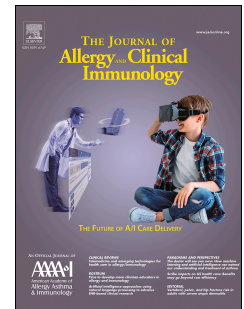


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Interrupting reactivation of immunological memory diverts the allergic response and prevents anaphylaxis

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TITLE:

Interrupting reactivation of immunological memory diverts the allergic response and prevents anaphylaxis

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30

ABSTRACT:

Background: IgE production against innocuous food antigens can result in anaphylaxis, a severe life-threatening consequence of allergic reactions. The maintenance of IgE immunity is primarily facilitated by IgG⁺ memory B cells (MBCs), as IgE⁺ MBCs and IgE⁺ plasma cells are extremely scarce and short-lived, respectively. **Objective:** To investigate the critical requirements for an IgE recall response in peanut (PN) allergy. **Methods:** We used a novel human peripheral blood mononuclear cell (PBMC) culture platform, a mouse model of PN allergy, and various experimental readouts to assess the IgE recall response in the presence and absence of IL-4R α blockade. **Results:** In human PBMCs, we demonstrate that blockade of IL-4/IL-13 signaling aborted IgE production upon activation of a recall response and skewed the cytokine response away from a dominant type 2 signature. Th2A cells, identified by single-cell RNA-sequencing, expanded with PN stimulation and maintained their pathogenic phenotype in spite of IL-4R α blockade. In allergic mice, anti-IL-4R α provided long-lasting suppression of the IgE recall response beyond antibody treatment and fully protected against anaphylaxis. **Conclusion:** The findings reported here advance our understanding of events mediating the regeneration of IgE in food allergy.

Clinical Implication: This work positions IL-4/IL-13 as a critical signaling pathway with disease-transformative potential.

CAPSULE SUMMARY:

In peanut-allergic human PBMCs and in peanut-allergic mice, we demonstrate that IL-4R α blockade inhibits IgE recall responses, prevents anaphylaxis, skews away from a Th2-dominant cytokine signature, and may facilitate long-lasting suppression of IgE production.

KEY WORDS:

IgE, food allergy, anaphylaxis, IL-4 receptor, memory response, Th2 immunity

ABBREVIATIONS:

Alum (aluminum hydroxide), CPE (crude peanut extract), IC (isotype control), MBC (memory B cell), NA (non-allergic), NS (no stimulus), OVA (ovalbumin), PBMC (peripheral blood mononuclear cell), PA (peanut-allergic), PN (peanut), s.c. (subcutaneous), scRNA-seq (single-cell RNA-sequencing), TFh (T follicular helper), TS (tape-strip), UMAP (uniform manifold approximation and projection).

MAIN TEXT:**Introduction:**

Allergens trigger the production of IgE, the key effector molecule in allergic reactions and, therefore, a critical therapeutic target. In contrast to Th1 immunity wherein infection or vaccination generates antigen-specific IgG titers that typically persist for decades¹, allergen-specific IgE levels drastically decline after extended periods without re-exposure. For example, in seasonal allergic rhinitis, serum allergen-specific IgE levels are cyclical, concordant to on- and off-season allergen peaks.^{2,3} A similar phenomenon has been observed in humans allergic to the common fish parasite *Anisakis* spp after strict avoidance of fish consumption for at least 10 months.⁴ This is challenging to observe in human food allergy where persistently increased peanut (PN)-specific IgE levels are likely the consequence of high rates of accidental exposures⁵ or even higher rates of subclinical exposures.⁶ In this regard, in a mouse model of PN allergy, PN-specific IgE titers became undetectable six months post-sensitization.⁷ Irrespective of declining or absent allergen-specific IgE titers, the capacity of allergic mice and humans to regenerate IgE following re-exposure can remain for a lifetime, presumably via reactivation of memory B cells (MBCs) and the subsequent re-emergence of IgE-secreting plasma cells (PCs).

Recent evidence in mice and humans has demonstrated that IgE⁺ MBCs are extremely rare.⁷⁻⁹ The predisposition of IgE⁺ B cells to terminally differentiate into IgE-secreting plasma cells (PCs), rather than quiescent MBCs, is thought to be a consequence of chronic membranous IgE signaling.^{10,11} Moreover, IgE⁺ PCs are shorter-lived than IgG counterparts, thus resulting in declining serum IgE titers over time. IgE-expression can be achieved through distinct pathways of class switch recombination, including direct class-switching from IgM and sequential class-

switch from IgM to one or more intermediate isotypes.¹² Analyses of human immunoglobulin heavy chain (IgH) repertoires identified IgG1⁺ B cells as the dominant intermediate of IgE⁺ B cells.¹³ *In vivo* experimentation corroborated a preferential switch through IgG and, therefore, positioned IgG⁺ MBCs as the chief reservoir of IgE memory responses.^{7,14–17} IgG⁺ MBCs class switching to IgE occurs under the influence of type 2 cytokines; however, the critical requirements that facilitate the regeneration of IgE⁺ PCs remain to be fully elucidated.

IL-4 and IL-13, hallmark type 2 cytokines, are involved in Th2 polarization, B cell proliferation, and IgE class switching. Both cytokines exert their biological activity through a shared heterodimeric receptor composed of IL-4R α and IL-13R α 1, with IL-4 having a second signaling receptor composed of IL-4R α and γ c.¹⁸ Whereas mouse models of Th2 immunity to helminth infection support a critical requirement of IL-4 signaling for primary and secondary IgE production^{19–22}, the function of IL-4/IL-13 signaling in class switching in human lymphocytes, as well as in the context of allergic (*i.e.* non-helminth) Th2 immunity, remains incompletely understood.

Here, we used a novel model system, comprehensive protein analysis, and single-cell transcriptomic analysis to interrogate the activation of Th2 cellular and humoral immune memory in humans and mice with food allergy. We first developed a novel *in vitro* human platform with peripheral blood mononuclear cells (PBMCs) which, for the first time, enabled joint T and B cell readouts. Our characterization of cytokine, cellular, and single-cell transcriptomic profiles in the human recall response to PN unveiled unique populations of Th2A cells, MAL⁺ CD4 T cells, and IGHE⁺ MBCs that were differentially regulated by IL-4 signaling.

We also show that these findings are clinically relevant *in vivo* because treatment of PN-allergic mice with anti-IL-4R α fully aborted the formation of PN-specific IgE and prevented anaphylaxis. These effects were sustained even beyond IL-4R α blockade, highlighting the potential to reprogram the pathogenic allergic response. Collectively, these data elucidate key events in human and mouse Th2 recall responses, which implicates anti-IL-4R α as a persuasive therapeutic approach for PN allergy.

Methods:

Study Population

A cohort of 12 PN-allergic and 5 non-allergic blood donors were recruited from McMaster University and McMaster Children's Hospital (Hamilton, ON). Allergy to PN was determined by PN-specific IgE ImmunoCap[®] performed at LRC Hamilton (McMaster Children's Hospital), by saline and histamine-controlled skin prick test, and clinical history of reactivity. PN-allergic individuals were considered for inclusion with PN-specific serum IgE levels >0.35 kU/L and skin prick test ≥ 3 mm greater than saline control. All allergy testing was performed within 6 months before blood was drawn for experimentation. Exclusion criteria for all recruited donors included: allergen immunotherapy, previous or current omalizumab (Xolair[®]), dupilumab (Dupixent[®]), or other systemic immunomodulatory treatments, or autoimmune/immunodeficiency diseases. Patient demographics and allergic indicators are summarized in Table S1. All donors were recruited with written consent and ethical approval from Hamilton Integrated Research Ethics Board (HiREB).

Mice

Female C57BL/6 mice (5-8 weeks old) were purchased from Charles River Laboratories. 4get mice and KN2 mice were a generous gift from Dr. Irah King (McGill University). Homozygous 4get (*Il4^{tm1Lky}*)²³ and KN2 (*Il4^{tm1(CD2)Mmrs}*) mice on C57BL/6 backgrounds were crossbred to generate 4get/KN2 mice, described previously.²⁴ All experimentation was in compliance with McMaster University Research Ethics Board.

Food Allergy Models

Mice were epicutaneously sensitized to- and challenged with either crude peanut extract (CPE, Greer) or ovalbumin (OVA, Sigma), as previously described.^{16,25,26} Briefly, 200 µg of CPE or OVA was applied on the shaved and tape-stripped (TS) backs of mice for 10 days. Two weeks after the final sensitization, 100 µg of CPE or OVA was injected subcutaneously (low back) 3 times in 1 week. For anti-IL-4Rα clearance experiments, 3 additional subcutaneous injections of CPE occurred six weeks after anti-IL-4Rα treatment. Two weeks after subcutaneous injections, mice were challenged through intraperitoneal injection of 5 mg CPE. Core body temperature and hematocrit were recorded, as previously described.²⁶ Clinical signs were graded on a scale of 0-5: 0 = No clinical signs, 1 = In-ear scratching, 2 = Decreased activity, 3 = Motionless, 4 = No response to whisker stimuli, 5 = Seizure, moribund, death.²⁷ Retro-orbital bleeds were performed to collect serum for analysis of PN-specific antibodies by ELISA.

