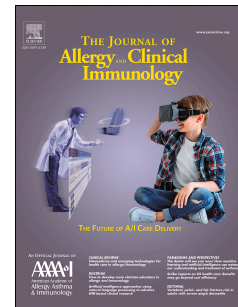


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Allergen-specific T cells and clinical features of food allergy: Lessons from CoFAR immunotherapy cohorts

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Abstract

Background: Allergen-specific IL-4⁺ and IL-13⁺ CD4⁺ cells (Type 2 cells) are essential for helping B cells class-switch to IgE and establishing an allergic milieu in the gastrointestinal tract. The role of T cells in established food allergy is less clear.

Objective: We examined the food allergen-specific T cell response in participants of two food allergen immunotherapy (IT) trials to assess the relationship of the T cell response to clinical phenotypes including response to IT.

Methods: Blood was obtained from 84 peanut-allergic and 142 egg-allergic participants who underwent double-blind placebo-controlled food challenges. Peanut and egg-responsive T cells were identified by CD154 upregulation after stimulation with the respective extract.

Intracellular cytokines and chemokine receptors were also detected. The response to peanut epicutaneous immunotherapy (CoFAR6, 49 participants receiving EPIT) and egg oral immunotherapy or baked egg diet (CoFAR7, 92 participants) was monitored over time.

Results: Peanut-specific Type 2 and CCR6⁺ T cells were negatively correlated with each other, and differently associated with immune parameters including specific IgE and basophil activation test. At baseline, Type 2 cells, but not CCR6⁺ cells, were predictive of clinical parameters including successfully consumed dose of peanut and baked egg tolerance. Exposure to peanut or egg immunotherapy was associated with a decrease in Type 2 cell frequency. At baseline, high egg-specific Type 2 cell frequency was the most predictive immune feature of oral immunotherapy failure.

Conclusions: Food-specific Type 2 T cells at baseline are informative of threshold of reactivity and response to immunotherapy.

Key Messages

- Egg and peanut-specific CD4⁺ T cells co-express IL-4 and IL-13, and type 2 cytokine expression negatively correlates with CCR6 expression.
- Type 2 CD4⁺ T cells and CCR6⁺ T cells are differently associated with IgE and basophil responses.

- Type 2 T cell responses are associated with key clinical parameters including successfully consumed dose, baked egg tolerance, and treatment with immunotherapy.
- A high frequency of egg-specific Type 2 cells predicts treatment failure with egg oral immunotherapy.

Capsule Summary

Berin et al show that high levels of food-specific Type 2 CD4+ cells making IL-4 or IL-13 not only decrease with food allergen immunotherapy, but high levels of these cells at baseline predict a poor response to immunotherapy.

Keywords

Th2 cells, Tfh cells, basophil activation test, immunoglobulins, oral immunotherapy, epicutaneous immunotherapy, double-blind placebo-controlled food challenge

Abbreviations

BAT	Basophil Activation Test
CoFAR	Consortium for Food Allergy Research
DBPCFC	Double Blind Placebo Controlled Food Challenge
EPIT	Epicutaneous immunotherapy
OIT	Oral immunotherapy
peTh2	Pathogenic effector Th2
SCD	Successfully consumed dose
Th2	T helper type 2
Tfh	T follicular helper
Tfh13	IL-13 producing T follicular helper
Treg	Regulatory T cell

Introduction

Allergen-specific CD4⁺ T cells play a key role in determining the outcome of tolerance versus allergy in response to allergen exposure. IL-4 is essential for IgE class-switching, and recent data demonstrate that IL-13 production specifically from T follicular helper (*Tfh13*) cells is essential for generation of high-affinity anaphylactogenic IgE ¹. Th2 associated cytokines also support the production of allergic effector cells including mast cells and basophils, and IL-4 increases the severity of anaphylaxis in mice ². In contrast, antigen-specific regulatory T cells (Tregs) play a critical role in the induction of oral tolerance, at least as demonstrated in mouse models ³. Peripheral Tregs, particularly ROR γ t⁺ Tregs and regulatory Tfh cells, suppress Th2 immunity and IgE class switch, while GATA3⁺ Tregs can exacerbate allergic disease ⁴⁻⁷.

Human food allergy is associated with a Th2-skewed cytokine profile, as measured by bulk cytokine secretion in recall assays ⁸, intracellular cytokine staining in combination with proliferative or activation markers ⁹⁻¹², and transcriptional analysis at the bulk and single cell level ¹¹⁻¹⁵. In addition to Tfh13 cells, highly differentiated pathogenic effector (peTh2) or Th2A cells produce Type 2 cytokines in peanut allergy ^{10-12, 15}. Dupilumab, which blocks IL-4 and IL-13 signaling through binding to IL-4R α , is currently in clinical trials for the treatment of peanut allergy, and it is hypothesized that targeting Type 2 immune responses will be clinically effective for the treatment of food allergy. However, we lack a thorough understanding of how the food allergen-specific T cell response relates to established food allergy, and whether these cells may contribute to clinical heterogeneity including successfully consumed dose, severity, or response to immunotherapy.

We developed harmonized antibody panels for immune profiling allergen-responsive CD4⁺ T cells in peanut and egg allergy, and applied these to two multi-center clinical trials conducted by the Consortium for Food Allergy Research (CoFAR). We detected allergen-responsive cells by upregulation of CD154 (CD40L) on CD4⁺ T cells after 6-18h of stimulation with allergen, and stained chemokine receptors, intracellular cytokines, and Foxp3 to identify CD4⁺ T cell subsets. We have previously reported that allergen-responsive CD4⁺ T cells, as measured by CD154 upregulation after short-term stimulation in vitro, were highly Th2 skewed at baseline in these two cohorts of egg and peanut allergic individuals, with a substantial expression of IL-4 and

IL-13, significant but low IL-10, and no statistically significant IFN- γ response in these cells ^{11, 16}. We previously found that allergen-responsive CD4⁺ T cells were enriched for expression of the Th2 marker/skin homing marker CCR4, the Th17/mucosal homing marker CCR6, and the Tfh marker CXCR5. We also identified the presence of peanut-specific Tfh13 cells in peanut allergic individuals but not controls ¹. In this manuscript, we report on the association of allergen-specific T cells with immune and clinical parameters. We provide evidence that two distinct T cell subsets, Type 2 cells and CCR6⁺ cells, are associated with immune parameters including IgE and basophil activation. Of these, Type 2 cells were most consistently associated with key clinical parameters including successfully consumed dose, baked egg tolerance, and outcome of response to oral immunotherapy.

