) cells from a milk-tolerant control subject (Control 1) who shared or approximated all HLA-DR and DQ alleles of EoE subject 1 by using lentiviral vectors in which the recombined *TRA* and *TRB* V-CDR3-J region was cloned upstream of the murine invariant gene segments.[5] Transduced T_{conv} cells (detected by using a murine *Trbc*-specific mAb) were tested for proliferation when cultured with protein-pulsed autologous monocyte-derived DCs. T_{conv} cells expressing eoeTCR-6 and eoeTCR-7 were not reactive to milk proteins (see Dilollo et al[6]). T_{conv} cells expressing eoeTCR-4 proliferated when stimulated with recombinant β-casein but not with other proteins (Fig 1, *C* and see Dilollo et al[6]). To identify the β-casein epitope recognized by eoeTCR-4, we purchased a peptide library spanning amino acids 59 to 224 and containing all β-casein–binding site predictions for the class II alleles shared by EoE subject 1 and Control 1.[7] Of these, we found that peptide 1 (amino acids 59-78) caused proliferation of eoeTCR-4–transduced T cells (Fig 1, *C*), whereas the remaining peptides did not (see Dilollo et al[6]).

To determine which HLA allele shared between EoE subject 1 and Control 1 was responsible for presenting antigen to eoeTCR-4, we recruited 3 additional control subjects who shared different class II alleles with these subjects. T_{conv} cells from donors lacking DRB1*07:01 failed to respond to β-casein when transduced with eoeTCR-4 (see Dilollo et al[6]). These experiments ruled out all alternative shared class II alleles and indicated that eoeTCR-4 was restricted to HLA-DRB1*07:01. To confirm the specificity and restriction of eoeTCR-4, a DRB1*07:01 MHCII tetramer loaded with the β-casein peptide 1 was generated by the National Institutes of Health tetramer core and used to stain eoeTCR-4–transduced T_{conv} cells. The transduced cells costained for both the murine invariant chain *and* the eoeTCR-4 tetramer, confirming that eoeTCR-4 is specific to peptide 1 and restricted to HLA-DRB1*07:01 (Fig 1, *D*).

Having established the milk specificity of eoeTCR-4, we revisited our single-cell RNA/TCR sequencing data. Unbiased clustering of T cells from unstimulated and milk-stimulated cultures (Fig 2, *A*) were overlayed with clone groups based on culture condition and expansion state, noting the 20 most milk-expanded and the eoeTCR-4^+ clones (Fig 2, *B*). Cluster 5 contained the highest proportion of milk-expanded clones and the majority of eoeTCR-4–expressing cells. Cluster Hallmark gene set enrichment analysis revealed increased IFN-α and IFN-γ response genes in clusters 3, 5, and 8 (Fig 2, *C*). To identify unique transcriptional features of eoeTCR-4^+ clones, we compared them with the other peT_H2 cell–high but non–milk-specific clones (eoeTCR-6 and eoeTCR-7). The EoeTCR-4^+ clones displayed elevated expression of genes related to activation (*TMSB4X* and *S100A4*), cytotoxicity (*SRGN*), exhaustion (*CAPG* and *S100A10*), and regulation (*NDFIP1*) (Fig 2, *D*).

Finally, we sought to determine whether eoeTCR-4 was a private or publicly shared TCR. To do so, we inferred *TRA* and *TRB* sequences from whole exome sequencing data of 132 patients with EoE milk allergy. Among these, we did not observe an amino acid sequence match for either the *TRA* or *TRB* gene sequence of eoeTCR-4. Further, we examined the paired *TRA* and *TRB* sequencing sequences of 21 patients with EoE milk allergy from a previously published data set that was enriched for potential specificity (eg, $CD154^+$ following *in vitro* stimulation, expansion in tissue during active EoE, or expression of $CD161^+CRTH2^+$ in circulation).[4] Using homology searching, we again did not detect eoeTCR-4 or related sequences. Therefore, eoeTCR-4 is likely either a private TCR or sufficiently rare that detection requires a larger cohort of patients with EoE milk allergy and the HLA-DRB1*07:01 allele. Further, the eoeTCR-4 antigen may or may not be shared among patients with EoE.