For intraperitoneal sensitization, 250 µL Alhydrogel 2% (alum, InvivoGen) with 250 µg CPE was delivered intraperitoneally twice, one week apart. After two weeks of rest, mice were bled and challenged, as described above.

Antibody Production and Administration

A hybridoma secreting monoclonal rat IgG2a anti-mouse IL-4R α (M1), generated by Immunex Corporation (Amgen), was generously provided by Dr. Fred Finkelman (University of Cincinnati, Cincinnati, OH). Hybridomas were expanded in tissue culture flasks with serum-containing media and then transferred to a spinner flask in serum-free media for up to 10 days. Antibodies were purified from culture supernatant with Protein G Sepharose (GE Healthcare). One mg anti-IL-4R α or an isotype control (rat IgG2a anti-trinitrophenol; Bio X Cell) was injected intraperitoneally the day before subcutaneous re-exposure, or 6 weeks, 2 weeks, or 1 day before sensitization. IgG-mediated anaphylaxis was blocked with rat IgG2b anti-mouse CD16/32 (Bio X Cell), as previously published.²⁷ CD4 and NK1.1 depletions were carried out with rat anti-mouse CD4 (GK1.5; Bio X Cell) and rat anti-mouse NK1.1 (PK136; Bio X Cell) antibodies, respectively. Two intraperitoneal injections of 200 μ g anti-CD4 or anti-NK1.1 were administered before subcutaneous re-exposure and once during re-exposures.

Tissue Processing

Mice were euthanized under inhalational anesthetic by cervical dislocation and, immediately after, inguinal lymph nodes and/or spleens were collected. Lymph nodes were crushed between frosted slides into a single-cell suspension with HBSS. Spleens were crushed through a 40 μ m filter using a 3 mL syringe plunger with HBSS. Cell suspensions were passed through a 40 μ m filter. Red blood cells in splenocyte suspensions were removed with ACK lysis buffer.

Splenocyte Culture

Three days after systemic allergen exposure, spleens were collected and processed under sterile conditions for culture. Cells were resuspended in complete RPMI (10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 55 μ M 2-mercaptoethanol) and plated in triplicate at 8×10^6 cells/mL. Cells were left unstimulated or were stimulated with 250 μ g/mL CPE. Cell-free supernatants were collected on day 5 and stored at -80°C until use.

PBMC Isolation and Storage

Up to 80 mL of peripheral blood was collected into heparinized tubes (BD). PBMCs were isolated via Ficoll-Paque (GE Healthcare) density gradient centrifugation, filtered, and resuspended in either enriched RPMI medium for culture, or in 10% dimethyl sulfoxide (DMSO)-supplemented fetal bovine serum (FBS) to be frozen at -80°C overnight in appropriate freezing containers and subsequently in liquid nitrogen for long-term storage. PBMCs used in this study were frozen for 0-6 months before usage.

PBMC Culture

Before culture, PBMC viability was assessed using automated viable cell counting (ADAM MC, Digital Bio). All fresh PBMCs were >95% viability and freeze-thawed PBMCs were >90% viability. Cells were resuspended in enriched RPMI 1640 (Gibco) supplemented with 10% heat-inactivated human AB serum (Corning), 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 55 μ M 2-mercaptoethanol, 1% L-glutamine, and 1% penicillin-streptomycin. Cells were plated in at least duplicate at a density of 1.5×10^6 per mL in 24-well plates for up to 12 days. On days 4 and 8, 1 mL of cell-free supernatant was collected, stored at -80°C , and replaced with 1 mL enriched medium. As a positive control for IgE production, cells

were stimulated with 68.7 ng/mL (8000 IU) rhIL-4 (Sigma-Aldrich) and 5 µg/mL anti-CD40 (BioXCell) at the start of culture. As a negative control, cells were incubated in medium alone. Allergen-stimulated cells were cultured with 2.5 ng/mL CPE. Cells incubated with neutralizing antibodies against IL-4Rα and keyhole limpet antigen (KLH, IgG2a isotype control for IL-4Rα antibody) received 50 µg/mL of antibody in the presence or absence of CPE.

ELISA and ELISpots

PN-specific mouse IgG1, IgE, and total human IgE ELISAs were performed as previously described.^{7,28} For the detection of human PN-specific IgE antibodies, MaxiSorp plates (Thermo Scientific) were coated overnight at 4°C with 4 µg/mL CPE in carbonate-bicarbonate buffer. Wells were blocked with 5% skim milk powder in PBS for 2 hours followed by 3 washes with 0.05% Tween-20. Samples were plated and incubated overnight at 4°C. Supernatant samples were lyophilized (Modulyo Freeze Dryer, Thermo Scientific) overnight and were reconstituted (10x concentrated) in 5% skim milk. After washing, biotin-conjugated goat anti-human IgE, cross-adsorbed (polyclonal; Invitrogen) was added at 0.25 µg/mL. Following a 2-hour incubation, wells were washed and streptavidin-HRP (BD) was added for 1 hour. ELISAs were developed with 3,3',5,5'-Tetramethylbenzidine (Sigma) and optical densities were acquired with a MultiskanTMFC (Thermo Scientific). Rat IgG2a ELISA (Invitrogen) and total IgE ELISpots (Mabtech) were performed with commercially available kits, as per manufacturer's recommendations.

Cytokine Analysis

Cytokines in PBMC culture supernatant were assayed using MILLIPLEX Immunology Multiplex Assays (HCYTOMAG-60K, Millipore Sigma) and analyzed using MAGPIX XMAP Technology (Luminex). Supernatants from day 4 of culture were assayed for either IL-4, IL-5 and IL-13 or a discovery panel including: sCD40L, EGF, Eotaxin/CCL11, FGF-2, Fit-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN- α 2, IFN γ , IL-1 α , IL-1 β , IL-1R α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL17A, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1 α , MIP-1 β , TGF- α , TNF- α , TNF- β , VEGF. Cytokines detected above background and falling within the detectable range (3.2-10000 pg/mL) were included in statistical analyses.

Cytokines in mouse splenocyte culture supernatant were assayed using MILLIPLEX Immunology Multiplex Assays (MCYTOMAG-70K, Millipore Sigma) and analyzed using MAGPIX XMAP Technology (Luminex). Supernatants from day 5 of culture were assayed for IL-4, IL-13, and IFN γ . Cytokines detected above background and falling within the detectable range (3.2-10000 pg/mL) were included in statistical analyses.

Tetramer Staining and Enrichment

PE-conjugated peptide:MHCII tetramers were prepared as previously described²⁹, and were generously provided to us by Dr. Marc Jenkins (University of Minnesota). Inguinal lymph node single-cell suspensions were pooled from 4-5 mice and incubated (1 hour, room temperature) in 100 μ L FACS buffer (0.5% BSA and 2% 0.5mM EDTA in PBS) with 1 μ L each of two peptide:MHCII tetramers containing the immunodominant OVA epitopes: OVA2C (OVA₃₂₅₋₃₃₅) and OVA3C (OVA₃₂₇₋₃₃₈).³⁰ Cells were washed with 10 mL FACS buffer to remove unbound

tetramer. Pellets were resuspended and incubated (30 minutes, on ice) with 50 μ L anti-PE microbeads (Miltenyi Biotec). Five mL FACS buffer was added to cells and the cell suspension was run through an LS Column (Miltenyi Biotec) on a magnetic stand. Tetramer-unbound cells (flow-through fraction) were collected. Columns were removed from the magnetic stand and 5 mL FACS buffer was plunged through the column to collect tetramer-bound cells (enriched fraction). Flow-through and enriched fractions were then counted, pelleted, and stained with fluorochrome-conjugated antibodies for flow cytometry.