Methods

Subject characteristics: Subject characteristics have been described elsewhere^{11, 16-18}. See Figure E1 for an overview of participants. Eighty-four peanut sensitized participants were screened, and of these 74 peanut allergic participants were enrolled in a 130-week treatment trial and randomized 1:1:1 to receive 250 ug peanut, 100 ug peanut, or placebo peanut Viaskin patch for 52 weeks followed by treatment of all participants with the 250 ug peanut dose (clinical trial referred to as CoFAR6¹⁷). Of 169 participants undergoing screening for the CoFAR7 study¹⁸, 92 egg allergic participants were enrolled in a 2 year treatment trial. Egg allergic participants were divided into baked egg reactive individuals (n=40) assigned to receive egg OIT, baked egg tolerant individuals randomized to receive egg OIT (n=24), and baked egg tolerant individuals randomized to receive baked egg diet (n=28). Blood samples for immune profiling were obtained in both studies at study baseline, throughout treatment, and at study endpoints. Double blind placebo-controlled food challenges before and after treatment were performed as previously described in the manuscripts describing primary outcomes^{17, 18}. Phenotypic analysis at baseline was performed on all participants screened and providing biospecimens (142 for CoFAR7 and 84 for CoFAR6, see Figure E1).

T cell assays: Samples obtained at clinical sites were shipped overnight in temperature-controlled shipping boxes (GreenBox, ThermoSafe, Arlington Heights, IL) to the Mount Sinai central laboratory. After removal of plasma, peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation with Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA) and cultured in AIM V[®] medium (Gibco, Grand Island, NY) with 2.5% autologous plasma. PBMCs were stimulated with 100 µg of crude peanut extract or 300 µg of egg extract as previously described^{11, 14, 16}. Time-matched unstimulated and anti-CD3/CD28 stimulated cells were used as controls. A single batch of egg or peanut extract was prepared prior to the start of the clinical trial. Endotoxin was removed from peanut or egg extract using Detoxi-Gel columns (Thermo Scientific, Waltham, MA). Residual endotoxin levels measured by LAL assay (Thermo Scientific) were 1.4 EU/ml for peanut and 0.3 EU/ml for egg. Extracts were aliquoted in single-use aliquots for storage at -80° C. Extracts did not activate non-allergic controls, or food-allergic controls tolerant to the food in question.

Cells were harvested at 6h (for effector cells) or 18h (for Tregs) after stimulation and stained as previously described^{11, 16} and as shown in Table E1 and E2.

At least 3.5×10^6 cells were acquired for analysis in each condition. Analysis was performed using FlowJo software (BD Biosystems, Ashland, OR). The frequency of CD154+ and cytokine+ cells per CD4+ T cells were gated based on unstimulated controls, and chemokine receptors were gated based on fluorescence-minus-one controls. All assays were performed on fresh PBMCs.

Basophil activation test: Whole blood was used for basophil activation tests performed at the clinical sites within 2 hours of blood draw, and stained fixed samples were shipped to the central laboratory for acquisition and analysis. Assays were performed as previously described^{17, 18}. Briefly, whole blood was stimulated with a dose titration of egg or peanut extract (as used for T cell assays) for 30 minutes, followed by stimulation and staining with anti-CD3, anti-CD14, anti-CD19, anti-CD41, and anti-HLA-DR (for exclusion), anti-CD123, and anti-CD63 and anti-CD203c to measure basophil activation.

Immunoglobulin Measurements. Frozen plasma samples were thawed and IgE, IgG, and IgG4 antibodies specific for food allergens (egg, ovalbumin, ovomucoid, peanut, Ara h 2) were measured by ImmunoCAP (Thermo Fisher Scientific).

Analysis: Analysis was performed as post-hoc analysis at Mount Sinai of datasets released from Emmes (data and statistical coordinating center for the studies). Number of datapoints available per category is shown in Tables E3-E5. All analysis was performed using GraphPad Prism Version 9. Correlation analysis of immune and clinical parameters was performed using Spearman correlation analysis, using baseline values of all individuals screened for the two studies. T cell responses over time were analyzed individually in each treatment group by a Mixed Effects model (REML, with time as fixed effect and individual as random effect), followed by Dunnett's multiple comparison test. Analysis of T cell response by treatment outcome in CoFAR7 was tested with Mann-Whitney Test. Treatment success for CoFAR7 was considered tolerating a full challenge of

egg while on treatment (desensitization) or off treatment (sustained unresponsiveness), while failing to reach full desensitization was considered treatment failure. Treatment success for CoFAR6 was considered reaching a successfully consumed dose of 300 mg of peanut (cumulative dose of 443 mg peanut) after treatment. P values less than 0.05 were considered statistically significant.

Data availability: All datasets are available through ImmPORT (SDY1520, SDY1550).

Results

Food allergen-specific CD4+ T cell subsets.

We identified peanut- and egg-responsive cells by detection of CD154 (CD40L) on CD4+ T cells after 6-18h of stimulation with allergen, and stained chemokine receptors, intracellular cytokines, and Foxp3 to identify CD4+ T cell subsets in CoFAR6, and CoFAR7, two cohorts of peanut and egg allergy, respectively. Here we looked at the relationship of allergen-specific T cell subsets to each other. All correlation analysis were performed at baseline, using all individuals screened for entry into CoFAR6 or CoFAR7. T cell data at baseline was available for 78 individuals screened for CoFAR6, and 137 individuals screened for CoFAR7.

We performed Spearman correlation analysis of T cell subset frequency, immune, and clinical parameters at baseline, which is shown in Figure E2 and Figure E3. Analysis included both frequency of T cell subsets as a frequency of CD3/CD4+ T cells (Figure E2) and as a proportion of specific (CD154+) T cells (Figure E3) which allows for an assessment of the proportion of the T cell subsets within the allergen-specific population. As shown in Figure 1A,D, there was an almost complete co-expression of IL-4 and IL-13 in both peanut and egg allergy. We refer to these cells going forward as Type 2 cells, as Th2, Th2A, and Tfh13 cells would be present within this population. Although CCR4 is commonly used as a marker of Th2 cells, CCR4+ cells were not perfectly associated with Type 2 cells (Figure 1B,E). CCR6, commonly used as a marker of Th17 cells, was positively correlated with Type 2 cells when examined as a frequency of total CD4+ T cells (Figure E1), but comparison of their proportions of specific cells demonstrated that Type 2 and CCR6+ cells were differentially present within the food-specific compartment (CD154+ after allergen stimulation) and were significantly and negatively correlated with each other (Figure E3, Figure 1C,F). Negative correlations were significant for peanut but only nominally significant for egg ($q=0.059$ after FDR correction). This negative correlation was not simply due to cells being CCR6+ if they were IL-4-negative. As shown in Figure E4, there is also a substantial population of antigen-specific cells negative for both CCR6 and IL-4.