Taken together, our data describe the first molecular details of food antigen presentation and recognition by T cells in a patient with EoE milk allergy. Notably, in this patient we detected antigen-expanded peT_H2 cells that were transcriptionally similar

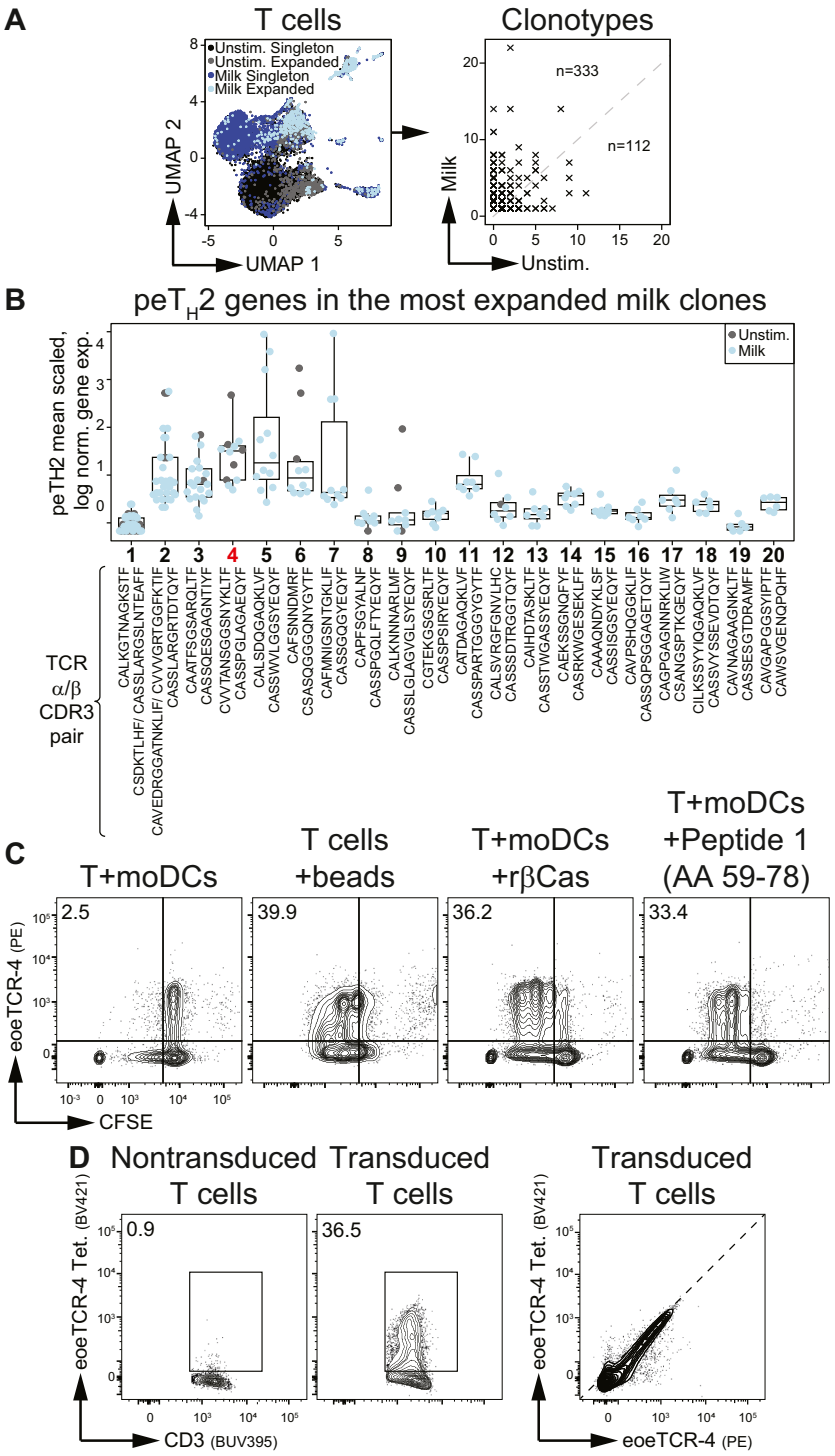

FIG 1. Identification of a β-casein–specific TCR clonotype from a child with EoE milk allergy. **A,** Uniform Manifold Approximation and Projection (UMAP) projection of singleton or expanded (≥ 2) $CD4^+$ T-cell clones from unstimulated (unstim.) or milk-stimulated cells. Each circle represents an individual cell, and each *x* represents an individual clonotype. **B,** Mean scaled, log-normalized (norm.) expression (exp.) of the peT_H2 gene signature per cell by the TCR pairs that were most expanded following milk stimulation in EoE subject 1. Boxplot elements are defined as the median (*center line*), upper and lower quartiles (*box limits*), and whiskers (1.5× interquartile range). **C,** Carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensity of T cells from Control 1 transduced with eoeTCR-4 and cultured with monocyte-derived DCs (moDCs), anti-CD3/28 activating beads (beads), or moDCs pulsed with recombinant β-casein (rβCas) or β-casein peptide 1. *AA,* Amino acid. **D,** Nontransduced T cells or T cells transduced with eoeTCR-4, stained with a β-casein peptide 1–loaded HLA-DRB1*07:01 tetrameric complex (eoeTCR-4 Tet.) and a mAb to the murine invariant chain (eoeTCR-4). Flow cytometric data representative of at least 3 independent experiments.