Flow Cytometry and Bulk Sorting

Cells (cultured human PBMCs or mouse inguinal lymph nodes) were plated in 96-well plates ($<5 \times 10^6$ cells/well) in FACS buffer prior to extracellular staining. Cells were incubated with an antibody cocktail and Fixable Viability Dye eFluor780 (eBioscience) for 30 minutes on ice. Fluorochrome-conjugated antibodies used in human flow cytometric analyses include: CD3-FITC (HIT3a), CD4-BV510 (OKT4), CD19-BV421 (SJ25C1), and CD45-AF700 (HI30). For apoptosis analysis, cells were stained with Annexin V-PE in Annexin V binding buffer (BioLegend). For mouse flow cytometric analyses, the following fluorochrome-conjugated antibodies were used: B220-BV711 (RA3-6B2), F4/80-BV711 (BM8), CD3-BV510 (17A2), CD8a-AF700 (53-6.7), CD4-PerCP-Cy5.5 (RM4-4), CD62L-BV605 (MEL-14), CD44-BV650 (IM7), CXCR5-BV421 (L138D7), PD-1-PE/Cy7 (RMP1-30), human CD2-APC (RPA-2.10, BD). All antibodies were purchased from BioLegend, unless otherwise specified. Data were acquired with an LSRFortessa (BD). Bulk cell sorting was performed on a MoFlo XDP Cell Sorter (Beckman Coulter) prior to single-cell sorting. All data were analyzed with FlowJo v.10 software (TreeStar).

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273 *Single-Cell RNA-Sequencing*

274 Single Cell 3' RNA Sequencing libraries were prepared using Chromium Single Cell V3
275 Reagent Kit and Controller (10X Genomics). Libraries were sequenced on HiSeq 4000
276 instruments (Illumina). The sequenced data were processed using Cell Ranger pipeline (v 3.0.1)
277 by 10X Genomics (<https://www.10xgenomics.com>). Sequencing reads were aligned to the
278 human transcriptome (GRCh38) followed by filtering and correction of cell barcodes and Unique
279 Molecular Identifiers (UMIs). Reads associated with retained barcodes were quantified and used
280 to build expression matrices.

281

282 The scRNA-seq R package Seurat (v3.1.1) was used for pre-processing, quality control and
283 downstream analyses.³¹ Genes not detected in at least 3 cells were filtered out. Low quality cells
284 expressing very few genes, and potential doublets or multiplets expressing aberrantly high
285 number of genes (>5000) were excluded. Dying or stressed cells with a high percentage of
286 transcripts (>20%) mapping to mitochondrial genes were removed. Samples from the four
287 libraries were merged for subsequent clustering and visualization. Data were normalized using
288 the log normalization method with a scaling factor of 10,000. Highly variable genes were
289 selected using the mean-variance method and data were scaled to regress out the effect of nUMIs
290 and percent mitochondrial genes. Dimensionality reduction was performed using principal
291 component analysis (PCA) and the most significant clusters were chosen for subsequent
292 clustering. Graph-based clustering was implemented by calculating k-nearest neighbors,
293 followed by modularity optimization to clusters cells (FindClusters function). Non-linear
294 dimensionality reduction and visualization was performed using UMAP (Uniform Manifold

Approximation and Projection). Clusters were annotated on the basis of canonical markers and differential gene expression testing was used to determine a gene set signature for each cluster using the Wilcoxon Rank Sum test.

Quantitative RT-PCRs

RNA was isolated from cultured PBMCs with RNeasy Mini Kit (QIAGEN) and was quantified with NanoVue Plus (GE Healthcare). cDNA was synthesized with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific), as per manufacturer's recommendations. Immunoglobulin epsilon (ϵ) heavy chain transcripts were amplified (StepOnePlus Real-Time PCR System, Applied Biosystems) using custom TaqMan gene expression kits (Applied Biosystems) with previously developed primer pairs and probes.³² Data was analyzed by $\Delta\Delta CT$, to represent data as fold change relative to 18S housekeeping gene (Applied Biosystems) and unstimulated (media only) control.

Statistical Analysis

Data were analyzed with Prism 8 (GraphPad Software). Comparisons were drawn using one- or two-way ANOVA, Student's *t*-test, or Pearson *r*. Benjamini-Hochberg (BH) correction for multiple testing was used in analysis of cytokine discovery panel. Nonlinear regression was interpolated for IgG1 dilution plots and EC_{50} values were calculated when possible. Data were considered significant when $p < 0.05$.

Results:

Induction of an IgE recall response in human PBMCs

Th2 responses to allergens follow a classical adaptive response, involving expansion of effector cell populations, a subsequent contraction phase, and memory persistence. However, a comprehensive understanding of the cellular and molecular interplay for recall responses remains to be fully elucidated. To this end, we developed an *in vitro* platform to interrogate allergen-dependent T and B cell responses concomitantly (Fig. 1A). Human PBMC cultures are widely used to assess cytokine production following allergen-stimulation, however, we found that these platforms did not support the generation of IgE responses in the absence of a polyclonal stimulus (*e.g.* IL-4 + anti-CD40).³³⁻³⁶ To overcome this limitation, we identified that the optimal dose of allergen that facilitates an IgE⁺ B cell responses is 5-log less than what has traditionally been used for the assessment of T cell responses.³⁴⁻³⁶ Thus, we proceeded under these modified culture conditions to validate the model with non-allergic (*n*=5) and PN-allergic (*n*=12) donors (Table S1).

To evaluate T cell responses, we performed a targeted proteomic analysis that included 38 analytes from 7 PN-allergic donors with the highest serum PN-specific IgE levels after either control (non-stimulated; NS) or PN exposure. Twenty-four analytes were detected above background, fell within the detectable range (3.2-10000 pg/ml; Fig. S1), and were included in subsequent analyses. Of these, IL-5, IL-13, IL-9, and IL-17A exhibited the highest upregulation in response to PN (Fig. 1B). Significant increases were also observed for GM-CSF, IL-2, IFN γ , TNF- α , IP-10, and IL-10 (Fig. 1B). These data demonstrate that the recall response to PN in this human platform involves a heterogeneous cytokine response with potent production of Th2 cytokines.

We next assessed the IgE memory response in culture. Treatment with recombinant IL-4 and anti-CD40, which induces IgE in human PBMCs³⁷, resulted in IgE responses detectable at the cellular, molecular, and transcriptional level, irrespective of allergic-status (Fig. S2A-C). PN stimulation increased IgE-secreting cells, PN-specific IgE, and *IGHE* transcripts as compared to NS cells only in allergic donors, with no change in non-allergic donors (Fig. 1C-E). The intensity of IgE readouts displayed a moderate (IgE-secreting cells; $r^2=0.553$) to strong (PN-specific IgE; $r^2=0.785$) correlation with serum PN-specific IgE levels used to ascertain allergic status (Fig. S2D and E). Production of *IGHE* transcripts did not correlate with serum PN-IgE ($r^2=0.275$, Fig. S2F).

To investigate the identity of B and T cells involved in PN recall responses at greater depth, we performed single-cell RNA-sequencing (scRNA-seq) on PBMCs isolated from non-stimulated (NS; 3272 cells) and PN-stimulated (3787 cells) culture at day 8 from a representative PN-allergic donor (P027; Fig. S3A). After quality control, removal of low-quality cells and doublets, Uniform Manifold Approximation and Projection (UMAP) was used to reduce the number of dimensions and visualize cell clustering. Fifteen discrete clusters were resolved, assigning nomenclature derived from recognized gene signatures (Fig. S3 B and C), including populations of CD4 T cells, B cells, NK cells, monocytes, and basophils (Fig. 1F and G). To assess heterogeneity between T cells populations, cells with high expression of *GATA3*, *FOXP3*, *IL7R*, and *MAL* were subclustered (Fig. 1H, Fig. S4A). These genes were selected to better resolve populations of Th2, Treg, and memory T cells, respectively, with the additional inclusion of *MAL*-expressing T cells due to changes in frequency observed in total cell clusters (C0 in Fig. 1G). A 2.3-fold increase in the proportion of *MAL*-expressing CD4 T cells (C0) was observed

with PN-stimulation (21.8% NS to 50.9% PN; Fig. 1I). In this cluster, 43/94 differentially expressed genes were ribosomal protein coding genes, suggesting a high degree of protein synthesis (Table S2). A pathway analysis (G:Profiler) of non-ribosomal genes displayed an enrichment in genes relating to lymphocyte differentiation, T cell activation, and response to IL-4 stimulus (Fig. S4B). Notably, *IL4R* was almost exclusively detected in the *MAL*^{hi} cluster, with *MAL* having been suggested to be induced by IL-4-signaling (Fig. S4C).³⁸ Due to the high frequency of these cells at baseline (NS), they are unlikely to be PN-specific, but rather expand as a result of bystander proliferation.³⁹ In contrast, cluster 2 (C2) was uniquely identified in PN-stimulated PBMCs (0.8% NS to 10.6% PN; Fig. 1I). Differentially expressed genes resembled a canonical activated Th2 phenotype, including *GATA3*, *IL17RB*, *KLRB1*, *IL2RA*, and *CTLA4* (Fig. 1J, Table S3). Additionally, C2 expressed genes encoding prostaglandin D synthase (*HPGDS*) and the prostaglandin D2 receptor, *CRTH2* (*PTGDR2*), which is consistent with a distinct population of Th2 cells specific to allergic disease, previously coined as “Th2A” cells.⁴⁰ C3 cells (Fig. 1H) expressed both regulatory- and Th2-related genes, including *FOXP3*, *CTLA4*, *IL2RA*, *LGALS3*, and *GATA3*, and thus is congruous with a Th2-like Treg phenotype (Fig. 1J, Table S3).⁴¹

