Successful immunotherapy induces previously unidentified allergen-specific CD4+ T-cell subsets

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Allergen immunotherapy can desensitize even subjects with potentially lethal allergies, but the changes induced in T cells that underpin successful immunotherapy remain poorly understood. In a cohort of peanut-allergic participants, we used allergen-specific T-cell sorting and single-cell gene expression to trace the transcriptional "roadmap" of individual CD4+ T cells throughout immunotherapy. We found that successful immunotherapy induces allergen-specific CD4+ T cells to expand and shift toward an "anergic" Th2 T-cell phenotype largely absent in both pretreatment participants and healthy controls. These findings show that sustained success, even after immunotherapy is withdrawn, is associated with the induction, expansion, and maintenance of immunotherapy-specific memory and naive T-cell phenotypes as early as 3 mo into immunotherapy. These results suggest an approach for immune monitoring participants undergoing immunotherapy to predict the success of future treatment and could have implications for immunotherapy targets in other diseases like cancer, autoimmune disease, and transplantation.

immunotherapy | T cells | gene expression | tolerance | anergy

Allergen immunotherapy (IT) is a process in which small amounts of allergen are given over time to the allergic individual until they can safely tolerate high amounts of allergen with no signs of clinical symptoms (1–9). In the regimen of oral IT for peanut-allergic patients (identified by an allergic reaction during a standardized blinded food challenge to peanut), small amounts of peanut flour protein are ingested and escalated to a servings-worth of peanut protein (4 g of peanut protein) over a period of 2–3 y (6-7). Most patients require continuous frequent (e.g., daily) exposure to such therapy for beneficial clinical outcomes. Mechanistic studies of oral IT to food allergens, although limited to date, show that plasma markers such as IgE and IgG4 immunoglobulins, skin test markers, component testing, and basophil activation tests are only weakly predictive of long-term clinical success (10–16). T cells are critical upstream regulators of allergic sensitization that are required to help B cells to synthesize IgE/IgG4 immunoglobulins, which then can activate or inhibit basophils and mast cells (10–17). Moreover, successful IT is associated with the development of regulatory T cells (Tregs) that are thought to dampen allergic reactivity to offending allergens (7). We therefore focused on finding T-cell markers of immune tolerance that could be detected early in the peripheral blood during IT. CD4+ T cells can be relatively long-lived (compared with plasma proteins and basophils) and changes detected early in populations of T cells could perhaps predict longer-lasting successful IT. For example, in one of the first studies in peanut allergen IT to withdraw therapy for more than 10 wk, we previously showed that despite negative skin tests to peanut, and high IgG4/low specific IgE levels to peanut, and decreased basophil reactivity to the allergen, some patients who withdrew from therapy for 3–6 mo were still reactive upon rechallenge with peanut (7). However, that study was limited because of uncertainty whether the T cells monitored were peanut

specific. Furthermore, many other T-cell markers which could play a role in immune tolerance (18) were not analyzed (7).

Therefore, in the current study, we focused our research on peanut-specific T cells by using tetramer technology, and we performed multiplex transcriptional profiling on single CD4+ T cells. We hypothesized that allergic individuals have a complex set of antigen-specific and nonspecific CD4+ T lymphocytes, including allergic, nonallergic, anergic, and regulatory subtypes that undergo induction and/or transitions during IT. In addition, we posit that there may be certain subsets of CD4+ T lymphocytes that could predict more permanent vs. transient vs. refractory outcomes in IT. To test this hypothesis, we chose a model of antigen-specific oral IT for peanut allergens, and studied peripheral blood samples from the peanut-allergic and control participants in a small, phase 1 IT clinical study (7), while excluding potentially confounding variables, such as simultaneous exposure to multiple antigens and use of concomitant immunosuppressive agents. To enable the recovery of comparable allergen-specific T-cell populations, we limited the analyses to those participants whose specific HLA haplotypes matched available allergen-specific HLA-dextramer sorting reagents. Allergen-specific T cells were collected at four time points during the first 18 mo of IT (Fig. 1). In accordance with a previously published protocol (7), participants who had no signs of clinical reactivity on a standardized food challenge (i.e., to 4 g of peanut protein) at 24 mo were

Significance

The mechanisms through which successful immunotherapy induces possible deletion, replacement, or reprogramming of T cells are unknown. By evaluating the expression of T-cell–related genes, and using appropriate multivariate statistical approaches, our data show that successful immunotherapy can induce previously unidentified CD4+ T-cell subtypes during treatment that could help to predict an "immune-tolerant" clinical phenotype identified after cessation of treatment. The ability to use "anergic" transcriptional phenotypes in single T cells to predict successful "immune tolerance" induction in the clinic setting, as suggested by our findings, could lead to transformative impacts in the field of immunotherapy.
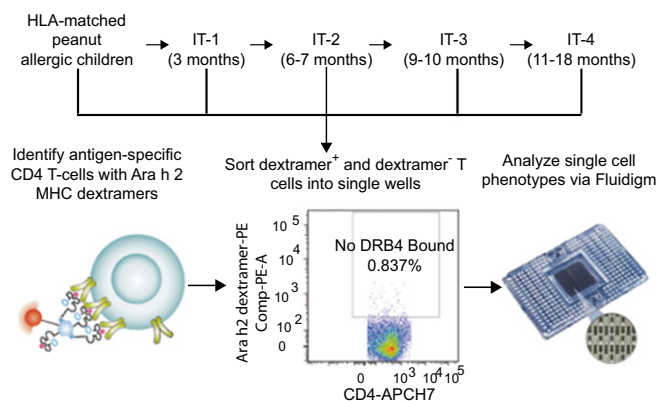
Fig. 1. Allergen-specific CD4+ T cells are collected before and throughout IT treatment and analyzed for gene expression at the single-cell level. Schematic of the overall oral IT study and the time-point analysis. Allergen-specific CD4+ T cells were sorted from peripheral blood monocytes from HLA-DR4+ and HLA-DR15+ peanut-allergic participants by HLA-matched Ara h 2-MHC dextramer reagents on FACS into individual wells, followed by transcriptional profiling of a targeted phenotypic marker panel by Fluidigm Biomark.

withdrawn from peanut IT for an additional 3 mo, and tested for any signs of allergic reactivity at 27 mo with the same standardized food challenge. Participants were defined functionally as either "immune tolerant" (i.e., no allergic reaction with the food challenge at 27 mo and thus possibly representing a more permanent clinical outcome), "desensitized" (i.e., any allergic reaction with the food challenge at 27 mo and thus possibly representing a more transient clinical outcome), or "refractory" (i.e., daily allergic symptoms to less than 300 mg peanut protein for at least 3 mo). Importantly, immunophenotyping of samples from these participants was done by blinded laboratory staff and were conducted before clinical outcomes of the participants had been determined, therefore permitting us to determine, once the study was completed, whether specific CD4+ T-cell subtypes could serve as possible predictors of immune tolerance.

Prior transcriptional profiling studies of lymphocytes have been based on analyses of bulk cellular populations, making it impossible to discern the cell-fate pathways and clonal relatedness of individual T cells or even clusters of T cells of a common phenotype (7, 19, 20). By contrast, functional phenotyping a single-cell level allows one to discern and quantify individual cell phenotypes among complex mixtures of T cells. We sorted dextramer+ and dextramer− CD4+ T cells for single-cell gene-expression profiling (21) to investigate their ontogeny in vivo. Transcript profiling was limited to 22 markers using Fluidigm Biomark technology (22). Biological controls were obtained to compare healthy controls vs. subjects with peanut allergy at pretreatment, healthy controls vs. patients undergoing IT treatment, patients at pretreatment vs. during IT treatment, and dextramer+ vs. dextramer− CD4+ T cells (Figs. 2 and 3). We first performed univariate analysis comparing gene profiles of the sorted CD4+ cells, which demonstrated significance ($P < 0.00057$, Table 1) for many individual immune markers such as IL-13, CD25, IL-17A, IL-4, and ITGα4β7 between comparison groups. Interestingly, during IT some markers (CD28, CD27) seemed to "normalize" to healthy control levels but others, such as CCR7, CD25, and forkhead box P3 (FOXP3), remained significantly different ($P < 0.00057$, Table 1).