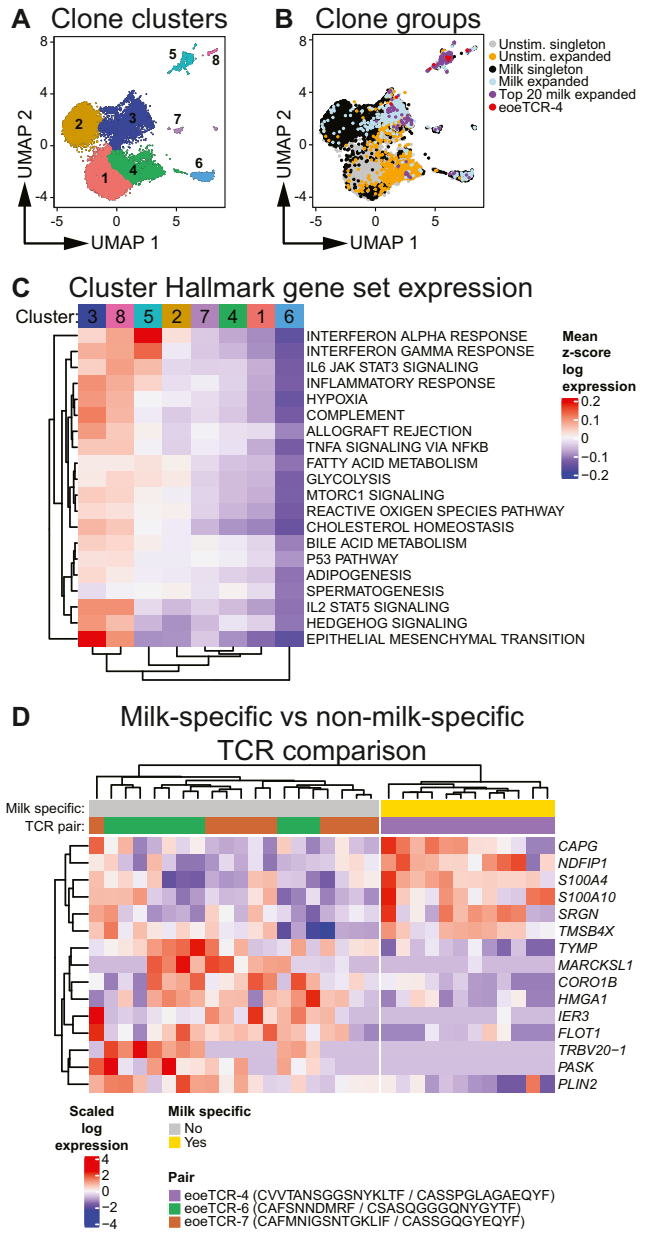

FIG 2. Milk-expanded T-cell clonotypes are enriched for interferon response gene signatures. **A,** Minimally biased clustering of CD4$^+$ memory T-cell clonotypes from unstimulated (unstim.) and milk-stimulated culture conditions. **B,** CD4$^+$ memory T-cell clonotype groups from unstimulated and milk-stimulated culture conditions. **C,** Mean *z* score log expression of the top 20 enriched Hallmark gene sets by *P* value in the CD4$^+$ memory T-cell clusters. False discovery rate ≤ 0.05. **D,** Differential gene expression analysis of eoeTCR-4–expressing T-cell clones and T-cell clones expressing non–milk-specific TCR clonotypes (eoeTCR-6 and eoeTCR-7). Scaled log expression; false discovery rate ≤ 0.05. *TNFA,* TNF-α; *UMAP,* Uniform Manifold Approximation and Projection.

to those in the inflamed esophageal mucosa.[4] Building from seminal studies of the EoE T-cell repertoire,[2-4] and taken in context with early studies indicating a very high response rate of EoE to elemental and milk elimination diets,[8] our findings further support the existing evidence that EoE is mediated by food antigen–specific T cells.

Moreover, milk-expanded peripheral T cells display evidence of interferon signaling. This finding is consistent with our prior observation of a conserved interferon response gene signature in the inflamed EoE mucosa,[9] and it further implicates interferons in EoE immunopathology. Of the genes distinguishing eoeTCR-4$^+$ clones from their non–milk-specific counterparts, *S100A4* expression may be relevant for identifying pathogenic cells, as it promotes allergic T-cell responses and is elevated in EoE.[10] Notably, our approach provides a platform for the discovery and study of additional eoeTCRs. Expansion of this line of investigation to other subjects with EoE milk allergy is necessary to determine the composition of the milk-specific eoeTCR repertoire.

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REFERENCES