Subclustering MBCs, which were identified by expression of the memory markers *CD27* and *AIM2* and low expression of *IGHM*, *IGHD*, and *TCL1A*⁴² (Fig. S5A and B), identified 3 distinct clusters (Fig. 1K). C0 and C1 contained heterogeneous IgH expression but did not express *IGHG4* and *IGHE* (highly expressed in C2 cells) (Fig. S5C). *IGHE*-expressing cells (C2) expanded with PN stimulation (Fig. 1L). As IgE⁺ MBCs are extremely rare in circulation, as measured by flow cytometry, and acquisition of tissue samples that may contain IgE⁺ MBCs is

challenging⁸, our detection of these cells at the single-cell level provided a valuable opportunity to elucidate their transcriptional profile. *IGHE*⁺ cells were transcriptionally unique (Fig. 1M and Table S3), expressing genes associated with co-stimulation (*e.g.* *CD40* and *CD83*) and IL-4-signaling^{43,44} (*e.g.* *IL4I1* and *GCSAM*), and high expression of *IL4R* (Fig. S5D). In sum, we have developed an *in vitro* platform that yields robust Th2 humoral responses with PN stimulation, positioning this as a novel system by which to study the human allergic recall response.

Application of scRNA-seq to *in vitro* stimulated PN-allergic PBMCs revealed the transcriptomic profile of PN-reactive B and T cell subsets. Cell populations that expanded with PN stimulation exhibited an upregulation of genes related to IL-4 signaling.

Of note, the scRNA-seq analysis also identified a population of *bona fide* basophils, a known contaminant to the PBMC fraction in density gradient-based isolation strategies. Although basophils encompass just a small percentage (<0.5%) of circulating leukocytes, basophil-derived IL-4 may contribute to the recall response *in vitro*. Differences in basophil frequency between donors may, therefore, provide an alternative explanation as to the intensity of IgE readouts. Too few basophils were detected by scRNA-seq (NS = 5, PN = 7) to make meaningful conclusions regarding activation status.

IL-4/IL-13 signaling blockade aborts IgE production and skews the type 2 cytokine response in human PBMCs.

Recent work using flow cytometry and single-cell sequencing methods have informed about the frequency and phenotype of human IgE-expressing cells in circulation. In food-allergic individuals, IgE-expressing cells are almost exclusively of a plasmablast phenotype⁹, with rare or

absent detection of IgE⁺ MBCs.^{8,9} This suggests that, similar to observations made in mice, IgG⁺ MBCs are the reservoir for IgE recall responses in humans and that IL-4/IL-13 signaling may be required to coordinate IgE class-switching. Moreover, the unique transcriptional signature we described in *IGHE*-expressing MBCs further suggests that the cells are responsive to IL-4. To assess the requirement of IL-4/IL-13 signaling in human IgE recall responses, we employed the above-mentioned *in vitro* platform with the addition of an IL-4R α blocking antibody or an isotype control (IC), in 7 PN-allergic donors with serum PN-IgE > 40 kU/L.

Remarkably, anti-IL-4R α resulted in a near complete inhibition of IgE recall responses, including IgE-secreting cells, PN-specific IgE, and *IGHE* transcripts (Fig. 2A-C). Suppression of IgE responses did not appear to result from apoptosis through IL-4 deprivation (Fig. S6). We applied the 38-plex cytokine array to investigate the implications of IL-4R α blockade on cytokine production (Fig. S7A). IL-5, IL-9, MDC, and IL-17A were significantly downregulated with anti-IL-4R α ; conversely, IL-10, IL-1 α , IL-1 β , MIP-1 α , G-CSF, and IFN γ secretion was upregulated (Fig. 2D). Th2:Th1 and Th2:IL-10 ratios were significantly reduced with anti-IL-4R α (Fig. 2E). Of note, IL-4 was excluded from these analyses as IL-4R α blockade results in secreted IL-4 accumulation.⁴⁵ Without anti-IL-4R α addition, no appreciable differences were detected in IL-4 secretion between NS and PN-stimulated PBMCs (Fig. S7B); however, IL-4R α blockade resulted in a 2.3-fold change increase over IC-treated PN-stimulated PBMCs (IC: 80 pg/ml, anti-IL-4R α : 184 pg/ml; Fig. S7B). This indicates that IL-4 is produced in response to PN stimulation, however rapid consumption of the cytokine likely impairs its detection.

We used scRNA-seq to compare the profiles of over 6000 human PN-stimulated PBMCs with or without anti-IL-4R α treatment (3427 PN + isotype control (IC) and 2718 PN + anti-IL-4R α). The PN-induced expansion of the *MAL*^{hi} population was completely inhibited by IL-4R α blockade (Fig. 2F and G). This was consistent with their high expression of *IL4R* and suggests that IL-4/IL-13 signaling is critical for their expansion. In contrast, IL-4/IL-13 signaling was dispensable for maintaining terminally differentiated Th2A cells (C2). With regards to MBCs, the expansion of *IGHE*⁺ cells was impaired by IL-4R α blockade, which coincides with upregulation of IL-4-signaling genes in this cluster (Fig. S8). These data establish the critical requirement of IL-4/IL-13 signaling in human IgE recall responses to PN.

CD4 T cells drive the recall response in vivo.

Given the critical requirement of IL-4/IL-13 signaling in the *in vitro* recall response, we used a mouse model of food allergy to validate and expand upon these findings *in vivo*. Here, we employed a previously described model of epicutaneous sensitization.¹⁶ This approach enables temporal control over the IgE response where sensitization initially produces allergen specific-IgG1-mediated anaphylaxis, which can be blocked with anti-CD16/32 treatment. Following contraction of the primary immune response, subclinical, non-sensitizing subcutaneous (s.c.) re-exposures activate a memory response and the conversion of IgG1 responses to IgE.¹⁶

First, we assessed the cellular sources of IL-4 in a recall response to PN in 4get/KN2 mice. In these mice, GFP and human CD2 (hCD2) expression report *Il4* gene transcription (GFP⁺ hCD2⁻) and IL-4 secretion (GFP⁺ hCD2⁺), respectively.³⁹ While the source of IL-4 upon Th2 sensitization has been extensively studied, the source of IL-4 in the recall response to food allergens remains unknown. In the inguinal lymph nodes, GFP⁺ hCD2⁺ and GFP⁺ hCD2⁻ cells peaked at 3 and 7 days post-re-exposure, respectively, and contracted by day 14 (Fig. 3A and B). Up to, and inclusive of, 7 days post-re-exposure, CD4 T cells accounted for over 92% of the IL-4-secreting and transcribing populations (Fig. 3A and B, shaded regions). Expectedly, almost all GFP⁺ hCD2⁺ CD4 T cells expressed the activation marker, CD44 (>95%, Fig. 3C) and 25% were T follicular helper (TFh) cells (Fig. 3D). Thus, in the draining lymph nodes CD4 T cells provide the overwhelming majority of IL-4, with contributions from both Th2 cells and TFh cells.

The requirement of CD4 T cells in IgE recall responses has been evaluated in the context of helminth infections and/or utilizing highly selective adoptive transfer models, but not in intact

food allergy models. Thus, we evaluated the contribution of CD4 T cells in the recall response to PN using an anti-CD4 antibody to deplete CD4 T cells prior to s.c. re-exposures. As CD4-targeted depletions also eliminate CD4-expressing NKT cells, a separate group that received anti-NK1.1 was included to discriminate the contribution of conventional CD4 T cells and CD4-expressing NKT cells. After induction of the recall response, PN-IgG1 production was similar between untreated and anti-NK1.1-treated mice, though depletion of CD4 T cells significantly impaired this response (Fig. 3E). Similarly, depletion of CD4 T cells, but not NK1.1⁺ cells, prevented the emergence of PN-IgE (Fig. 3F). Accordingly, anti-CD4-treated mice were protected from IgE-mediated anaphylaxis (Fig. 3G-I). Thus, in the absence of conventional CD4 T cells, other cells (*e.g.* NKT cells, basophils, etc.) are insufficient at mounting an IgE recall response. Taken together, these data highlight a critical role of CD4 T cells for the IgE recall response *in vivo* and positions these cells as key therapeutic targets.