We next performed multivariate analysis of multiple immune markers simultaneously to detect possible novel CD4+ T lymphocyte subtypes. Phenotype clustering of single-cell gene-expression profiles obtained over the course of IT revealed distinct phenotypic clusters of CD4+ T cells, with marker combinations characteristic of Th2 "allergic" (IL4+/IL13+), "nonallergic" (IFN-γ+), "regulatory" (FOXP3+/CD25+/IL10+), and "anergic"

(CD28-/CD38-/IFN-γ/IL4-/IL13-/IL10-) CD4+ T-cell subsets (Figs. 4 and 5). In summary, our data show, for the first time to our knowledge, that during the course of IT, antigen-specific CD4+ T cells of diverse T-cell receptor (TCR) clonal origin expanded in frequency, and transitioned from allergic and regulatory to anergic and nonallergic phenotypes, changes that were associated with decreased allergic symptoms and the development of operationally defined immune tolerance.

Results

CD4+ T-cell Transcriptional Profiling. We performed transcriptional profiling of individual dextramer+ and dextramer− CD4+ T lymphocytes throughout the course of IT in vivo, using a regimen of peanut oral IT to test our hypothesis. IT was given to peanut-allergic participants, who had no other known allergies, under a published protocol (7), and peripheral blood was collected from these participants at different time points before treatment (pretreatment time points) and during IT at 3 mo (IT-1), 6–7 mo (IT-2), 9–10 mo (IT-3), and 11–18 mo (IT-4) (Fig. 1). One IT-3 blood draw was performed at 9 mo and the other was performed at 10 mo, whereas one IT-4 blood draw was performed at 11 mo and the other at 18 mo. Participants from whom blood was drawn pretreatment are the same individuals from whom blood was drawn during IT. CD4+ lymphocytes from each participant were labeled with dextramers specific for the peanut-derived antigen Ara h 2 23 (Fig. 1), the most widely recognized peanut antigen among allergic individuals (23) and dextramer+ and dextramer− CD4+ T cells were sorted separately into single-cell wells, followed by profiling of genes expressed in T cells like CD69, Ki67, CD28, CD38, CD27, CD127, IL-4, IL-13, IFN-γ, ITGα4β7, FOXP3, and IL-10 and others (Table S1) to generate heat maps and determine immunophenotyping of CD4+ T-cell subtypes (Fig. S1) (24).

t tests of individual gene expression for dextramer+ CD4+ T cells between healthy controls vs. pretreatment (all pretreatment time points), healthy controls vs. IT treatment (all IT time points), pretreatment vs. IT treatment, and dextramer+ vs. dextramer− CD4+ T cells, identified several shared significant markers ($P < 0.00057$) across two or more comparisons, particularly CD28, IL-10, FOXP3, IL-17a, ITGα4β7, IL-13, CCR7, CCR8, and CD25 (Table 1). The most frequent statistically significant changes ($P < 0.00057$) were detected in the pretreatment vs. IT treatment comparison. In addition, there were several markers that were statistically different between dextramer+ and dextramer− CD4+ T cells (Table 1). Notably, the elbow method for gap statistics performed on all data (including all healthy, pretreatment, and IT cells) identified seven clusters of CD4+ T cells with distinct gene-expression patterns (Fig. 2A). The elbow method for gap statistics looks at the percentage of variance explained as a function of the number of clusters in a data set, seeking to choose a number of clusters so that adding more clusters does not significantly improve the modeling of the data (25). Each of the seven clusters identified had a particular "phenotype" assigned using the expression level, or absence, of specific transcripts (Fig. 2 B and C, Table 2, and Fig. S2). Within most clusters, there were allergen-positive CD4+ T lymphocytes and, to a lesser extent, negatively sorted (dextramer−) cells (which represent a cell population that is over 90% nonspecific because these cells do not have a TCR cognate for the peanut peptide-MHC used in the staining reagent) (26) (Fig. 2B). Comparison of the dextramer+ vs. dextramer− composition of each cluster by *t* tests showed statistically significant ($P < 0.01$) different proportions of antigen-specific CD4+ T cells in each cluster, except cluster 7 (Fig. 2B). Cluster 1, IFNγ-expressing cluster 2, and nonallergic cluster 3 were primarily composed of dextramer− or "antigen-nonspecific" cells. The allergic cluster 4 and regulatory cluster 5 consisted exclusively of dextramer+ or "antigen-specific" cells, whereas anergic memory cluster 6 was primarily composed of dextramer+, antigen-specific cells. IL-10 expressing cluster 7 exhibited a roughly equal mixture of antigen specific and antigen nonspecific cells (Fig. 2B).

Dextramer+ cells are strongly enriched for allergy-associated phenotypes, demonstrating the specificity of the dextramer reagents
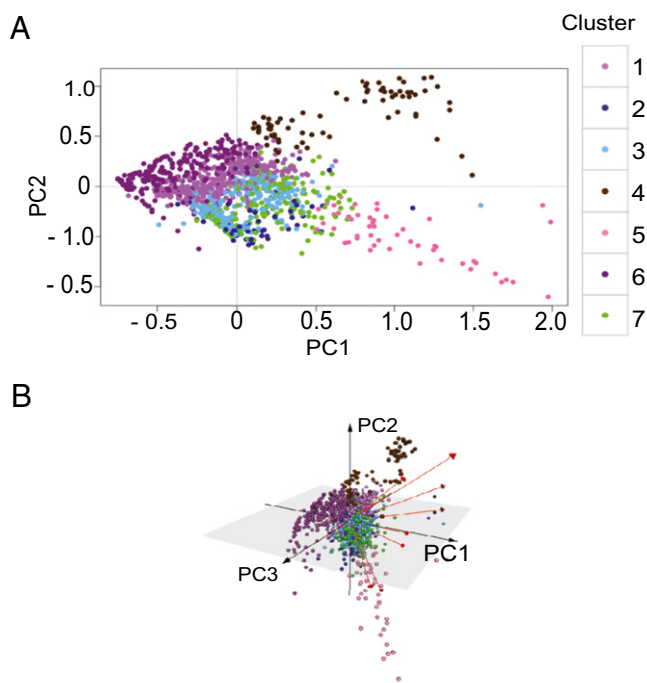
Fig. 2. CD4+ T cells form clusters with distinct gene expression, with allergen-specific cells preferentially occupying IL4+/IL13+/CD69+ cluster 4 and FOXP3+/IL10+/CD25+ cluster 5. (*A*) Gap statistics graph illustrating the elbow method for determining the number of *k*-means clusters that best represents the variance observed in single-cell gene-expression data. Seven clusters were chosen. (*B*) Fraction of dextramer+ and dextramer− CD4+ T cells which belong to each cluster, and a heat map profile of relative gene expression for each cluster. *$P < 0.0073$, ns = not significant (χ^2 tests with Bonferroni-corrected *P*-value cutoff for multiple testing). (*C*) Interquartile ranges of relative gene expression for each of the seven clusters.

used (Fig. 2*B*). Nonspecific binding of dextramer reagents would assume to sample randomly from antigen-specific and antigen-nonspecific T cells, and therefore to resemble the phenotypic distribution of the background population and not to enrich specifically for allergy-related phenotypes. However, the phenotypic characterization of the dextramer+ cells is consistent with what is currently assumed regarding the phenotypes of allergen-specific T cells, including being greatly enriched IL4+/IL13+ cells as well as FOXP3+/IL10+/CD25+ cells compared with dextramer-cell types (Fig. 2*B*).