Relationship of T cell subsets to immune parameters

We next assessed the relationship between baseline T cell subsets to immune parameters including specific IgE and basophil activation test. We observed that the frequency and

proportion of peanut- and egg-specific Type 2 cells were significantly and positively associated with the respective specific IgE, including peanut- and egg-specific IgE as well as the components Ara h 2 and ovomucoid (Figure 2A,D). A positive correlation with peanut or egg-specific Type 2 cells was also observed for peanut or egg basophil activation test, either measured as area under the curve of CD63 expression (Figure 2B,E) or as percent of basophils expressing CD63 at different concentrations of peanut or egg allergen¹⁷ (Figure E2, E3). This association was statistically significant for peanut, but only nominally significant for egg ($p=0.066$ after FDR correction). Finally, the proportion of CCR6+ T cells specific for peanut was negatively and significantly correlated with peanut -specific IgE (Figure 2C). A similar trend not reaching the level of statistical significance was observed for egg (Figure 2E).

T cell subsets and clinical parameters

We examined the relationship between T cell subset frequency and clinical parameters measured during a double-blind placebo-controlled food challenge at study baseline. Immune and oral food challenge (OFC) data were available for 78 treatment-naïve individuals undergoing a screening food challenge for peanut allergy (CoFAR6), and 137 treatment-naïve individuals undergoing screening food challenges for egg allergy (CoFAR7). Individuals were all avoiding peanut or egg exposure at time of study entry. No relationship of T cell frequency was found to age, sex, or presence of other atopic disease.

A significant and negative association of peanut-specific Type 2 cell proportion (% of CD154) was found with successfully consumed dose (SCD), the highest cumulative dose achieved prior to development of symptoms and a measure of the sensitivity to the food (Figure 3A). These data indicate that the higher the Type 2 response within the peanut-specific response, the lower the amount of antigen required to trigger symptoms. Frequency of Type 2 cells (% of CD3/CD4) was also significantly and negatively associated with SCD. The Type 2 response was not different between those who were administered epinephrine or not, a loose proxy for reaction severity (not shown).

We observed nominally significant associations between SCD and egg-specific Type 2 responses (Figure E2, E3). We also examined the relationship of T cell subsets to tolerance to

baked egg. The egg allergic subjects included baked egg reactive and tolerant subjects, and those with baked egg tolerance had a significantly lower frequency of Type 2 cells among their egg-specific T cells (Figure 3B).

Dynamics of the T cell response during allergen immunotherapy

The frequency of allergen-specific T cells was initially analyzed as part of the statistical analysis plan for the clinical trials as reported¹⁸⁻²⁰. Analysis of the entire cohort by group and timepoints showed no statistically significant difference between treatment groups in CoFAR6 or CoFAR7 with respect to any T cell subset frequency. Thus, as clinical trial biomarkers, the specific T cell responses were of limited utility. Here we performed post-hoc analysis on pooled treatment groups in order to explore the biology of response during food allergen immunotherapy.

In CoFAR6, peanut allergic individuals were randomized into 3 treatment groups for epicutaneous immunotherapy (EPIT) with 250 µg peanut, 100 µg peanut, or placebo Viaskin patches²⁰. We examined how the T cell response to peanut evolved in all initially treated individuals (250 µg and 100 µg groups) over the course of the treatment. Shown in Figure 4A, the frequency of peanut-responsive Type 2 cells (CD154+IL-4+ or CD154+IL13+) were significantly reduced compared to baseline in individuals treated with peanut EPIT. In contrast, the frequency of CD154+IL10+ cells increased at one year post-treatment. The placebo group was monitored over the first year before crossing over to treatment, and showed no significant change in type 2 cytokines or IL-10 (Figure 4B). The frequency of peanut-responsive cells producing IFN-γ or peanut-responsive Foxp3+ Tregs identified after 18h of stimulation were not significantly altered by peanut EPIT (data not shown).

The phenotype of peanut-responsive cells after immunotherapy was also examined by expressing data as the percent of CD154+ cells (Figure E5). IL-4+ and IL-10+ cells decreased and increased, respectively within the CD154+ population. Interestingly the proportion of CCR6+ T cells within the peanut-responsive population significantly increased at 24 months, indicating that there was an alteration in the phenotype of the peanut-specific CD4+ T cell response in those treated with EPIT. The frequency of these cells (% of CD3/CD4) did not change, suggesting that

these cells were resistant to immunotherapy and that they increased in proportion due to deletion of other cell subsets (such as type 2 cells).

In CoFAR7, egg allergic individuals were divided into 3 treatment groups. Baked egg reactive individuals were assigned to receive egg OIT, while baked egg tolerant individuals were randomized to receive OIT or baked egg diet (the primary comparison of the clinical trial, reported by Kim et al ¹⁸). In Figure 5, all individuals receiving OIT were grouped together (A) while baked egg is shown for comparison (B). OIT was associated with a significant reduction in frequency of Type 2 CD154+ T cells. Unlike in CoFAR6, a reduction in frequency of IL-10+ CD154+ T cells was observed with oral immunotherapy (Figure 5). No change was observed in IFN- γ + CD154+ T cells or CD154+ Tregs over time (data not shown). In comparison, those on a baked egg diet showed no effect on frequency of cells (or proportion of CD154+ cells) expressing Type 2 cytokines (Figure 5B). When examined as a proportion of egg-specific T cells (Figure E6), there was no reduction or increase in any subset, indicating that cell subsets were lost proportionally in response to egg OIT, and that surprisingly there was not a reprogramming of the T cell response.

We next examined the association of egg OIT outcome with immune parameters. Dividing participants into treatment failure (not tolerating a full egg challenge while on treatment or terminating treatment early), desensitization (tolerating a full egg challenge while on treatment), and sustained unresponsiveness (tolerating a full egg challenge after stopping treatment), we found that only baseline Type 2 responses were significantly associated with treatment failure (Figure 6A). The frequency of IL-4+CD154+ cells was significantly higher in treatment failure compared to treatment success, which included both desensitization and sustained unresponsiveness groups (Figure 6B). Furthermore, division of participants into tertiles of IL-4+CD154+ response to egg at baseline demonstrated a 60% rate of treatment failure (measured at 2 years) in the high tertile compared to 30% in the middle and low tertiles (Figure 6C). Peanut EPIT has a low rate of treatment failure due to side effects, and does not typically result in full desensitization, and therefore treatment failure and success could not be examined in the same way in CoFAR6 as in CoFAR7. Instead, we examined the frequency of individuals who reached a SCD of 300 mg after EPIT treatment (Figure E6) among those in the highest tertile of IL-4+CD154+

cell frequency. After 2 years of treatment, only 17% of those in the highest tertile had reached a SCD of 300 mg of EPIT treatment compared to 38% of those with lower levels of IL-4+CD154+ cells (Figure E7).