Th2 polarization is maintained in allergen-specific CD4 T cells

Next, to elucidate the impact of anti-IL-4 α on T cell phenotype *in vivo* during recall and validate findings revealed by scRNA-seq *in vitro*, we employed the same epicutaneous sensitization model in 4get/KN2 mice. For these experiments, mice were sensitized to ovalbumin (OVA), an allergenic protein in egg. Epicutaneous sensitization to OVA yields the same allergic phenotype as PN sensitization¹⁵ and enables the use of peptide:MHC II tetramers for detection of OVA-specific CD4 T cells. Draining (inguinal) lymph nodes were collected 3 days after the final s.c. injection, at the peak of IL-4 production from CD4 T cells. Cells were stained with two peptide:MHC II PE-conjugated tetramers loaded with the immunodominant OVA peptides:

OVA2C (OVA₃₂₅₋₃₃₅) and OVA3C (OVA₃₂₇₋₃₃₈).⁴⁰ Tetramer-positive (OVA⁺) cells were enriched with anti-PE magnetic microbeads, then stained with antibodies for flow cytometry analysis.

The impact of anti-IL-4R α on IL-4 transcript and protein expression was assessed in B220⁺ F4/80⁺CD3⁺CD8⁺CD4⁺ cells. An average of 61 and 33 OVA⁺ CD4 T cells were detected in IC- and anti-IL-4R α -treated mice, respectively, which was not significantly different ($p = 0.2998$; Fig. 4A). As TFh cells produce IL-4 independent of the type of immune response (*e.g.* Th1, Th2, etc.)⁴⁶, we separately analyzed TFh cells (CD44^{hi}CD62L⁺PD-1⁺CXCR5⁺) and activated non-TFh CD4 T cells (CD44^{hi}CD62L⁺CXCR5⁺) (Fig. 4B); previous literature has described the GFP⁺ non-TFh cells to be the Th2 cell population.^{39,41} In both the OVA⁺ TFh and OVA⁺ Th2 populations, *IL4* transcription and IL-4 secretion were unchanged by IL-4R α blockade (Fig. 4C-F). This is in agreement with the observation *in vitro* where human Th2A cells maintained their phenotype in the presence of anti-IL-4R α blockade. Overall, these data demonstrate that, although anti-IL-4R α abrogates IgE production and anaphylaxis in a recall response, Th2-polarized allergen-specific cells maintain their pathogenic phenotype in the absence of key polarizing signals via IL-4 and IL-13.

IL-4R α blockade inhibits IgE recall response and anaphylaxis in epicutaneously-sensitized mice.

Last, we evaluated the functional impact of IL-4R α blockade *in vivo* by challenging mice after anti-IL-4R α (or isotype control) treatment and s.c. re-exposures. All groups of mice that underwent epicutaneous sensitization presented with similar levels of PN-specific IgG1 and background levels of PN-specific IgE (Fig. 5A). Following s.c. PN re-exposures, PN-IgG1 levels remained similar and an IgE response was generated, which was fully abrogated by anti-IL-4R α

treatment (Fig. 5B). To assess the functional impact of IL-4R α blockade, mice were challenged intraperitoneally with PN. Mice receiving anti-IL-4R α exhibited clinical reactivity upon challenge, though to a lesser extent than untreated mice (Fig. 5C-E). We reasoned that this half-way response could be a consequence of IgG-mediated alternative anaphylactic pathways, as anti-IL-4R α -treated mice develop normal IgG1 responses. To this end, groups of mice were treated with anti-CD16/32 before challenge to block IgG binding to Fc γ receptors.²⁷ Mice co-treated with anti-IL-4R α and anti-CD16/32 displayed no clinical reactivity upon challenge, as evidenced by a maintenance of homeostatic core body temperature and hemoconcentration (Fig. 5C-E). To confirm that anaphylaxis was inhibited due to anti-IL-4R α -mediated events occurring prior to challenge (*e.g.* preventing IgE production) and was not due to the blockade of IL-4R α during challenge, we assessed the impact of anti-IL-4R α administered the day before challenge. Although IL-4 has been shown to exacerbate anaphylaxis⁴⁷, we found that blockade of IL-4R α before challenge did not impact clinical reactivity (Fig. S9). Thus, IL-4R α blockade specifically impacts the recall response to allergens and, as a consequence, prevents anaphylaxis.

To establish if IL-4R α blockade exhibited long-lasting impact on the allergic response, we conducted experiments involving tertiary re-exposures following anti-IL-4R α clearance. Clearance of anti-IL-4R α was ascertained by two independent approaches. First, IL-4 is known to be critically required for allergic sensitization.^{28,48} To this end, naïve mice were treated with anti-IL-4R α 1 day, 2 weeks, or 6 weeks prior to sensitization. We reasoned that any residual antibody would, expectedly, prevent or stunt allergic sensitization. By 6 weeks post-anti-IL-4R α treatment, clinical reactivity of anti-IL-4R α mice was not different than that of untreated mice (Fig. S10A and B). Secondly, we measured rat IgG2a from the serum of antibody-treated mice

and found that it was undetectable after 6 weeks (Fig. S10C). Thus, to establish the lasting impact of IL-4R α blockade, allergic mice were treated with anti-IL-4R α and received s.c. re-exposures (as per experiments in Fig. 5A-E), followed by a 6-week wait for antibody clearance and an additional round of s.c. re-exposures (tertiary exposure). A sustained and complete inhibition of IgE production in mice previously receiving anti-IL-4R α was observed (Fig. 5F). Moreover, at this time point, anti-IL-4R α -treated mice exhibited a significantly dampened production of IL-4, but not IL-13, in *in vitro* stimulated splenocytes (Fig. 5G). The reduction in IL-4 was paired with a significant increase in IFN γ production. Thus, anti-IL-4R α appears to transform IL-4/IL-13 signaling where, over time, IL-4 production is lessened in conjunction with an increase in the Th1 cytokine, IFN γ .

Discussion:

Food allergies, such as those to PN, routinely onset before the age of two and persist for a lifetime in most patients. The current standard of care is strict allergen avoidance; however, accidental exposures estimated to occur in 14% of PN-allergic people each year pose a continual risk of life-threatening reactions.⁵ The lifelong nature of some food allergies is thought to result from repeated activation, through clinical or sub-clinical allergen exposures, of lifelong allergen-specific memory cells which regenerate transient IgE-secreting cells. It is thus necessary to elucidate critical requirements for memory cells to generate IgE recall responses against allergens. This has remained elusive in humans in part due to a lack of platforms that facilitate the *in vitro* regeneration of IgE and IgE-secreting cells. To this end, we established a novel human PBMC culture that yields both T and B cell readouts following allergen stimulation exclusively in allergic patients. We surmise that this model is the first-of-its-kind to enable rapid

screening of human biologics with the potential to inhibit recall responses in food allergy and other allergic diseases.

As IL-4/IL-13 signaling is central to the initiation of food allergy²⁸ and other Th2-mediated disease (*e.g.* helminth infection)⁴⁸, we probed the requirement of this signaling pathway in the context of a recall response. Application of an IL-4R α blocking antibody entirely abrogated IgE transcription and production upon PN stimulation of human PBMCs from PN-allergic donors. Moreover, Th2 cytokine production was broadly suppressed with a remarkable inhibition of IL-9 secretion, which has been demonstrated to be critically required for secondary antibody responses.⁴⁹ The advent of single-cell technology has provided an unparalleled depth of understanding into biological processes. Here, we applied scRNA-seq to generate novel insights into the transcriptomic profile of different populations of PN-reactive CD4 T cells and B cells during a recall response in the presence or absence of IL-4/IL-13 blockade. Moreover, despite the extreme rarity of IgE⁺ MBCs in circulation as measured by flow cytometry, we were able to detect these cells at the single-cell level following culture with PN to elucidate their transcriptional profile. In concordance with Wambre *et al.*⁴⁰, a unique population of Th2 cells thought to be pathogenic in allergy, termed Th2A cells, expanded upon PN stimulation compared to the non-stimulated control. We extend on this work by demonstrating that Th2A cell proliferation as well as the maintenance of their phenotype during allergen exposure occurs independent of IL-4/IL-13 signaling.

A number of studies have described a role of IL-4/IL-13-secreting cells in the allergic cascade. For example, basophils have been shown to supply IL-4 and IL-13 in an IgD-dependent

manner⁵⁰ and ILC2-derived IL-4 constrains the production of regulatory T cells⁵¹, both of which amplify the IgE response. Thus, there appears to be a number of IL-4/IL-13-secreting cells that have seemingly relevant effects on the IgE response. In a recall response, however, the essential source of IL-4 remained unknown. Using IL-4 dual-reporter mice, we demonstrate that Th2 and TFh cells provide the vast majority of IL-4 locally. Through antibody-mediated depletion of CD4⁺ cells which, unlike adoptive transfer of B and T cells into immunodeficient mice, leaves CD4⁺ cells (*e.g.* basophils and ILC2s) intact, we demonstrate that CD4 T cells are critically required for the differentiation of IgG1⁺ MBCs to IgE-secreting cells. Moreover, NKT cells, which also secrete IL-4 and may be critical for the development of allergen-induced airway hyperreactivity⁵², were not critically required, nor did they amplify the recall response.