Differential Cluster Gene Expression. Gene-expression profiling allowed the phenotypic categorization of particular CD4+ T-cell clusters, including cluster 5, which exhibited increased IL-10, CD25, and FOXP3 expression, features associated with Tregs (27, 28), cluster 4, with increased IL-4 and IL-13 and low CD27, features linked to allergic Th2 cells (2), cluster 6, with low CD28 and K_i-67, features linked to anergic T cells (29), cluster 3, with high CD27 and low IL-4 and IL-13 expression, features linked to nonallergic cells (2), and cluster 2, with increased IFN-γ, a feature linked to Th1 cells (30) (Fig. 2 *B* and *C*, Table 2, and Fig. S2). Interestingly,

A

B

Fig. 3. Antigen-specific CD4+ T cells form seven cluster phenotypes with distinct gene expression. PCA representation of variations in cell phenotypes, plotting cells along (*A*) PC1 and PC2; (*B*) PC1, PC2, and PC3. The cells of all participants at all time points are colored according to the CD4+ T-cell clusters to which they were assigned using *k*-means. Variance accounted for by each PC: PC1 = 32.8%, PC2 = 15.8%, PC3 = 8.83%, PC4 = 6.34%.

only the anergic cluster 6 had a memory phenotype, exhibiting low CD45RA expression (Fig. 2 *B* and *C*, Table 2, and Fig. S2).

We next used principal components analysis (PCA) to globally visualize gene expression of single CD4+ T cells. PCA allows for data visualization by reducing the dimensionality of the data by deriving principal components (PCs) that account for the variation in the data. Plotting the individual cells along the first 2–3 PCs showed a clear separation of distinct CD4+ T-cell clusters, including separation and clustering of allergic cluster 4, regulatory cluster 5, and anergic memory cluster 6 (Fig. 3 *A* and *B*). Plotting along PC2 and PC3 continued to show distinct separation of CD4+ T-cell clusters, despite accounting for a smaller percentage of the data's variance than PC1 and PC2 (Fig. 3*B*).

IT Changes Cluster Proportion. Because previous studies showed that IT induces phenotypic changes in bulk T cells (31), we visualized the changing composition of all seven clusters in antigen-specific CD4+ T cells from individual participants during the course of IT. Compared with healthy controls, IT participants had significantly increased frequencies of antigen-specific CD4+ T cells over the duration of treatment ($P < 0.01$) (Fig. 4*A*). In Fig. 4, it appears that whereas healthy individuals have a distribution of phenotypes in their allergen-specific CD4+ T cells that are similar to allergic patients, it is the number of these cells that is significantly different at some time points during IT (Fig. 4*A*). The allergic individuals have less cluster 4 (IL4+/IL13+) cells at baseline, but more cluster 1 (also IL4+/IL13+) cells than healthy controls.

Antigen-specific CD4+ T-cell clusters were recognized that were associated with IT vs. pretreatment (Fig. 4*B*). At IT-1 (3 mo into IT), compared with pretreatment, there was an increase in CD4+ T cells in the allergic cluster 4 and anergic memory cluster 6, and a decrease in CD4+ T cells in regulatory cluster 5 (Fig. 4*B*). As IT progressed, the anergic memory cluster 6 markedly increased, which was associated with a decrease in allergic cluster 4, beginning at IT-2 (6–7 mo into IT), and a reduction of allergy symptoms (Fig. 4*B*). Interestingly, at IT-2 there was an increase

in nonallergic cluster 3 and a maintenance of IL-10-expressing cluster 7. Importantly, over time, the anergic memory cluster 6 increased at later IT time points (IT 3 and 4) compared with earlier IT time points (pretreatment, IT-1, and IT-2) (Fig. 4*B*). Further, CD4+ T cells in regulatory cluster 5 decreased as IT progressed, and were undetectable at IT-4 (at 11–18 mo into IT) compared with pretreatment.

Antigen nonspecific cells did not exhibit significant changes in cluster distribution during IT, indicating that IT induced changes predominantly among antigen-specific CD4+ T cells (Fig. S3*A*). When observing the distribution of antigen-specific CD4+ T cells in an individual participant, the relative contribution of anergic memory cluster 6 continued to increase as IT progressed (Fig. S3*B*). Peanut-allergic participants at pretreatment and healthy controls maintained an equal distribution of cells across the different clusters over time, although some nonstatistically significant ($P > 0.05$) fluctuations in clusters were observed (Fig. S3 *C* and *D*).

Importantly, we juxtaposed the aggregated clinical symptoms of the participants undergoing IT with the same time points in which immune monitoring occurred (Fig. 4*C*). As a participant ingests the food allergen as part of their IT regimen, we found that allergic symptoms such as skin rash, abdominal pain, and respiratory symptoms decreased in frequency over the time course of IT. This decrease in allergic reaction frequency over time in IT is associated with the concomitant increase in the anergic memory CD4 antigen-specific immunophenotype (Fig. 4 *B* and *C*).

Furthermore, other immune markers were examined in these same participants, including skin tests, basophil activation tests, and levels of IgG4 and IgE antibodies. Tests in healthy controls did not show significant activation of their basophils upon stimulation with peanut compared with media (less than 3% difference in percentage of CD63+ cells for all patients), nor did healthy controls have high levels of IgE specific to peanut (<0.35 kUA/L for all individuals) or IgG4 specific to peanut (<0.14 mgA/L for all individuals). Although there were differences in basophil activation, IgE levels, skin tests, and IgG4 levels among allergic individuals, we found that in the small group of subjects analyzed, none of these markers were specifically associated with immune-tolerant vs. desensitized vs. refractory clinical phenotypes (Fig. S4). Importantly, changes in certain T-cell phenotypes occurred before changes in levels of IgE or IgG4, or in results of basophil activation (Fig. S5).

Distinct Clustering Linked to Tolerance. We next tested whether the changes in phenotypes of antigen-specific CD4+ T cells could be used to predict individual clinical outcomes following IT. To do this, we visualized transitions in CD4+ T-cell phenotypes during the course of IT among individual participants for whom we later determined clinical phenotypes of refractory, immune tolerant, or "desensitized." PCA permitted us to detect distinct T-cell clusters over time during IT, with distinct individual gene-expression patterns for T cells in each cluster at each IT time point (Fig. 5 *A* and *B* and Table 1). At pretreatment, there was a diversity of clusters represented for all participants. Interestingly, in both the refractory and immune-tolerant patients, there were no allergic cells at pretreatment but at IT-1 there was a significant transition toward allergic cluster 4 cells for the refractory clinical phenotype with an indistinct scattering of cells across several clusters as IT progressed (Fig. 5 *A* and *B*). Whereas cells remained partly scattered across clusters in the desensitized clinical phenotype during IT, there was some transition toward nonallergic and anergic memory clusters as IT progressed (Fig. 5*B*). In contrast, for the immune-tolerant clinical phenotype, cells transitioned distinctly to nonallergic cluster 3 and anergic memory cluster 6 during IT (Fig. 5*B*).

A comparison of levels of each biomarker per cluster demonstrated significant differences ($P < 0.00057$) in the pretreatment vs. IT groups and a shift over time toward the anergic memory cluster (Table 1). The phenotypic shift distance, a measure of variation in all markers for each cell from one IT time point to

Table 1. Pairwise comparison of individual gene expression

Gene	Healthy vs. pretreatment	Healthy vs. IT	Pretreatment vs. IT	Dextramer+ vs. dextramer−
CCR7	0.5963	0.0003	0.0001	0.1180
CCR8	0.0015	0.0410	<0.0001	<0.0001
CD127	0.7228	0.5096	0.2863	0.5007
CD25	0.1125	<0.0001	<0.0001	0.0057
CD27	0.0127	0.5430	0.0011	0.0222
CD28	<0.0001	0.3085	<0.0001	<0.0001
CD38	<0.0001	0.0442	<0.0001	0.3122
CD45RA	0.0383	<0.0001	<0.0001	0.1107
CD69	0.0119	0.2122	<0.0001	<0.0001
CD90	0.0001	0.2854	<0.0001	<0.0001
FOXP3	0.3602	<0.0001	<0.0001	0.4124
IFNγ	0.0414	0.4841	0.1087	0.4301
IL-10	0.9659	0.0171	0.0244	0.0416
IL-13	0.0074	<0.0001	<0.0001	<0.0001
IL-4	0.6381	0.0055	0.0020	<0.0001
IL-5	0.4080	0.1204	0.0249	<0.0001
IL-17A	0.0066	0.0337	<0.0001	<0.0001
IL-17F	0.0051	0.0321	<0.0001	<0.0001
CXCL10	0.0050	0.0209	<0.0001	<0.0001
ITGA4	0.0023	0.7393	0.0003	0.0284
ITGB7	0.0001	0.0446	<0.0001	0.0076
KI-67	0.0006	0.0291	<0.0001	<0.0001