1. Chang JW, Kliewer K, Haller E, Lynett A, Doerfler B, Katzka DA, et al. Development of a practical guide to implement and monitor diet therapy for eosinophilic esophagitis. Clin Gastroenterol Hepatol 2023;2:1690-8.
2. Wen T, Aronow BJ, Rochman Y, Rochman M, Kc K, Dexheimer PJ, et al. Single-cell RNA sequencing identifies inflammatory tissue T cells in eosinophilic esophagitis. J Clin Invest 2019;129:2014-28.
3. Dilollo J, Rodríguez-López EM, Wilkey L, Martin EK, Spergel JM, Hill DA. Peripheral markers of allergen-specific immune activation predict clinical allergy in eosinophilic esophagitis. Allergy 2021;76:3470-8.
4. Morgan DM, Ruiter B, Smith NP, Tu AA, Monian B, Stone BE, et al. Clonally expanded, GPR15-expressing pathogenic effector TH2 cells are associated with eosinophilic esophagitis. Sci Immunol 2021;6:eabi5586.
5. Linsley PS, Barahmand-Pour-Whitman F, Balmas E, DeBerg HA, Flynn KJ, Hu AK, et al. Autoreactive T cell receptors with shared germline-like α chains in type 1 diabetes. JCI Insight 2021;6:e151349.
6. Dilollo J, Hu A, Qu H, Canziani KE, Clement RL, McCright SJ, et al. Companion to: A molecular basis for milk allergen immune recognition in eosinophilic esophagitis. bioRxiv 2024;2024.11.13.623408.
7. Reynisson B, Barra C, Kaabinejadian S, Hildebrand WH, Peters B, Nielsen M. Improved prediction of MHC II antigen presentation through integration and motif deconvolution of mass spectrometry MHC eluted ligand data. J Proteome Res 2020;19:2304-15.
8. Kelly KJ, Lazenby AJ, Rowe PC, Yardley JH, Perman JA, Sampson HA. Eosinophilic esophagitis attributed to gastroesophageal reflux: improvement with an amino acid-based formula. Gastroenterology 1995;109:1503-12.
9. Ruffner MA, Hu A, Dilollo J, Benocek K, Shows D, Gluck M, et al. Conserved IFN ignature between adult and pediatric eosinophilic esophagitis. J Immunol 2021;206:1361-71.
10. Bruhn S, Fang Y, Barrenäs F, Gustafsson M, Zhang H, Konstantinell A, et al. A generally applicable translational strategy identifies S100A4 as a candidate gene in allergy. Sci Transl Med 2014;6:218ra4.

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