Discussion

We performed profiling of food allergen-specific T cells in two multi-center allergen immunotherapy trials with extensive clinical phenotyping. As the studies featured baseline double-blind placebo-controlled food challenges, we were able to examine how T cell responses related to clinical phenotype. We found that allergen-specific Type 2 cell responses, identified as CD154+IL-4+ and CD154+IL-13+ cells after short stimulation with allergen extract *ex vivo*, were associated with a number of immune and clinical parameters, including serum IgE and basophil activation, successfully consumed allergen dose on challenge, baked egg tolerance, and treatment with immunotherapy. Most compelling was our finding that Type 2 cell frequency at baseline was associated with poor outcome in response to egg OIT.

There are increasing numbers of T cell subsets that have been identified as increased in food allergy (peTh2 or Th2A cells)^{11, 12, 15}, or have functional data demonstrating a key role in anaphylaxis (Tfh13 cells)¹. These cells have in common the production of IL-4 and IL-13. We observed near total correlation of IL-4 and IL-13 expression within our food-specific CD4+ T cells, and we refer to all CD4+ T cell subsets producing these cytokines as Type 2 cells. We had previously described that the antigen-specific T cell response was enriched for cells expressing CCR6¹¹. Here, we found that the proportion of cells expressing CCR6 was inversely related to the proportion of cells expressing Type 2 cytokines. Although CCR6 is a hallmark of IL-17 expression, we have not found significant production of IL-17 by intracellular cytokine staining or by single cell transcriptional analysis¹¹. Ruiter et al reported a high induction of IL-17 genes (IL17A, IL17F, IL22) in peanut-specific T cells, but without accompanying Th17 protein expression¹⁵. It was recently reported that IL-17+ cells can readily transform into Th2 cells, even under non-polarizing conditions²¹, suggesting that these cells may be a precursor or immature form of the allergen-specific Type 2 cells. We do not yet have data to determine the function of these CCR6+ cells, which is essential to identify their contribution to allergen immunotherapy.

The proportions of allergen-specific Type 2 cells and CCR6+ cells positively and negatively correlated, respectively, with allergen-specific IgE and basophil activation. It has been shown in mice that T cells contribute to IgE maintenance in a STAT6-dependent manner by continuously providing help to IgG+ memory cells for class-switch to IgE+ plasma cells^{22, 23}. However, long-lived IgE-plasma cells have also been demonstrated in mice after chronic allergen stimulation²⁴. It was not clear in humans if a T cell response was still relevant after sensitization had already occurred. While our results are observational, the significant association between allergen-specific Type 2 cells and allergen-specific IgE suggests an ongoing role of T cells in maintenance of specific IgE levels. A recent study from Bruton et al observed that peanut-specific IgE production after allergen exposure of PBMCs from peanut allergic individuals *in vitro* was dependent on Type 2 cytokines²⁵. In our cohorts, individuals were carefully avoiding allergen exposure at baseline and therefore the factors driving Type 2 cell maintenance are poorly understood. The association with basophil activation was not as strong as with IgE, suggesting an indirect effect through IgE production.

CCR6+ cells as a proportion of the allergen-specific T cell response, but not independently expressed as a frequency of total CD4+ T cells, inversely correlated with allergen-specific IgE. If CCR6 was indeed a marker of immature or precursor Th2 cells, a high proportion of these cells may indicate a weakly differentiated allergen-specific T cell response that is less able to support IgE class-switch. Alternatively, they may promote class-switch to another isotype. We did not observe a positive correlation with allergen-specific IgG, but other isotypes such as IgA were not examined. Studying the functional relationship between these two subsets, and following their frequency together with Type 2 cells in infants developing allergy or tolerance to foods should be performed to examine their contribution to allergy or tolerance to foods.

We found that food-specific (CD154+) Type 2 cells were negatively associated with successfully consumed dose of peanut. This correlation was relatively weak, however we still think merits discussion. We are examining the Type 2 cell response in peripheral blood as we do not have access to tissues from individuals with food allergy unless they would be undergoing endoscopy for another indication. Therefore the measured Type 2 response is distal from the site where we anticipate that it would be contributing to anaphylaxis response. IL-4 contributes to

anaphylaxis in mouse models by potentiating the effect of vascular mediators². IL-13 increases intestinal epithelial permeability^{26, 27}, and drives the development of secretory cell associated passages²⁸, providing an additional mechanism for the negative association between Type 2 cytokines and successfully consumed dose. Highly differentiated Type 2 cells express receptors for cytokines such as IL-25 and IL-33^{11, 12, 29, 30}, suggesting a potential mechanism for constitutive activation at inflamed sites such as the gastrointestinal mucosa. Neither the number nor the proportion of CCR6+ T cells correlated with SCD. Type 2 cells were also increased in those with reactivity to baked egg, indicating an important role in phenotypic heterogeneity of food allergy.

Individuals receiving immunotherapy, either EPIT or OIT, experienced a reduction in Th2 cytokines. This was not observed in the initial statistical analysis plans of the clinical trials where all treatment groups were compared over time and between each other. Here we performed post-hoc analysis, combining all EPIT-treated individuals, and all OIT-treated individuals. While we believe that these findings support an important role for Type 2 cells in clinical responses, our findings also demonstrate that allergen-specific T cell responses perform relatively poorly as large-scale biomarkers of desensitization. We observed a substantial heterogeneity in the frequency of peanut and egg-specific T cell responses, with many individuals being under the threshold of detection. This heterogeneity makes it difficult to identify significant differences with small treatment groups. However, as a discovery tool to probe the biology of the food-specific T cell response, they can be illuminating. For example, we found that a high baseline egg-specific Type 2 response was the best immune marker associated with egg OIT treatment failure. A higher-throughput and more standardized assay of this response may be a useful biomarker. Being able to identify those who would not benefit from immunotherapy before starting a two-year treatment course would be of very high value, and this may offer one screening approach. We speculate that high Type 2 responses may contribute to gastrointestinal inflammation leading to termination of treatment^{31, 32}, or may prevent up-dosing during oral immunotherapy by acting to lower the threshold dose. Alternatively, IL-4 has been shown to contribute to Treg reprogramming and reduced regulatory function⁴. Screening for an antigen-specific Type 2 response could identify a subset who might benefit from Type 2 targeting (with Dupilumab or JAK inhibitors) together with immunotherapy. There are also some interesting differences in the

response to OIT and EPIT which may relate to a difference in mechanism of action (IL-10 and CCR6 induced by EPIT but not OIT, global suppression of T cell responses in OIT but not EPIT); it would be of interest to compare different modalities of therapy across a single food.

Our studies are limited by the use of the single activation marker CD154 to detect antigen-responsive T cells. Other markers such as CD137, OX40, and CD25 have been used to identify different subsets including regulatory T cells and Tfh cells^{33 34}. However it should be noted that food-responsive Tregs were not detected well using CD137 compared to aeroallergens or microbial antigens³³. Others have found that CD137 detection on Tregs in the context of peanut allergy does not discriminate between reactive and non-reactive individuals³⁵, however this has not been examined in the context of immunotherapy for foods. We did not find that CD154 expression on Tregs detected 18h after stimulation was a useful biomarker of any clinical feature. However, because we used a single snapshot of immune activation, we may have missed key regulatory subsets that could only be detected by a careful kinetic analysis and functional assays. Furthermore, the functional role of the CCR6+ T cell subset that negatively correlates with IgE is not understood and could potentially have a regulatory role. Finally, a limitation of our studies is the use of peripheral blood and our inability to detect cells that have homed to the gastrointestinal mucosa.