P values for t tests of pairwise comparisons of individual gene expression of CD4+ cells for healthy vs. pretreatment cells, healthy vs. IT cells, pretreatment vs. IT cells, and dextramer+ vs. dextramer− cells. Bonferonni-corrected P-value cutoff: $P < 0.00057$. Pretreatment cells include all cells from all pretreatment time points, and IT cells include all cells from all IT time points (IT-1, IT-2, IT-3, IT-4). Pretreatment cells and IT cells are from the same individuals.
*Bonferroni-corrected P-value cutoff for each gene is 0.00057.
†Significant differences in gene expression (shaded).

the next, revealed that immune-tolerant and desensitized clinical phenotypes had statistically significant ($P < 0.001$) reduced variance in the later stages of IT (Fig. 5C). The refractory clinical phenotype, however, exhibited greater phenotypic shifting throughout IT ($P < 0.001$) (Fig. 5C). Overall, antigen-specific CD4+ T cells in immune-tolerant and desensitized individuals appeared to settle on anergic memory or nonallergic immunophenotypes during IT, whereas those in the refractory clinical phenotype continued to represent several different phenotypes.

CD4+ T-Cell TCR and Gene Expression. Because successful IT induced significant increases in anergic memory and nonallergic CD4+ T-cell clusters, we sought to define the characteristics of CD4+ T-cell clones in a representative immune-tolerant clinical phenotype. We sequenced TCRs and determined additional gene expression in individual antigen-specific and nonspecific CD4+ T cells (21) from an immune-tolerant participant at IT-2, when several of the phenotypic transitions began to emerge (Figs. 4B and 5B). By doing this, we gained insight into the clonal TCR and additional gene-expression changes linked to tolerance induction.

Both antigen-specific and nonspecific CD4+ T cells were observed to be polyclonal, indicating no particular TCR bias (Fig. 6). TGF-β1, which has been linked to tolerance induction and Th2 inhibition, was expressed by several clones (Fig. 6) (32, 33). Runt-related transcription factor, RUNX1, which represses Th2 programming (34), was expressed in multiple clones (Fig. 6). Interestingly, GATA-3, the transcriptional regulator of Th2 development, was expressed in several antigen-nonspecific clones, often in association with TGF-β1, and not IL-13, a Th2 cytokine (Fig. 6). The Th1 cytokine, TNFα, was expressed by many clones, often concurrently with TGF-β1, but not necessarily with T-BET, the transcriptional regulator of Th1 development (Fig. 6). Expression of TNFα is consistent with presence of Th1 cells; however, it is difficult to draw definitive conclusions based on a limited number of cells. Few other cytokine transcripts were expressed by the selected clones, including limited expression of

IFN-γ, IL-13, IL-12, and IL-21. The follicular helper T-cell (Tfh) lineage commitment factor BCL-6, was expressed by some antigen-specific clones, raising the possibility that Tfh may have a role in tolerance induction during IT (Fig. 6). Additionally, one FOXP3-expressing clone was observed (Fig. 6). Although all dextramer+ cells were unique, in 1,000 repeat random subsamplings of 13 CDR3 sequences from 5′RACE-derived, naive TCR repertoire, four motifs (GLT, PTG, LTD, and RVA) were found to be significantly elevated above expectation in the dextramer+ set ($P < 0.01$). These four motifs occur in partially overlapping regions in the middle of TCRb CDR3, shared across 4 of the 13 recovered dextramer+ single cells. Overall, during successful IT, it is possible to observe marked expression of tolerogenic TGF-β1 and indistinct lineage commitment or cytokine expression by CD4+ T cells.

Discussion
Our goal in this study was to identify mechanisms involved in IT by using single-cell gene profiling, combined with multivariate statistical analyses. Quantifying single-cell gene expression has applications across many biological fields (35–37). High-throughput transcriptional profiling of single cells and computational modeling enabled us to track, on an unprecedented level, the molecular details of CD4+ lymphocytes during IT in vivo. We found evidence of both antigen-specific and -nonspecific CD4+ lymphocytes belonging to seven different phenotypic clusters with distinct gene-expression profiles. Many of our computational analyses of phenotypic transitions were possible only with data derived from single cells. In particular, our data showed significant distinct transitions in antigen-specific CD4+ T cells that were not observed in antigen-nonspecific CD4+ T cells. Notably, shifts in T-cell populations appeared before significant changes in basophil activation, IgE levels, or IgG4 levels, and were more predictive than such tests of an individual participant's clinical outcome (Figs. 4 and 5 and Fig. S4). These findings demonstrate the potential
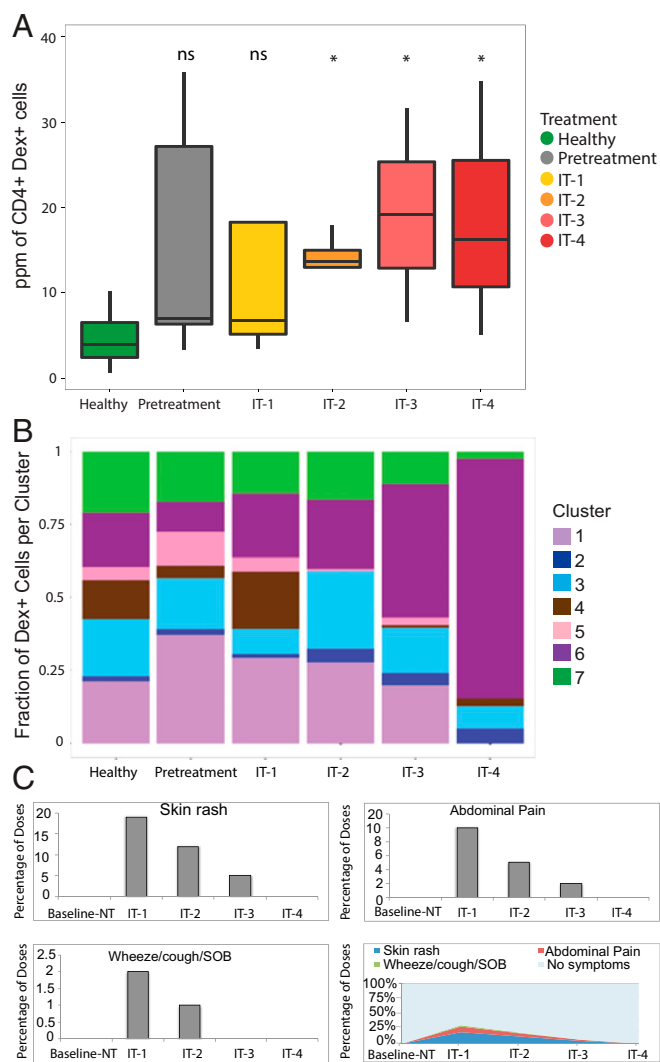
Fig. 4. Cluster 6 T-cell receptor nonresponsive (CD28-/CD38-) noninterleukin secreting (IL13-/IL4-/IL10-/IL5-/IFNγ-) antigen-specific CD4+ T cells expand and allergy symptoms diminish over the course of IT. (A) Interquartile ranges for ppm of antigen-specific CD4+ T cells from pooled healthy (n = 7), pretreatment (n = 5), IT-1 (n = 5), IT-2 (n = 5), IT-3 (n = 2), and IT-4 (n = 2) participants. *P < 0.01, ns = not significant (t tests comparing each time point to healthy controls with Bonferroni-corrected p-value cutoff for multiple testing). (B) The fractional proportion of dextramer+ CD4+ T cells in each phenotype cluster from all participants at each IT time point IT-1 (n = 5), IT-2 (n = 5), IT-3 (n = 2), and IT-4 participants (n = 2), pretreatment (n = 5), and healthy controls (n = 7). (C) Percentage of doses resulting in allergy symptoms observed within 2 h of daily peanut ingestion within each IT time frame drawing from all IT participants.

importance of monitoring T cells during IT and suggest that single-cell approaches may be useful for future studies on the effects of IT and the immune monitoring of individuals undergoing IT. Further, significant differences in CD28 and CD38 expression between experimental groups suggest that it would be interesting in future studies to monitor other cell types, such as B cells and natural killer cells, which express the same markers as the T cells we monitored in our study.