We next sought to determine whether blockade of IL-4/IL-13 signaling impaired the pathogenic Th2 response *in vivo* by altering IL-4 production and preventing IgE recall responses. We administered an IL-4R α blocking antibody to PN-allergic mice following contraction of the primary immune response and immediately preceding the induction of the recall response via s.c. injections. Production of IL-4 from Th2 and TFh cells was unchanged during IL-4R α blockade, although IgE production was abrogated resulting in complete protection from anaphylaxis upon allergen challenge. Therefore, it is likely that the immediate effects of anti-IL-4R α are a result of IL-4R α blockade on IgG1⁺ MBCs preventing IgE class switch recombination and not due to interruption of IL-4/IL-13 signaling on T cells. Importantly, however, the inhibition of IgE regeneration was sustained following clearance of anti-IL-4R α demonstrating that the immunological changes imparted by anti-IL-4R α persist beyond the duration of therapy. We posit that this sustained effect could be due, at least in part, to the decrease of IL-4 and

substantial increase in IFN γ seen *in vitro* given that IFN γ has been shown to potently suppress primary IgE responses, both *in vitro* and *in vivo*.^{53,54} Recently, IL-21 was demonstrated to negatively regulate IgE responses more so than IFN γ in an alum adjuvanted model.⁵⁵ In our experiments, IL-21 was undetectable in *in vitro* stimulated splenocytes, though this does not negate the potential for IL-21-mediated effects *in vivo*. Ultimately, the precise mechanism by which anti-IL-4R α confers sustained protection from IgE-regeneration is complex and remains an active area of investigation.

The human monoclonal antibody targeting IL-4R α is licensed for the treatment of moderate-to-severe atopic dermatitis, eosinophilic asthma, chronic rhinosinusitis with nasal polyposis, and remains in clinical trials for other atopic diseases. Anti-IL-4R α treatment in humans comprehensively improves disease scoring (*e.g.* FEV₁ for asthma) and decreases total IgE levels by 35-38% after 12 weeks of treatment^{56,57}, however, its therapeutic potential in food allergy remains largely unexplored. Herein, we demonstrated that signaling through IL-4R α is critically required for the regeneration of IgE in both allergic mice and humans. Importantly, this resulted in protection from anaphylaxis on allergen challenge. Moreover, blockade of IL-4/IL-13 signaling skews the Th2-dominant cytokine response and imparts lasting protection from the regeneration of IgE upon allergen re-exposure beyond the duration of therapy. These results spotlight IL-4R α blockade as a therapy with disease-transformative potential for the treatment of food allergy.

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Figure 1. Induction of an IgE recall response to PN in human PBMCs. (A) Schematic of culture with PN-allergic or non-allergic donor PBMCs. (B) Volcano plot of cytokines detected with 38-analyte array in PN-stimulated PBMCs from PN-allergic donors. Benjamini-Hochberg correction was applied to correct for multiple comparisons. Data are plotted as upregulated cytokines (red), downregulated cytokines (blue), and unchanged (grey). (C) Enumerated IgE-secreting cells detected at end of culture with representative images. (D) PN-specific IgE measured from culture supernatant. (E) Fold change of *IGHE* mRNA expression relative to housekeeping gene (*18S*) and non-stimulated control. Data are representative of 5 non-allergic donors (C-E) and 7-11 PN-allergic donors (B-E) and are plotted as mean $\pm$ SEM (C-E). * $P < 0.05$, ** $P < 0.01$. (F) UMAP identifying distinct leukocyte populations from a PN-allergic donor. (G) Frequency of cells in each cluster. (H) UMAP of subclustered T cells. (I) Stacked bars of subcluster frequency (%) in H. (J) Heatmap of top 20 differentially expressed genes in T cell subclusters. (K) UMAP of subclustered MBCs. (L) Stacked bars of subcluster frequency (%) in K. (M) Heatmap of top 20 differentially expressed genes in MBC subclusters.

Figure 2. IL-4/IL-13 signaling blockade aborts IgE recall response and skews cytokine response in human PBMCs. (A) Enumerated IgE-secreting cells detected at the end of culture with representative images. (B) PN-specific IgE measured from culture supernatant. (C) Fold change of *IGHE* mRNA expression relative to housekeeping gene (*18S*) and non-stimulated control. (D) Volcano plot of cytokines detected with 38-analyte array in IC or anti-IL-4R α -treated PN-stimulated PBMCs from PN-allergic donors. Benjamini-Hochberg correction was applied to correct for multiple comparisons. Data are plotted as upregulated cytokines (red), downregulated cytokines (blue), and unchanged (grey). (E) Ratio of type 2 cytokine (IL-5, IL-9,

IL-13) to type 1 cytokines (IL-2, IFN γ , IL-12(p40)) or IL-10 concentration. Data are plotted as mean $\pm$ SEM (**E**) or individual donors (**A-C**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (**F**) UMAP of subclustered T cells. (**G**) Stacked bars of subcluster frequency (%) in F.

Figure 3. CD4 T cells are critically required for IgE recall response. (**A-B**) Frequency of *Il4* transcribing cells (**A**) and IL-4-secreting cells (**B**) in draining lymph nodes of 4get/KN2 mice after s.c. re-exposures. Shaded region is percent of CD4 T cells from GFP⁺ hCD2⁺ (**A**) and GFP⁺ hCD2⁻ (**B**) cells. (**C-D**) Representative contour plots of CD44 and CD62L expression (**C**) and T follicular helper cells (**D**) from GFP⁺ hCD2⁺ cells at 3 days post-re-exposure. (**E-F**) Serum PN-specific IgG1 (**E**) and IgE (**F**) following sensitization. (**G**) Core body temperature at 10-minute intervals during systemic challenge. (**H**) Hemoconcentration at 40-minutes post-challenge. (**I**) Scoring of clinical signs during systemic challenge. Data are representative of 2 experiments with 4-5 mice per group, plotted as mean $\pm$ SEM or median (I). Anti-NK1.1 group is from one experiment only. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. IC, isotype control.

Figure 4. Th2 phenotype persists with IL-4R α blockade. (**A**) Identification of OVA-specific CD4 T cells in OVA-allergic mice at 3 days post-subcutaneous re-exposure. (**B**) Gating strategy for detection of IL-4 production in OVA-specific TFh cells and non-TFh cells from pooled inguinal lymph nodes. (**C, D**) Frequency of GFP⁺ hCD2⁻ cells (**C**) and GFP⁺ hCD2⁺ cells (**D**) from OVA-specific TFh cells. (**E, F**) Frequency of GFP⁺ hCD2⁻ cells (**E**) and GFP⁺ hCD2⁺ cells (**F**) from OVA-specific non-TFh cells. Data are representative of 2 experiments with 4-5 mice per experimental group and are plotted as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$. FMO, fluorescence minus one (flow cytometry control); TS, tape strip; IC, isotype control.

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833 **Figure 5. IL-4R α blockade inhibits IgE recall response and anaphylaxis in epicutaneously-**
834 **sensitized mice. (A)** Serum PN-specific IgG1 and IgE following sensitization. **(B)** Serum PN-
835 specific IgG1 and IgE following PN re-exposure. **(C)** Core body temperature at 10-minute
836 intervals during systemic challenge. **(D)** Hemoconcentration at 40-minutes post-challenge. **(E)**
837 Scoring of clinical signs during systemic challenge. **(F)** Serum PN-specific IgG1 and IgE
838 following anti-IL-4R α clearance and s.c. re-exposures. **(G)** Cytokines measured from splenocyte
839 culture after systemic challenge. Data are representative of 2-3 experiments with 3-5 mice per
840 experimental group, plotted as mean $\pm$ SEM or median (E). * P <0.05, ** P <0.01, *** P <0.001.
841 TS, tape strip; IC, isotype control.