In conclusion, our studies find that food-specific Type 2 cells expressing IL-4 or IL-13 are associated with key clinical parameters, including successfully consumed dose, and importantly the frequency of these cells at baseline are associated with treatment failure in the context of egg oral immunotherapy. Our studies also demonstrate an intriguing role for CCR6+ T cells which negatively correlate with pro-allergic immune parameters (IgE, Type 2 immunity) and are expanded as a proportion of cells post-EPIT. The functional role of these cells, for example to determine if they are regulatory or are precursors of Type 2 effector cells, requires further study. In spite of the limitations of the observational nature of the data, this is the largest multi-center clinical study to quantify and phenotype food-specific T cell responses by multiparameter flow cytometry, providing single cell resolution, in the context of rigorous clinical phenotyping.

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Figure Legends

Figure 1: Correlation of peanut or egg-specific T cell subsets. Allergen-specific (CD154+ after 6h of allergen stimulation) CD4+ T cells were gated and phenotyped by cytokine and chemokine receptor expression. Correlation of CD154+IL-4+ cells with CD154+IL13+ cells (A,D), CD154+CCR4+ cells (B,E), or CCR6+CD154+ cells (C,F) after stimulation with peanut (A-C) or egg (D-F). A,B,D,E are expressed as frequency/CD3+CD4+ T cells, E,F expressed as percent of CD154+ cells. Analysis by Spearman Correlation Analysis, correlation and q value (FDR corrected) are indicated on the graphs.

Figure 2: Correlation of peanut or egg-specific T cell subsets with immune parameters Allergen-specific (CD154+ after 6h of allergen stimulation) CD4+ T cells were gated and phenotyped by cytokine and chemokine receptor expression. Correlation of IL-4+CD154+ (A,B,D,E) or CCR6+CD154+ (C,F) cells with specific IgE (A,B,C,F) or basophil activation test (B,E) in peanut allergic (A-C) or egg allergic (D-F) participants. Analysis by Spearman Correlation Analysis, correlation and q value (FDR corrected) are indicated on the graphs.

Figure 3: Relationship of T cell subsets to clinical parameters. Specific (CD154+ after 6h of allergen stimulation) CD4+ T cells were gated and phenotyped for cytokine expression. During a baseline (pre-treatment) DBPCFC, the last successfully consumed dose (SCD) before reacting was recorded. Relationship of peanut-specific IL-4+ CD154+ cells with SCD (A). Analysis by Spearman Correlation Analysis, correlation and q value (FDR corrected) are indicated on the graph. B: IL-4+CD154+ cells as frequency of egg-specific T cells in comparing baked egg tolerant (BET) or baked egg reactive (BER) individuals. Mann Whitney Test, * $p < 0.05$.

Figure 4: Frequency of CD4+ T cell subsets during peanut EPIT. Allergen-specific (CD154+ after 6h of allergen stimulation) CD4+ T cells were gated and co-stained for IL-4, IL-13, and IL-10 at baseline, 26, 52, or 130 weeks after treatment with EPIT daily (A). Shown on the right for comparison are individuals treated for up to 1 year with placebo (B). Mixed Model Test with Dunnett's multiple comparison test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 5: Frequency of CD4+ T cell subsets during egg oral immunotherapy or baked egg diet.

Specific (CD154+ after 6h of allergen stimulation) CD4+ T cells were gated and phenotyped by cytokine and chemokine receptor at baseline, 12, 26, 52, or 130 weeks after treatment with OIT (A) or baked egg diet (B). Mixed Model Test with Dunnett's multiple comparison test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 6: Relationship of Type 2 cells at baseline to egg OIT treatment outcome. A: Mean levels of immune parameters at baseline in those with sustained unresponsiveness (SU), desensitization (DS), or treatment failure (TF) after egg OIT. (Units are kU/L for IgE, % CD63+ for BAT, and cells/ 10^6 CD4+ T cells for cytokine-producing cells). This includes both baked egg reactive and tolerant individuals. Tukey's Multiple Comparison Test, * $p < 0.05$, ** $p < 0.01$. B: IL-4+CD154+ and IL-13+CD154+ cells in those with TF or treatment success (TS, SU+DS groups). Mann Whitney test, * $p < 0.05$. C: Treatment outcome in individuals in top and bottom tertile of IL-4+CD154+ cells.