Although Ara h 2 is only one among multiple clinically relevant peanut allergens, it is of great significance that this single representative allergen allows for complex insights into the cellular mechanisms of the allergic process. It is significant that strong statistical statements and predictive observations can be made using Ara h 2 as a single representative allergen. It is

possible that the population of dextramer− cells could contain some CD4+ T cells specific against other peanut allergen peptides displayed on the same or other HLA molecules. However, given the frequency of the known immunodominant
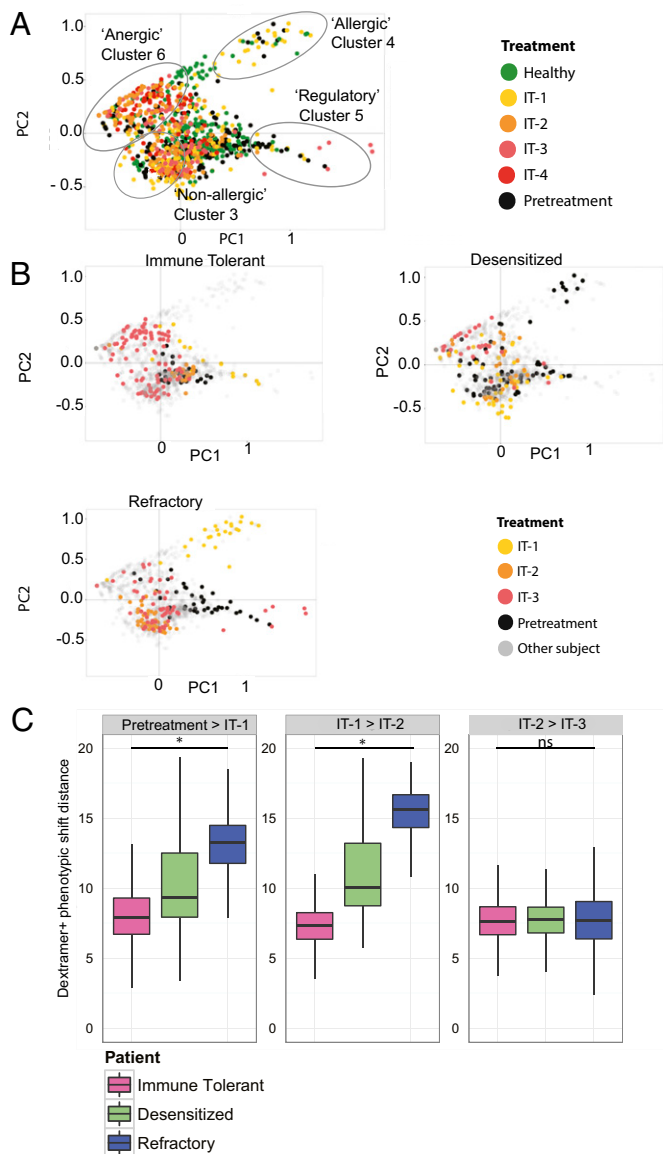

Fig. 5. Temporal expression of CD4+ T-cell clusters reveals individualized patterns associated with clinical phenotypes. (A) Two-dimensional PCA of antigen-specific CD4+ T cells from all healthy subjects (green), and all pretreatment time points (black), IT-1 (yellow), IT-2 (orange), IT-3 (pink), and IT-4 (red) time points for IT participants. Variance accounted for by PCs: PC1 = 32.8%, PC2 = 15.8%. (B) Representative phenotypic shifts across IT time points from one immune tolerant subject, one refractory subject, and one desensitized subject, displayed via 2D-PCA for antigen-specific CD4+ T cells from immune-tolerant, desensitized, or refractory participants over the course of IT. Gray dots represent cells from other participants. (C) Interquartile range of "phenotypic shift" distances for dextramer+ CD4+ T cells from individual participants between IT time points. The one immune-tolerant subject, one refractory subject, and one desensitized subject are the same individuals that appear in B. Higher shift values indicate greater average change in CD4+ allergen-specific phenotype between time points. Phenotype shifts were for months 0–18 during IT, whereas "desensitized/immune tolerant/refractory" status was determined at month 27. *P < 0.001, ns = not significant (one-way ANOVAs). The calculations were performed from every cell in one time point to every cell in the next time point within an individual. The total number of cell–cell comparisons are summarized in Table S2.

Table 2. CD4+ T-cell cluster characteristics

Color:	Light purple	Royal blue	Light blue	Brown	Pink	Dark purple	Green
Clusters:	Group 1 Nonspecific	Group 2 "IFNγ-Expressing"	Group 3 Nonallergic	Group 4 Allergic	Group 5 Regulatory	Group 6 "Anergic memory"	Group 7 "IL-10-Expressing"
Antigen-specific:	Negative	Negative	Positive	Positive	Positive	Both	Both
Markers							
CD69	0	0	0	↑↑ increase	↑ increase	↓↓ decrease	0
CD38	0	0	0	↓↓↓ decrease	↑ increase	↓↓↓↓ decrease	decrease
CD28	0	0	0	↓↓↓↓↓↓↓↓ decrease	↑ increase	↓↓↓↓↓↓↓↓↓↓ decrease	decrease
CD45RA	0	0	0	↑↑ increase	↑ increase	↓↓↓↓↓ decrease	0
CD25	↑ increase	↑↑ increase	↑ increase	↑ increase	↑↑↑↑ increase	0	↑ increase
FoxP3	0	0	0	0	↑↑ increase	0	0
IL-4	0	decrease	0	↑↑↑ increase	↑ increase	decrease	decrease
IL-13	decrease	decrease	↓ decrease	↑↑ increase	↑ increase	↓ decrease	decrease
CCR7	↑ increase	↑↑ increase	↑ increase	↑↑ increase	↑↑↑ increase	0	↑↑ increase
ITGA4	↑ increase	↑↑ increase	↑ increase	↑↑ increase	↑↑ increase	0	↑ increase
IL-10	negative	↑↑ increase	negative	0	↑↑↑ increase	decrease	↑↑↑ increase
IFN-γ	negative	↑↑↑↑↑ increase	negative	0	↑↑↑ increase	decrease	decrease
CD27	↑ increase	↑ increase	↑ increase	↓ decrease	↑↑ increase	0	↑ increase
IL-5	0	↑ increase	0	↑ increase	↑↑ increase	↑ increase	↑ increase
CCR8	0	0	0	decrease	↑↑ increase	decrease	0
CD127	0	↑↑ increase	↑↑ increase	decrease	↑↑ increase	0	↑↑ increase
CD90	0	0	0	0	↑↑↑ increased	0	0

Notes: (*i*) All expression is relative to 18S housekeeping control, 0 = no change. (*ii*) Most distinguishing markers for each cluster phenotype (shaded/bold). (*iii*) ↑ or ↓ indicate relative magnitude of marker expression increase or decrease. (*iv*) Seventeen out of 22 markers shown here because these were the markers significantly associated with cell clusters.

peanut-specific cells (5–20 cells per million) (Fig. 4*A*), even with 1,000 different peanut epitopes just as immunodominant as the one we investigated, with each represented at 20 parts per million, we would expect less than 1 in 50 T cells would be allergen-specific from the circulating CD4+T-cell repertoire, and thus would not affect our statistical results in any meaningful way. Further, it is worth noting that although cells were obtained from the peripheral blood rather than the gut, studies in celiac disease have indicated that several days after ingesting bread, gliadin-specific T cells appear in the peripheral blood (38). Therefore, we speculate that daily exposure to peanuts through oral IT may contribute, via egress of peanut-specific T cells from the gut to the blood, to elevations in the frequencies of peanut-specific T cells in the peripheral blood of patients during IT.