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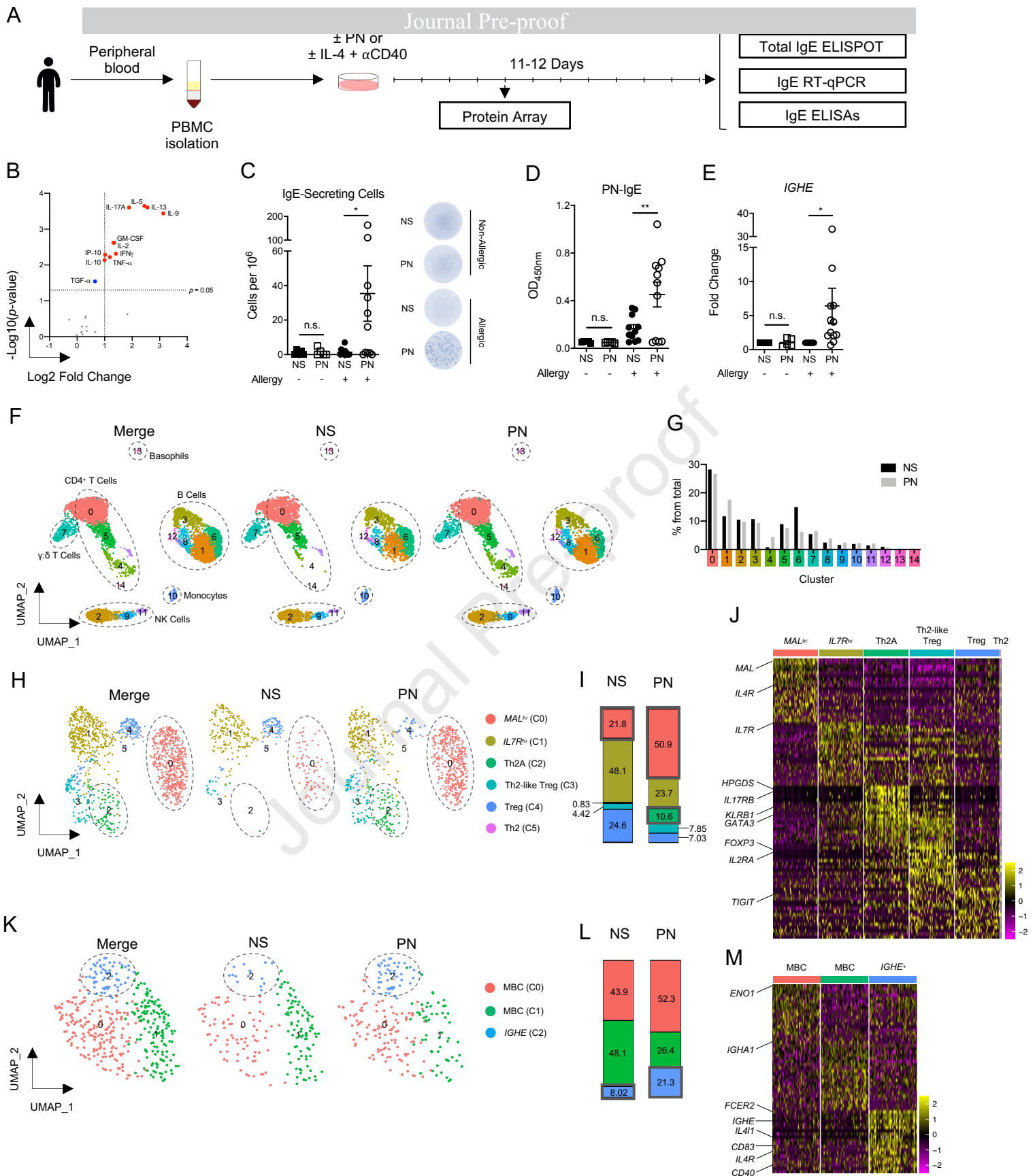


Figure 1

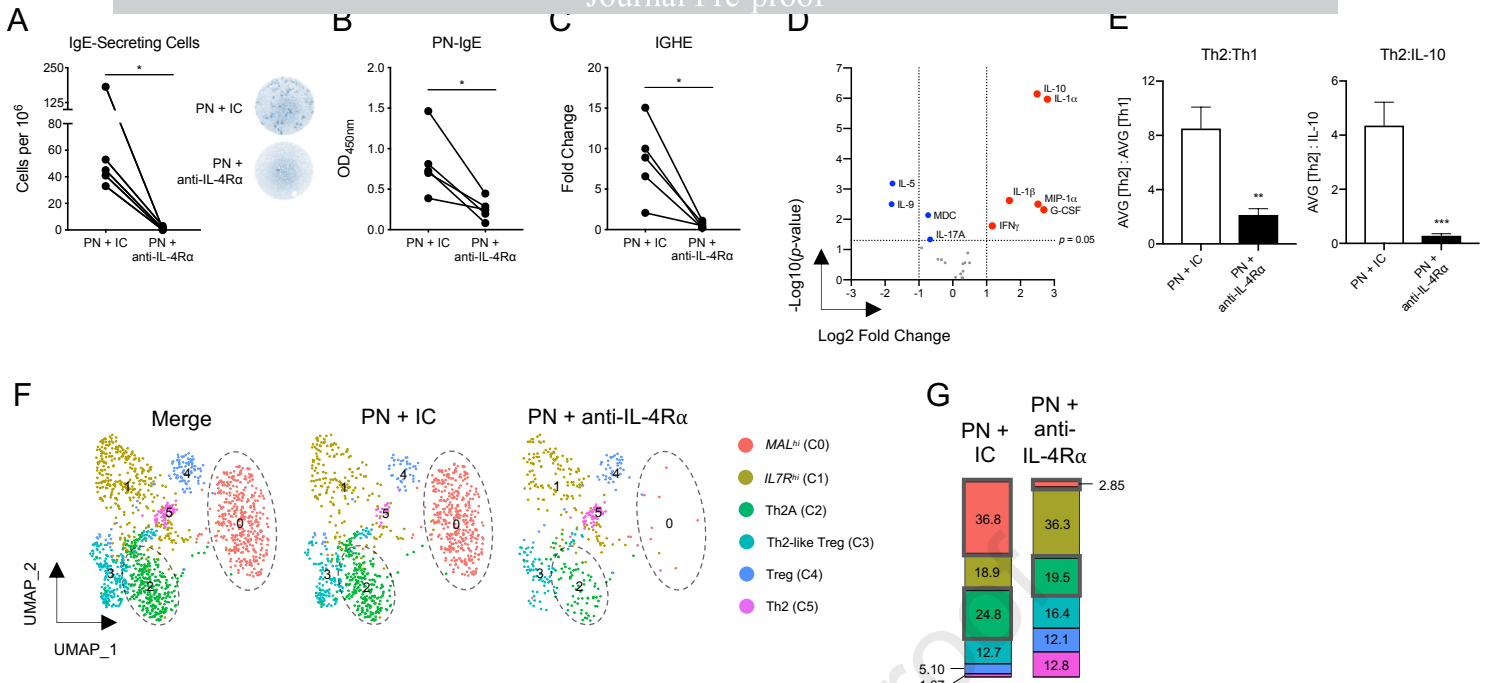


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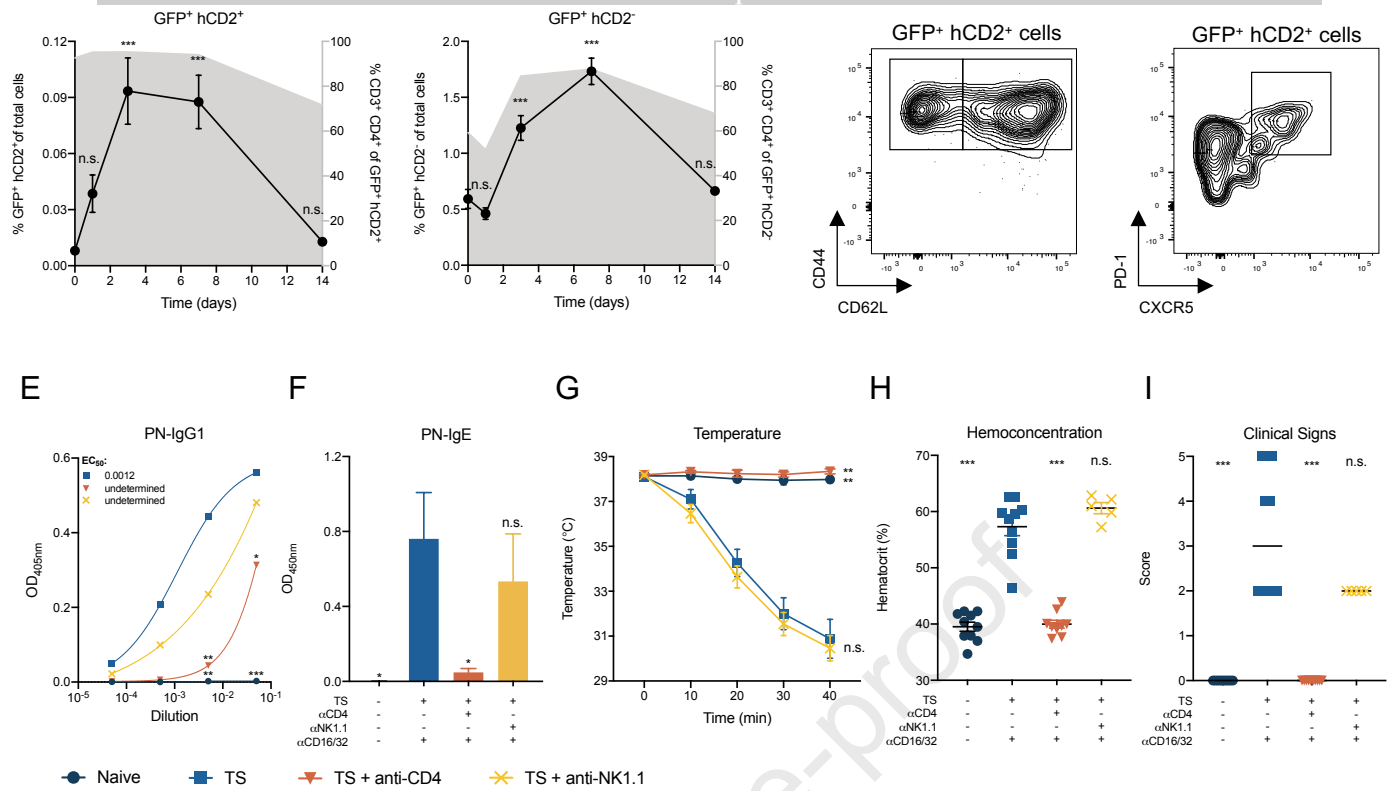