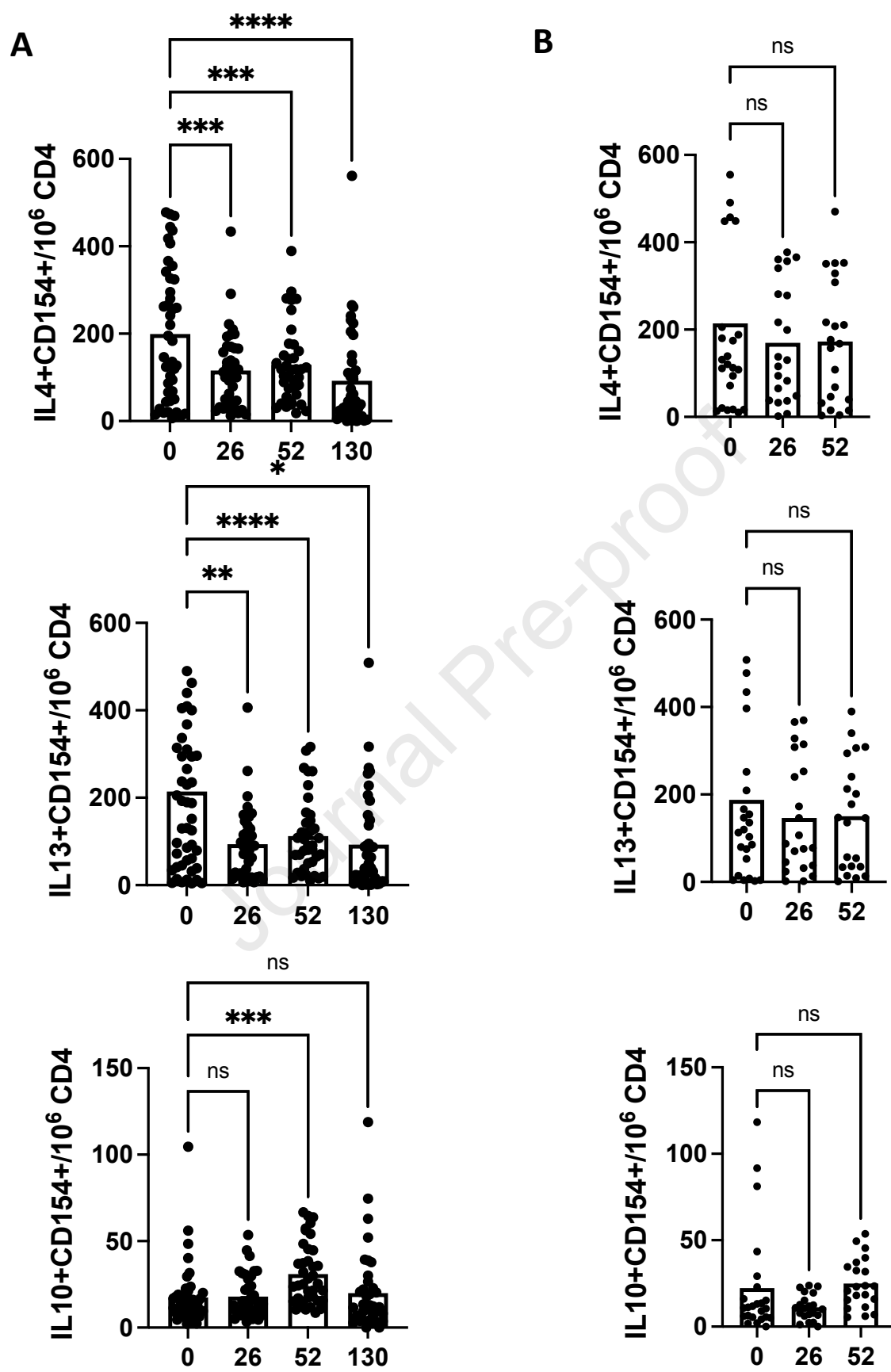


Figure 4

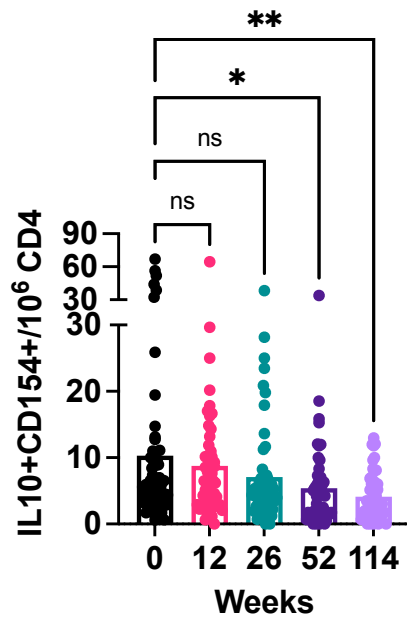
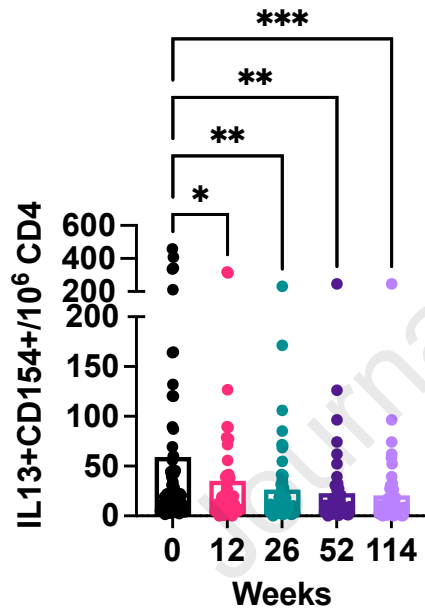
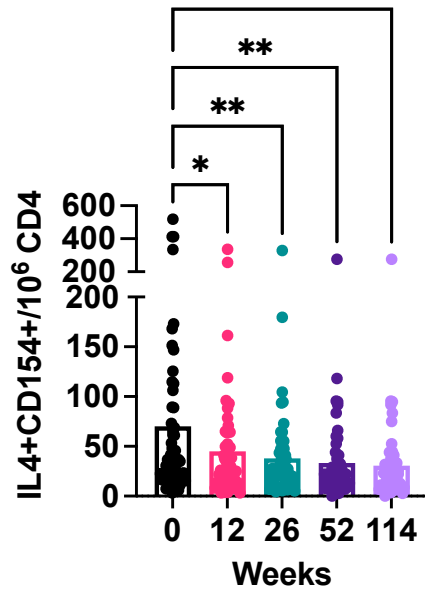
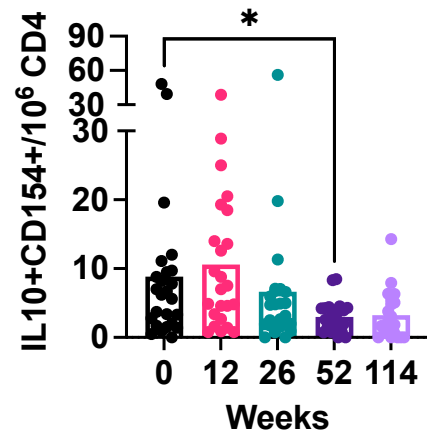
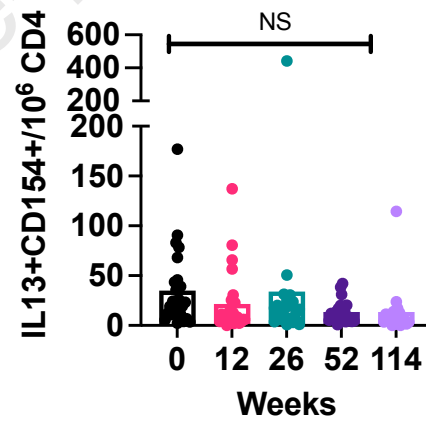
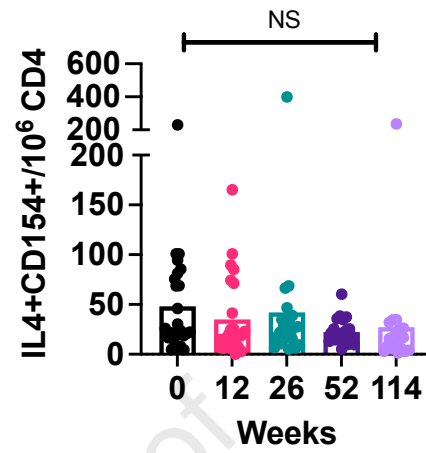
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Figure 5