Although there was a relatively small sample size of participants in our study, we were able to follow their single cells sequentially over time and therefore perform detailed, multidimensional comparisons against a previous time point. To address the small sample size, we conducted studies in many control settings (i.e., health controls vs. allergic controls, pretreatment vs. treatment, negative-sorted vs. positive tetramer-sorted cells) to best determine the biological significance of our findings. Sequential single-cell gene-expression measurements in CD4+ T lymphocytes during the course of IT identified unique sets of T-cell clusters that were significantly linked to the immune-tolerant clinical phenotype. As IT progressed, anergic memory and nonallergic antigen-specific CD4+ T lymphocytes were significantly induced only in the immune-tolerant individuals. A regulatory CD4+ T-lymphocyte cluster was also observed as having CD45RA expression. Perhaps there is meaningful variation in the intermediate region of the CD45RA expression and further studies will be done to characterize these potential regulatory populations. The regulatory cluster expressed CD127, suggesting that it is likely an induced regulatory T-cell subset, not a thymic-derived regulatory subset (3, 27, 39. At the same time points, the desensitized clinical phenotype exhibited a greater dispersion of cells across the different clusters, with only some transitions toward nonallergic and anergic memory clusters. Notably, the desensitized subject was the only one of these three subjects to have allergic cells pretreatment. This might be predictive of clinical outcome. In contrast, in the refractory clinical

phenotype, cells spread indistinctly across several different clusters as IT progressed. Of interest is a shift in the refractory patient from no allergic cells at pretreatment to a large number of allergic cells at IT-1 (correlating with the peak of allergic symptoms) before transitioning toward anergic and nonallergic phenotypes at later IT time points. This spike in allergic cells coinciding with IT-1, which was not seen in either the immune-tolerant or desensitized patient, may be predictive of the refractory clinical phenotype. By using current single-cell sorting technology, we were able to distinguish distinct anergic and nonallergic cellular phenotypes that could not have been identified using traditional immunophenotyping methods (39). Defining phenotypic transition patterns in antigen-specific and antigen-nonspecific lymphocytes during successful IT might allow us to infer the timing of expression changes in key genes associated with induction of tolerance.

We defined the anergic memory phenotype by the lack of co-stimulatory receptor CD28 and the proliferation-associated antigen K_i-67, and low-to-absent expression of the early activation marker CD69, indicating a nonproliferating, nonactivated phenotype with diminished CD28 signaling potential, which has been linked to anergic CD4+ T cells (40). However, our current single-cell gene-expression study was technically limited by the number of markers that could be used for the comprehensive detection of anergic cells. Using a more expansive panel of anergy markers (i.e., Cbl-b, GRAIL, program-death-1, and cytotoxic T-lymphocyte-associate protein-4) (20, 41–45) may elucidate in greater detail the transitioning phenotypes of anergic cells during IT.

Although the generation of long-lived memory lymphocytes would seem to be an essential feature of IT, we observed several changes in the naive antigen-specific and -nonspecific CD4+ T cells. Most importantly, the predominant anergic cluster 6 lymphocytes induced during IT had a memory phenotype and were increased substantially over all other clusters. Although there is background variability between subjects at pretreatment time points, the expansion of the anergic cluster 6 can be seen to be much larger than any background variation (Fig. 4) and was statistically significant compared with background ($P < 0.01$). This may indicate that repeated antigen treatments during the course of IT may be critical for anergy induction in memory CD4+ T cells, and for the promotion of a tolerogenic environment that
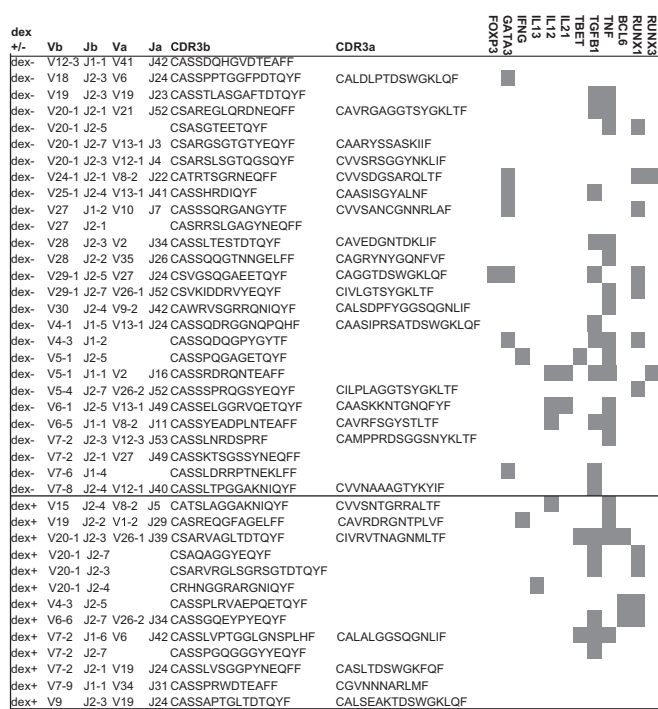
Fig. 6. Single-cell TCR sequencing and gene expression during successful IT demonstrates tolerogenic gene expression without defined lineage commitment. TCR sequencing of CDR3 regions and Vβ, Vα, Jα, and Jβ use, and RT-PCR of transcript expression, for individual sorted dextramer+ or dextramer− CD4+ T cells at IT-2 from one participant later determined to be immune tolerant. Shaded boxes represent gene expression of FOXP3, GATA3, IFN-γ, IL-13, IL-12, IL-21, T-BET, TGF-β1, TNFα, BCL-6, RUNX1, or RUNX3. Horizontal line separates data from dextramer− cells (above the line) and dextramer+ cells (below the line).

dex +/-	Vb	Jb	Va	Ja	CDR3b	CDR3a
dex-	V12-3	J1-1	V41	J42	CASSDQHGVDTEAFF	
dex-	V18	J2-3	V6	J23		CALDLPTDSWGKLQF
dex-	V19	J2-3	V19	J23	CASSTLASGAFTDTQYF	
dex-	V20-1	J2-1	V21	J52	CSAREGLQRDNEQFF	CAVRGAGGTSYGKLTF
dex-	V20-1	J2-5			CSASGTEETQYF	
dex-	V20-1	J2-7	V13-1	J3	CSARGSGTGTYEQYF	CAARYSSASKIIF
dex-	V20-1	J2-3	V12-1	J4	CSARSLSGTQGSQYF	CVVSRSGGYNKLIF
dex-	V24-1	J2-1	V8-2	J22	CATRTSGRNEQFF	CVVSDGSARQLTF
dex-	V25-1	J2-4	V13-1	J41	CASSHRDIQYF	CAASISGYALNF
dex-	V27	J1-2	V10	J7	CASSQRGANGYTF	CVVSANCGNNRLAF
dex-	V27	J2-1			CASRRSLGAGYNEQFF	
dex-	V28	J2-3	V2	J34	CASSLTESTDTQYF	CAVEDGNTDKLIF
dex-	V28	J2-2	V35	J26	CASSQQGTNNGELFF	CAGRYNYGQNFVF
dex-	V29-1	J2-5	V2	J24	CSVGSQGAEETQYF	CAGGTDSWGKLQF
dex-	V29-1	J2-7	V26-1	J52	CSVKIDDRVYEQYF	CIVLGTSYGKLTF
dex-	V30	J2-4	V9-2	J42	CAWRVSGRRQNIQYF	CALSDPFYGGSQGNLIF
dex-	V4-1	J1-5	V13-1	J24	CASSQDRGGNQPQHF	CAASIPRSATDSWGKLQF
dex-	V4-3	J1-2			CASSQDQGPYGYTF	
dex-	V5-1	J2-5			CASSPQGAGETQYF	
dex-	V5-1	J1-1	V2	J16	CASSRDRQNTEAFF	
dex-	V5-4	J2-7	V26-2	J52	CASSSPRQGSYEQYF	CILPLAGGTSYGKLTF
dex-	V6-5	J2-5	V13-1	J49	CASSELGGRVQETQYF	CAASKKNTGNQFYF
dex-	V6-5	J1-1	V8-2	J11	CASSYEADPLNTEAFF	CAVRFSGYSTLTF
dex-	V7-2	J2-3	V12-3	J53	CASSLNRDSPRF	CAMPPRDSGGSNYKLTF
dex-	V7-2	J2-1	V27	J49	CASSKTSGSSYNEQFF	
dex-	V7-6	J1-4			CASSLDRRPTNEKLFF	
dex-	V7-8	J2-4	V12-1	J40	CASSLTPGGAKNIQYF	CVVNAAAGTYKYIF
dex+	V15	J2-4	V8-2	J5	CATSLAGGAKNIQYF	CVVSNTGRRALTF
dex+	V19	J2-2	V1-2	J29	CASREQGFAGELFF	CAVRDRGNTPLVF
dex+	V20-1	J2-3	V26-1	J39	CSARVAGLTDTQYF	CIVRVTNAGNMLTF
dex+	V20-1	J2-7			CSAQAGGYEQYF	
dex+	V20-1	J2-3			CSARVRGLSGRSGTDTQYF	
dex+	V20-1	J2-5			CRHNGGRARGNIQYF	
dex+	V4-3	J2-5			CASSPLRVAEPQETQYF	
dex+	V6-6	J2-7	V26-2	J34	CASSGQEYPYEQYF	
dex+	V7-2	J1-6	V6	J42	CASSLVPTGGLGNSPLHF	CALALGGSQGNLIF
dex+	V7-2	J2-7			CASSPGQGGGYYEQYF	
dex+	V7-2	J2-1	V19	J24	CASSLVSGGPYNEQFF	CASLTDSWGKFQF
dex+	V7-9	J1-1	V34	J31	CASSPRWDTEAFF	CGVNNNARLMF
dex+	V9	J2-3	V19	J24	CASSAPTGLTDTQYF	CALSEAKTDSWGKLQF