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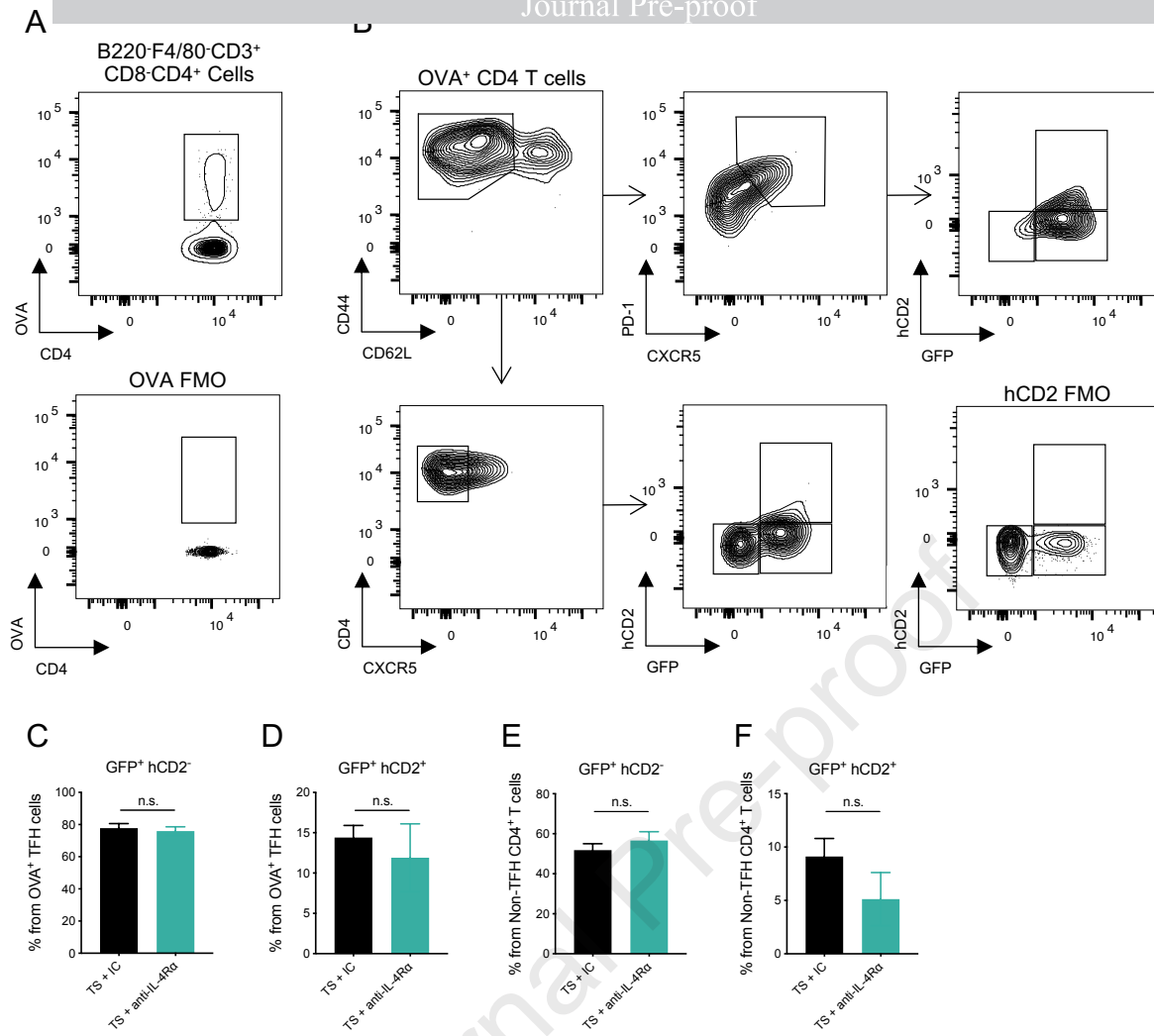


Figure 4

A

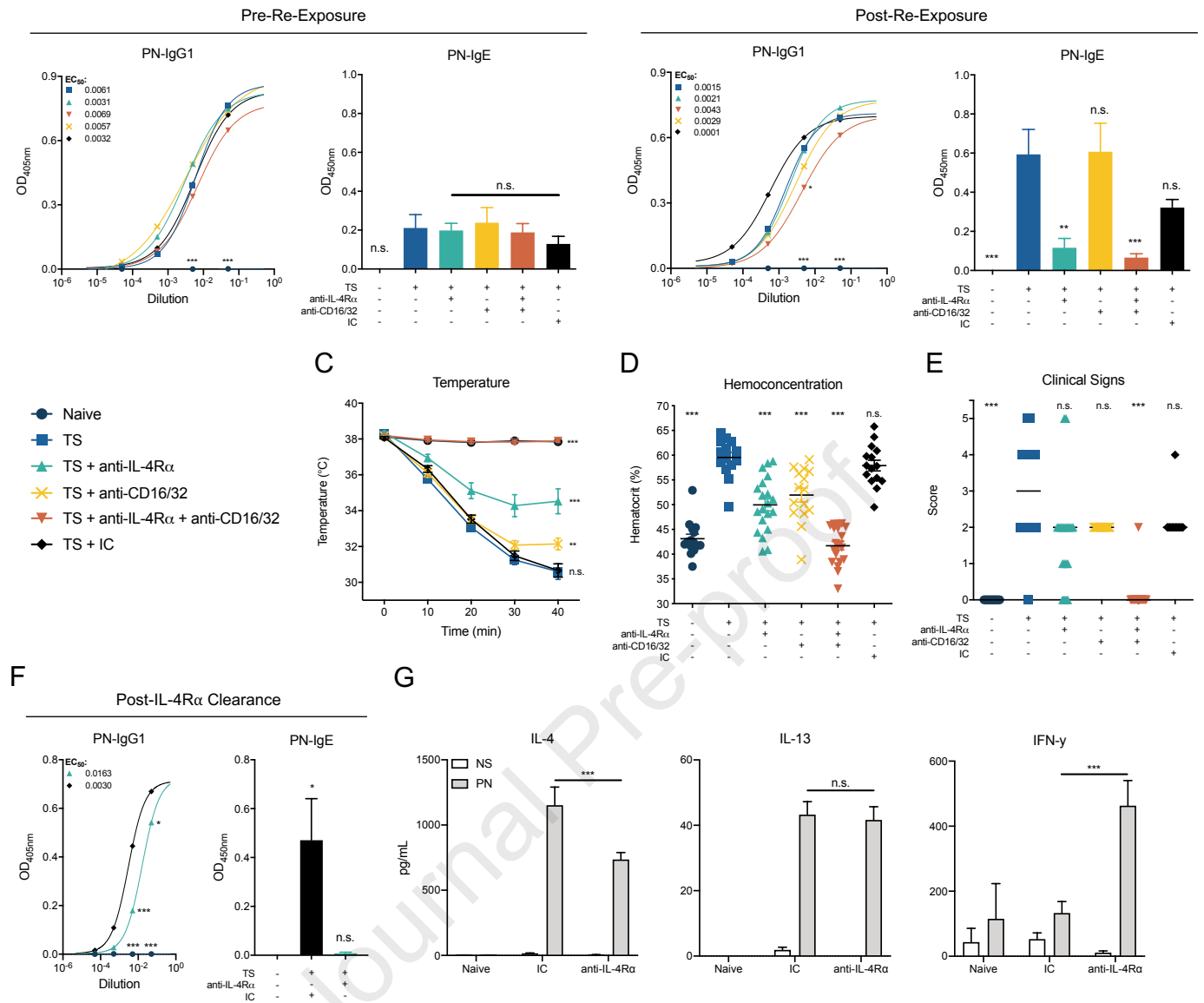


Figure 5