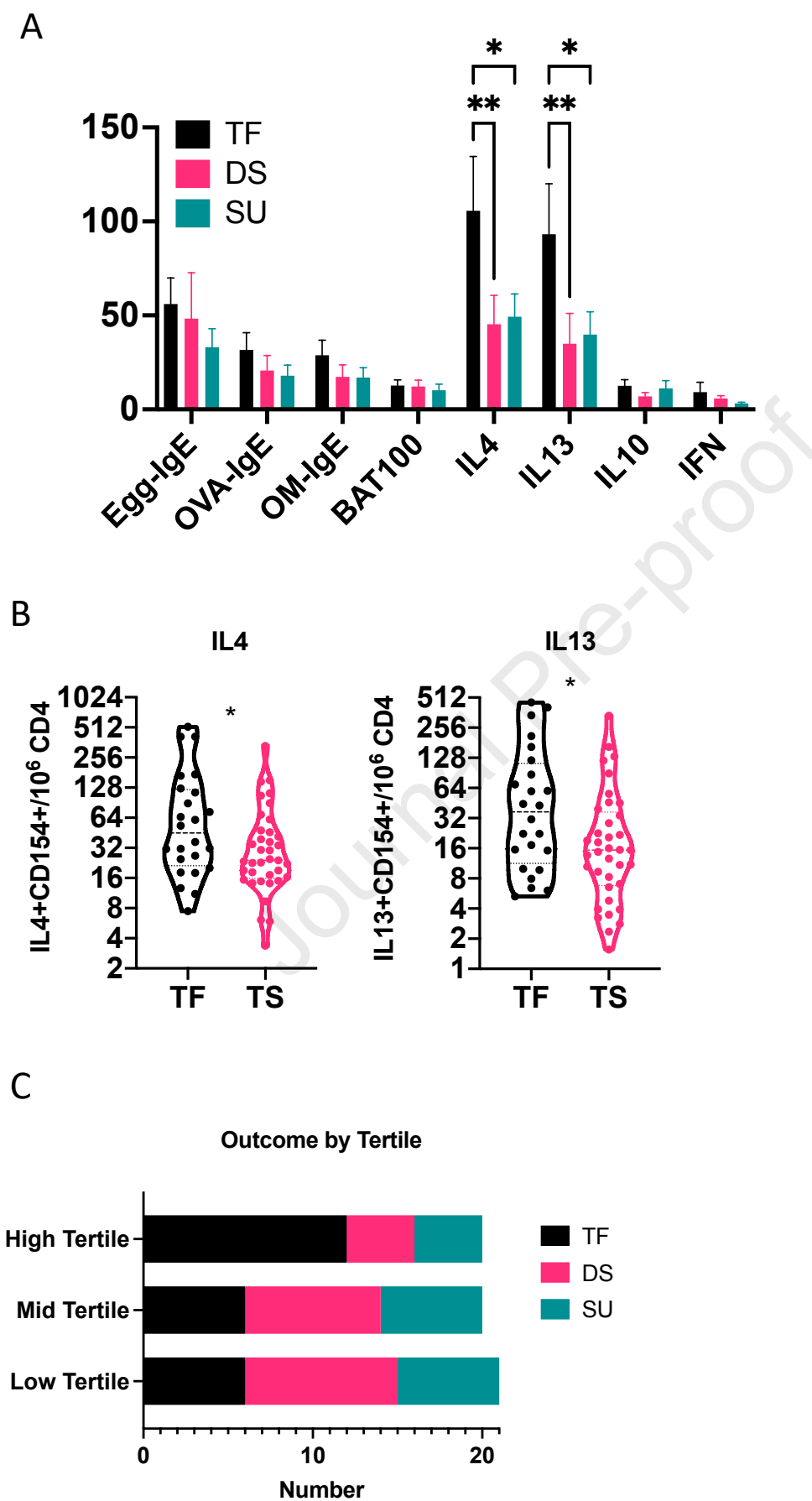


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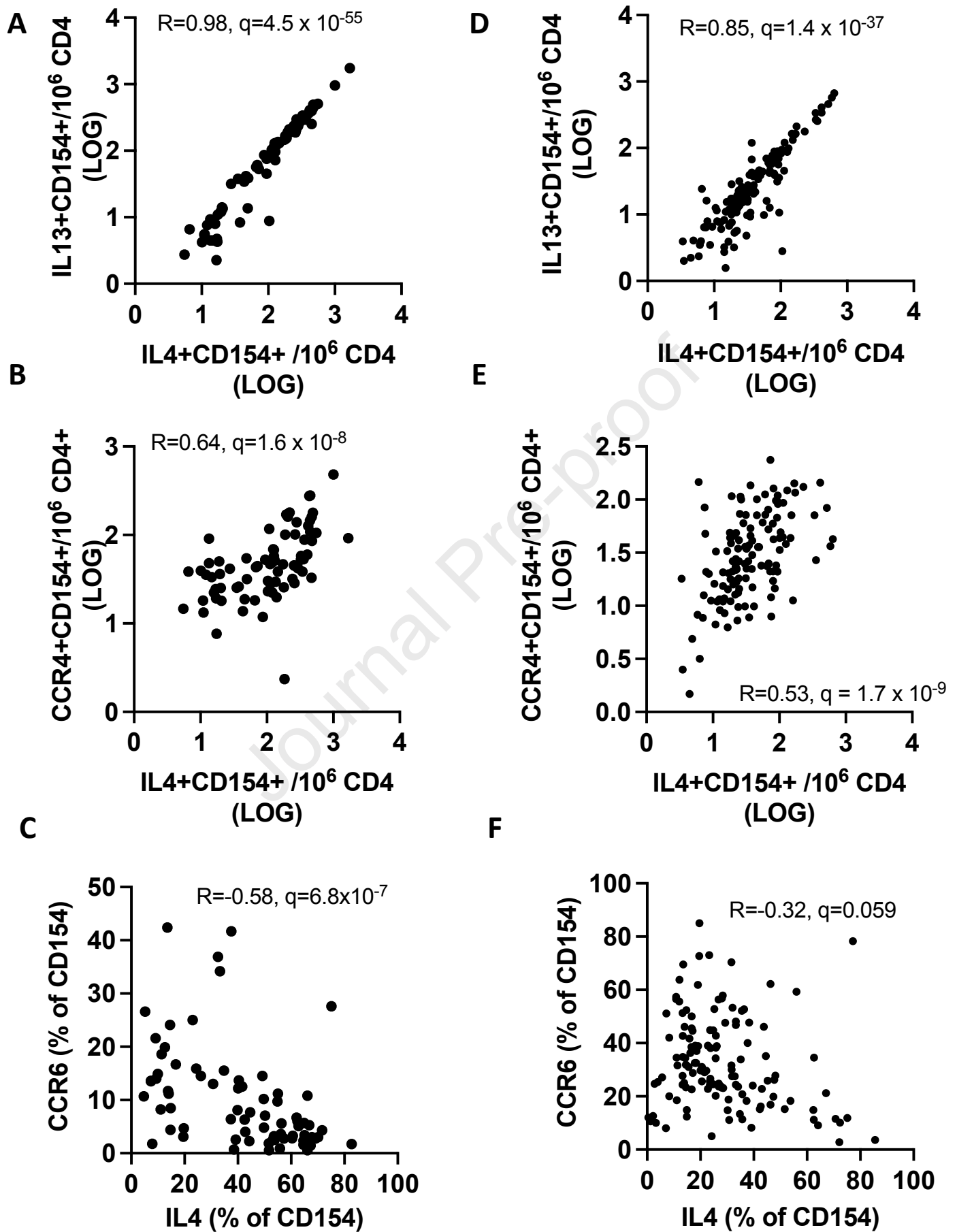