ther, clones expressing GATA-3 also largely coexpressed TGF-β1, which inhibits GATA-3–driven Th2 differentiation (33). This may be another indication of transitory CD4+ T-cell phenotypes within a successfully treated individual, with allergic Th2 cells undergoing transcriptional reprogramming to tolerogenic TGF-β1-expressing cells. Additionally, several clones expressing TNFα also coexpressed TGF-β1, again indicating functional plasticity and transitory phenotypes of CD4+ T cells during IT.

The results of the TCR sequencing gives confidence that the cells we sequenced are representative of a larger pool of antigen-specific T cells and do not display only clones that were greatly expanded. Initially when performing our phenotype analysis we did have some concerns that there might be large clonal expansions that would bias our analysis of T-cell phenotypes toward the most expanded T cells. However, in our TCR sequencing analysis, we determined that the recovered populations are quite diverse. This implies that the total number of allergen-specific clones is considerably larger than the number of T cells that we characterized, and that the single-cell phenotyping analysis performed was sampling phenotypes from almost entirely non-redundant clones. Further, evidence of specific shared and overlapping CDR3 motifs could possibly indicate that a diverse population of clones converges to recognize pMHC complexes with similar selected amino acid motifs. In future studies, this suggests that such motifs could be used to recognize allergen-specific T cells from primary sequence (21). Although we only acquired TCR sequencing data during treatment, it would be interesting in future studies to match TCR sequencing data obtained during treatment to TCR sequencing data acquired before treatment begins to answer important questions pertaining to clonal transformation during IT treatment.

Studies of antigen-specific IT have implicated FOXP3-expressing Treg induction in association with immune tolerance in allergy, diabetes, and multiple sclerosis (7, 46–49), and a subversion of Th2 or Th1 responses in allergy and diabetes, respectively (47, 48, 50). However, we observed a reduction in allergic Th2 cells and a reduction in antigen-specific regulatory cells in the peripheral blood over time during IT. Preferential deletion of antigen-specific Th2 cells has previously been observed following IT (1) and decreased Treg over time in IT has been reported (7, 15). Further repertoire analysis of CD4+ T cells during IT may reveal the preferential deletion or reprogramming of T cells based on factors like functional avidity or structural TCR avidity. Additionally, several IT studies, particularly for allergy, have identified changes in antigen-specific IgE and IgG responses, regulatory B cells, and basophils associated with tolerance induction (13, 17, 39, 46, 51).

In conclusion, our study is the first, to our knowledge, to show complex phenotypic transitions in CD4+ T cells during IT. By analyzing the gene-expression patterns of individual CD4+ lymphocytes, we were able to reconstruct the pathways of these cells as they transitioned to "tolerant" or "nontolerant" states. This approach has yielded previously unidentified insights into the potential mechanisms of tolerance induction during oral IT. Although our study has used oral IT in food allergy as a model treatment, the mechanisms we have uncovered and the methods we have applied could be relevant to other forms of IT and disease states associated with modulation of the immune system, such as cancer, autoimmune

inhibits pathogenic CD4+ T-cell recruitment and aberrant responses (43). Although we do not usually think of anergic cells as a population that proliferates, there are some plausible explanations of this observed phenomenon. A change in IL-2 production could account for this expansion. Also, it is plausible that through the course of IT other nonanergic cell types are activated and proliferate before becoming anergic. These cells may then act upon subsequent allergen exposure, suppressing the allergic response. It would be interesting in future studies to further elucidate the mechanism of this observed expansion, and to define in greater detail the phenotype of these cells, measuring the presence of cytokines (such as IL-2) and markers of effector memory T cells (such as CD62L).

Our results also suggest that successful IT can induce polyclonal antigen-specific and -nonspecific CD4+ T cells, with indistinct lineage commitment, possibly indicating their functional plasticity and transitory phenotype. During successful IT, several clones expressed TGF-β1, which has been linked to anergy and tolerance induction during IT, typically by way of Tregs (31). Fur-

Table 3. Summary of demographics of participants

Participant type	Number of participants	IT time points, mo	Frequency of CD4+Dex+, median (range)	Age, median (range)	Sex	HLA type
Healthy controls	7	−3, 0	22.5 (3–55)	38 (32–47)	Males = 1, Females = 6	1501 = 4, DRB4 = 3
Pretreatment participants	5	−6, −3, 0	27.5 (9–48)	10 (8–15)	Males = 1, Females = 4	1501 = 2, DRB4 = 3
IT participants	5	3, 6–7, 9–10, 11–18	44 (14–78)	10 (8–15)	Males = 1, Females = 4	1501 = 2, DRB4 = 3

diseases, and transplantation. The data presented here provide important insights into the changes in gene expression and T-cell phenotypes that may occur during successful IT.

Materials and Methods

Study Design. The study was designed to discern the changes in CD4+ T-cell phenotypes that underpin the induction of immune tolerance in oral IT. To this end, we isolated CD4+ T cells from IT participants and healthy controls (individuals who have no known allergies and have not taken drugs that are known to influence peripheral T-cell response) at predefined time points (Table 3), sorted individual T cells, and performed single-cell gene-expression analyses and TCR sequencing and phenotyping on these isolated cells. The protocol for this study was reviewed and approved by the Institutional Review Board (IRB) of Stanford University. Written informed consent was obtained for all participants before entering the study.

Participants and IT. Participants with matching HLA types, HLA-DRB1*1501 and HLA-DRB4, compatible with peanut-derived Ara h 2 peptide dextramers from a previous recent study (7), were enrolled in this pilot study. Double-blind, placebo-controlled food challenges (DBPCFCs) occurred at screening and clinical reactivity was determined.

Clinical reactivity is defined as any sign of allergic reaction. The oral IT protocol was conducted in a hospital setting with trained staff. Healthy controls were proven to be nonallergic via serum IgE less than 0.35 kU/L and no clinical symptoms consistent with atopy and no positive food challenges. Peanut allergy participants were confirmed using DBPCFCs conducted under an IRB-approved protocol at Stanford University School of Medicine. Participant demographics can be found in Table 3. Further subject information, including eligibility criteria, and further IT details, including food challenge dosing protocol, have been previously published (7).