Figure 1

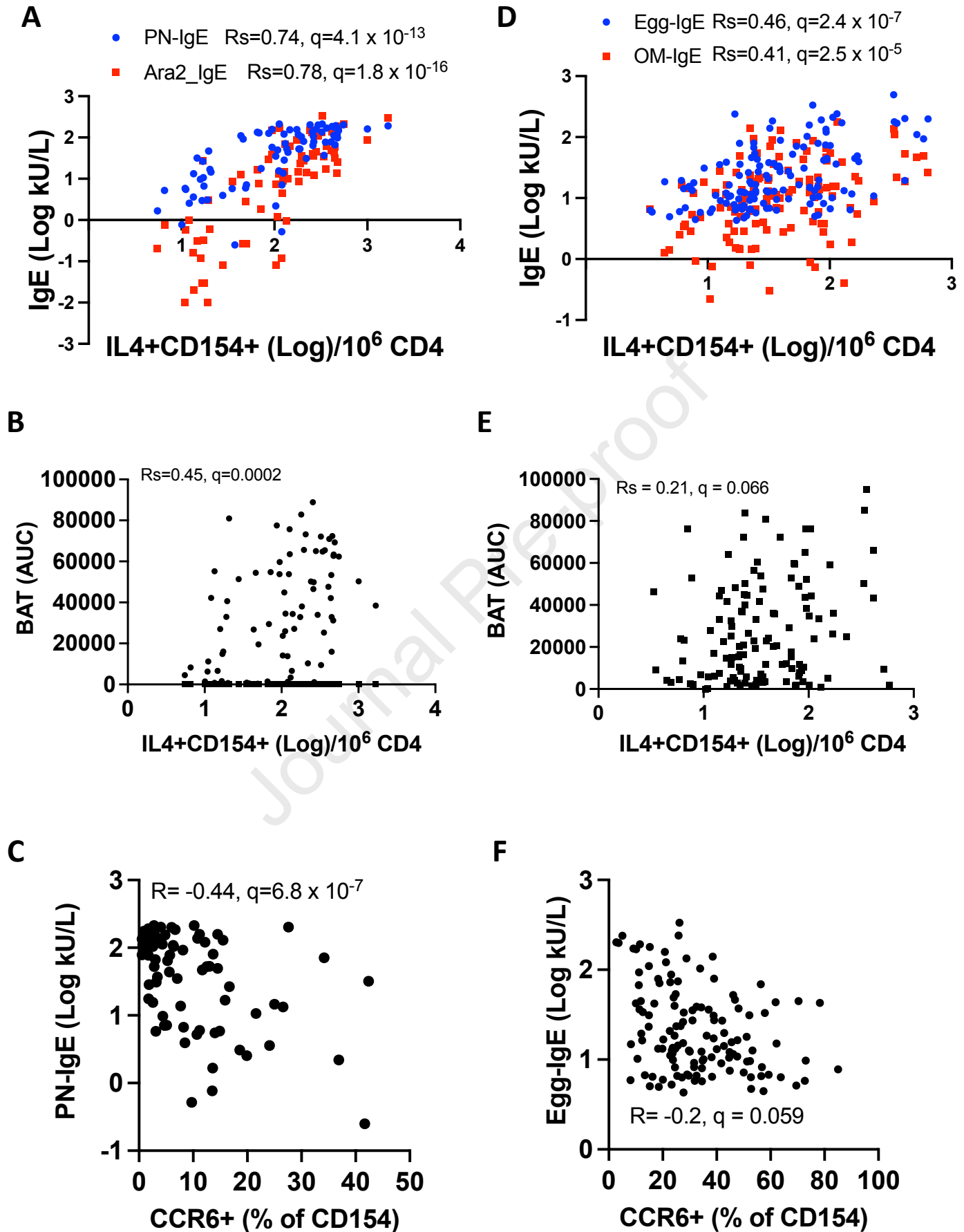
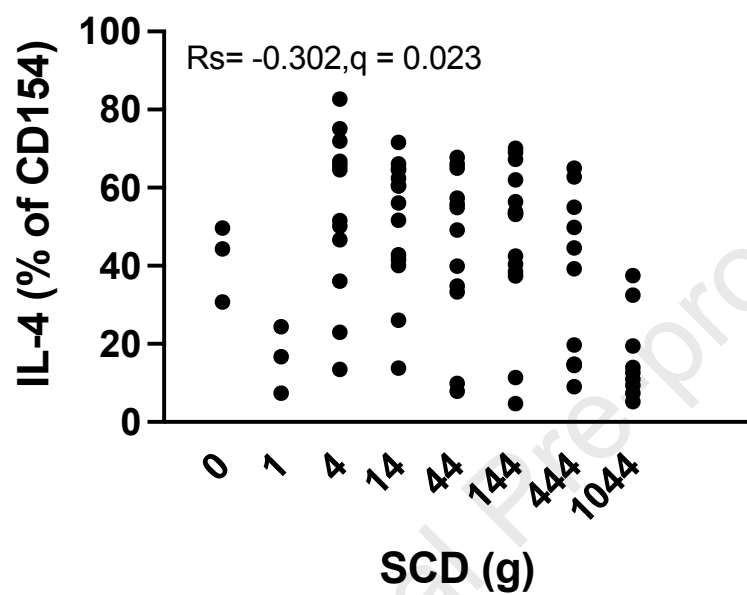


Figure 2

A



B

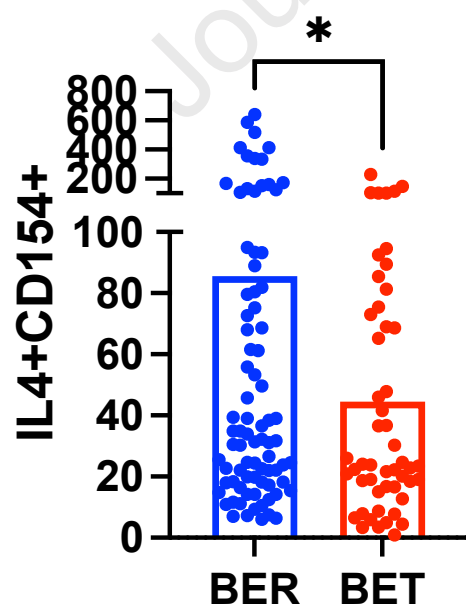


Figure 3