Cell Preparation and Sorting. From each subject, 20–40 mL of blood was obtained during the afternoon and placed on a rotator overnight before being processed in the morning the following day. Basophil activation assays were performed as previously described (52). Specific IgE and IgG4 were measured (Stanford Clinical Laboratories). CD4+ T cells were isolated using the Human CD4+ T-cell enrichment kit (StemCell Technologies), according to the manufacturer's protocol. CD4+ T cells were activated with phorbol myristate acetate (PMA) at 20 ng/mL and Ionomycin at 1 µg/mL at 37 °C for 1.5 h to up-regulate cytokine expression. After washing twice, CD4+ T cells were stained with PE-labeled Ara h 2 dextramers [DRB1*1501 and DRB4; Ara h 2 (120-139, RQQEQQFKRELRNLPQQCGL)] (Immudex) at room temperature for 45 min. Cells were then stained with anti-CD3 V500, anti-CD4 APC-H7, anti-CD8 FITC, anti-CD14 FITC, anti-CD19 FITC (BD Biosciences), anti-CD45RA brilliant violet 421 (BioLegend), anti-CD56 FITC, and anti-CD294 Alexa Fluor 647 (BD Biosciences) for 20 min at 4 °C. Cells were washed and incubated with anti-PE magnetic beads (Miltenyi Biotec), according to the manufacturer's protocol, and a 1/20 fraction was saved for analysis. The other fraction was passed through a magnetic column (Miltenyi Biotec). The bound, PE-labeled cells were flushed and collected. Cells in the bound fraction and the fraction not passed through the column were stained with 7-AAD (BD Biosciences) for 10 min before flow cytometry. Dextramer-stained T cells were sorted as single cells into individual wells of a 96-well plate. Cells were stored in reverse transcriptase reaction buffer at −80 °C until use. Flow cytometric data were acquired on a BD Fluorescence Activated Cell Sorting (FACS) Aria (BD Biosciences) and the data analyzed using FlowJo (FlowJo).

Fluidigm. This assay was performed by the Human Immune Monitoring Center at Stanford University. For single sorted cells, RT-PCR was performed directly in a 96-well PCR plate (ABI) containing lysis buffer (Invitrogen) by using SuperScript III One-Step RT-PCR System with PlatinumTaq (CellDirect kit, Invitrogen). PreAmp was performed on a thermocycler using the TaqMan PreAmp Master Mix Kit (Invitrogen) added to cDNA and 0.2× pooled Taq-man assays. RT enzyme was inactivated and the Taq polymerase reaction was started by bringing the sample to 95 °C for 2 min. The cDNA was pre-amplified for 18 cycles by denaturing at 95 °C for 15 s, annealing at 60 °C for 4 min. The resulting cDNA product was diluted 1:2 with 1× TE buffer (Invitrogen). Next, 2× Applied Biosystems Taqman Master Mix, Fluidigm Sample Loading Reagent, and preamplified cDNA were mixed and loaded into the 48.48 Dynamic Array (Fluidigm) sample inlets, followed by loading 10× assays into the assay inlets. Manufacturer's instructions for chip priming, pipetting, mixing, and loading onto the BioMark system were followed. Real-time PCR was carried out with the following conditions: 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Data were analyzed

using Fluidigm software. All reactions were performed in duplicate or triplicate, and Ct values were normalized to the 18S positive control.

TCR Sequencing and Phenotyping. TCR sequencing and phenotypic analyses were performed as previously described (21). Briefly, PCR sequence and gene-expression analysis from single cells were obtained by a series of nested PCRs for multiple Vα, Vβ, Jα, Jβ, Cα, and Cβ regions and multiple genes, including *FOXP3, GATA-3,* IFN-γ, *IL-13, IL-12, IL-21, T-BET, TGF-β1, TNFα, BCL6, RUNX1,* and *RUNX3.* Bar-coding PCRs were used to track PCR products from individual cells that were sequenced using the Illumina MiSeq platform (Illumina Inc.).

Initial Data Transformation. Initial Fluidigm output consists of marker expression levels ranging from 0 to 40, which represent the number of amplification cycles necessary to obtain a threshold level of the marker. A score closer to 0 indicates a greater expression level, and a score closer to 40 indicates a lesser expression level. Any markers not expressed at a high enough level to be detected after 40 cycles receive a score of N/A (nonapplicable). We transformed them by subtracting each score from 40, so that higher transformed scores correspond to higher expression level. All N/A values were set to 0.

Normalization. To control for interplate variance, we normalized using the expression of 18S, a housekeeping gene, to establish a baseline level of activity for each plate and adjust accordingly. We found the median 18S expression of all of the cells on each plate and then found the median of these 35 medians. An "activity level" for each plate was calculated by dividing the median 18S expression of that plate by the median-of-medians. Then, each marker expression level for each cell on the plate was divided by this activity level.

Cluster Analysis. We started by using the gap statistic to compare within- to between-cluster sums of squares for various numbers of clusters (25). After plotting these and looking for an elbow, we divided the cells into seven clusters. Before clustering, the data for each marker were centered by the overall median and scaled by the range. Cells were then clustered using *k* means. To ensure that all presenting phenotypes were represented and contributed to cluster designation, all cells (both dextramer+ and dextramer−) were included for cluster analysis at all time points. All statistical analysis and graphs were performed using R. *K*-means clustering (53) was performed using the R package "kmeans" (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/kmeans.html). Heat map visualization was performed using the R package "heatmap 2" (www.inside-r.org/packages/cran/gplots/docs/heatmap.2). The agglomerative clustering method used for dendrogram construction was complete-linkage clustering, using a Euclidean metric. The clustering method passed to the clusGap function was kmeans, with *k* ranging from 1 to 7.

PCA was performed using the single-cell gene-expression data to visualize the relationship among the individual cells. PCA is an unsupervised method that generates a new set of unrelated variables (PCs) that represent the most variation in the data set (54). PCA was run using the "prcomp" function in R (stat.ethz.ch/R-manual/R-patched/library/stats/html/prcomp.html) to attribute the variance in the data to a reduced set of variables (PCs). For 3D-PCA, we projected the high-dimensional immunophenotype data onto the first three PCs and mapped each element into a 3D viewer using the R "pca3d" package (cran.r-project.org/web/packages/pca3d/index.html). For 2D-PCA we used the R package "ggplot2" (cran.r-project.org/web/packages/ggplot2/index.html) and mapped each element.

Variance/cluster composition bar graphs were plotted using the R package "ggplot2." The fractional cluster composition at each stage of IT was calculated as the number of cells in that stage of IT that were statistically determined to sort into each cluster (*k*-means) divided by the total number of cells, yielding a fraction between 0 and 1.

Phenotypic Shift Distance. Within each participant, we calculated the degree of phenotypic shift of all observed CD4+ dextramer+ peanut-specific T cells between progressive time points. Phenotypic shift is reported as the root-mean-square deviation (RMSD) (55) of all phenotypic markers between each cell at an IT time point to each cell observed at the following time point. All phenotypic shift distance calculations were performed in Perl (Version 3.20.1) (https://www.perl.org).

Statistics. To determine which markers were informative in determining the effect of IT on individual T cells, we used *t* tests to compare: cells from healthy controls against cells from pretreatment participants, cells from healthy controls against cells from IT participants (all IT time points), cells from pretreatment participants against cells from IT participants (all IT time points), and dextramer+ cells vs. dextramer− cells. *t* tests were performed using R.

Comparison of phenotypic shift distances by one-way analysis of variance (ANOVA) was performed with Prism software (GraphPad). Comparison of proportion of dextramer+ and proportion of dextramer− cells belonging to each cluster was done using χ^2 tests in Excel. Comparison of number of dextramer+ cells at each IT time point was performed in Excel using t tests. Bonferroni-corrected P-value cutoffs were computed by setting a significance level of $\alpha = 0.05$ and dividing by the number of tests performed.

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