Discovery and characterization of dietary antigens in oral tolerance

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Abstract: Food antigens elicit immune tolerance through the action of regulatory T cells (Tregs) in the intestine. Although antigens that trigger common food allergies are known, the epitopes that mediate tolerance to most foods have not been described. Here, we identified murine T cell receptors specific for maize, wheat, and soy, and used expression cloning to de-orphan their cognate epitopes. All of the epitopes derive from seed storage proteins that are resistant to degradation and abundant in the edible portion of the plant. Multiple unrelated T cell clones were specific for an epitope at the C-terminus of 19 kDa alpha-zein, a protein from maize kernel. An MHC tetramer loaded with this antigen revealed that zein-specific T cells are predominantly Tregs localized to the intestine. These cells, which develop concurrently with weaning, constitute up to 2% of the peripheral Treg pool. Bulk and single-cell RNA sequencing revealed that these cells express higher levels of immunosuppressive markers and chemokines compared to other Tregs. These data suggest that immune tolerance to plant-derived foods is focused on a specific class of antigens with common features, and they reveal the functional properties of naturally occurring food-specific Tregs.

Introduction:

Humans consume nearly 100 g of protein a day from varied sources. Despite being non-self, these foods typically result in a tolerant immune response, a phenomenon called oral tolerance (*1*). Oral tolerance is an intrinsic function of the immune system resulting from continuous surveillance of intestinal contents. The immune interpretation of food is mediated by unique T cell receptors (TCRs) on CD4+ T cells. Antigens encountered through food are typically interpreted as innocuous and trigger the development of regulatory T cells (Tregs), which attenuate immune activation (*2, 3*).

Repertoire level analyses have shown that antigen-free diets change the abundance and composition of intestinal T cells (*2, 4*). In one report, nearly half of the peripherally induced Tregs emerge in association with dietary protein, and thus presumably recognize a food epitope (*2*). In addition to suppressing responses to their cognate antigen, these food-responsive intestinal Tregs foster an environment that broadly promotes tolerance. For example, when mice encountered a new oral antigen (OVA), most of the OVA-responsive cells became Tregs (*2*). In contrast, mice consuming an antigen-free diet lack food-responsive Tregs and are impaired in developing OVA-specific Tregs. Tregs mediate immune suppression through varied mechanisms including anti-inflammatory cytokine secretion (e.g., IL10, TGFβ), competition for growth factors (e.g., IL2 binding by CD25), and cell-cell inhibition (e.g., Lag3, LAP, CTLA4). These mediators can suppress conventional T cell activation directly or via reduced antigen presentation (*5*).

The induction of durable and specific immune tolerance is critical for allergy prevention, and its restoration is the primary goal of allergy immunotherapy. Despite the interest in programming antigen-specific Treg responses, few dietary proteins have been identified that mediate tolerance, and none in an untargeted manner that reveals naturally selected epitopes (*6*). Most studies of oral tolerance use model antigens in adoptive transfer paradigms – these antigens differ in dose, route, and timing of exposure, possibly eliciting transient phenotypes; and focusing on an adoptively transferred cell occludes the epitope selection step, which is integral to initiating an immune response (*7-11*). Recently, in studies to identify immune epitopes from gut resident bacteria, our team serendipitously identified TCRs responsive to a component of mouse chow (*12*). Here, we expand and characterize this set of dietary antigen-TCR pairs and phenotype the identified T cells *in vivo* to reveal an in-depth profile of a normal tolerance response.

Results:

Identification of dietary antigens from corn, soy, and wheat

As a first step towards elucidating a mechanism for naturally evolved immune tolerance to food, we sought to identify the food epitopes recognized by T cells. We adopted a strategy recently developed for the discovery of T cell epitopes from the gut microbiome. In this prior work, mice were colonized with a complex defined bacterial community, T cells were isolated from intestine, and single cell-sequencing was used to identify TCRs (*12*). We then constructed T cell hybridomas bearing these receptors and performed an *in vitro* stimulation assay, incubating them

individually with each bacterium in the community. We observed four T cell hybridomas that were unresponsive to any of the bacterial strains but were strongly induced by germ-free stool or chow, suggesting the possibility that the corresponding TCRs respond to a food-derived epitope. Notably, each of these TCRs were found predominantly (or exclusively) on Tregs, suggesting they mediate a tolerogenic response. These data are consistent with our, and previously reported, repertoire level analyses of mice consuming chow (protein-containing) or amino acid defined diets (fig. S1) (*2*).

Here, we further screened TCRs found on sequenced Tregs, selected using a variety of strategies (Table S1), for chow responsiveness – resulting in a total set of 7 food-responsive TCRs. Next, we set out to identify the antigens recognized by these TCRs. We began by incubating the T cell hybridomas with homogenized preparations of each of the 7 protein-containing components of mouse chow, using dendritic cells for antigen presentation (Fig. 1A-B). Five of the TCRs are activated by corn, while the other two are specific for soy and wheat. Food-responsive TCRs were cloned from Tregs obtained from both germ-free and colonized animals, suggesting development of food tolerance occurs independently of the gut microbiome.

We used an untargeted approach to identify the cognate antigen of each TCR, starting with corn-responders. We expressed a corn cDNA library in *E. coli* (Fig. 1A), with the anticipation this library would approximate the seed proteome (estimated 10,000 proteins (*13*)). Approximately 17,280 total clones were selected and initially tested in 576 bins of 30. *E. coli* cells were heat-killed and the resulting lysates were tested for their ability to activate food-responsive hybridomas. Bins that stimulated a response were retested as individual clones, and those yielding signal were sequenced. All five of the corn-specific TCRs were restimulated by an isoform of 19 kDa alpha-zein, a member of a family of closely related maize proteins that differ in sequence and size (*14*) (receptors: henceforth, αZ_TCRs). We tested a set of tiled synthetic peptides to identify the epitope recognized from within the protein (Fig. 1A). Strikingly, we found that the C-terminal sequence FYQQPIIGGAL ($\alpha Zein_{223\text{-}233}$) is the epitope for all 5 αZ_TCRs (Fig. 1B-C; Fig. 2A). To our knowledge, this epitope has not previously been associated with a T cell response in mice or humans. The $\alpha Zein_{223\text{-}233}$-responsive TCRs are no more similar in sequence to each other than they are to other food responsive TCRs (fig. S2A). The sole exception - αZ_TCR_1 and αZ_TCR_5 – differ by only 1 amino acid. However, they derived from different mice, suggesting independent convergence on a nearly-identical TCR sequence. Taken together, these data suggest that $\alpha Zein_{223\text{-}233}$ is an immunodominant epitope.

Notably, seed storage proteins were also the targets of the soy and wheat-responsive TCRs. We identified the epitope EYVSFKTNDT from the soy protein glycinin G1 ($Glycinin_{430\text{-}439}$) using a screening approach similar to that described for corn. The soy epitope resides partially in an internal beta sheet of this protein (Fig. 1B; Fig. 2B; fig S2B). This epitope is orthogonal to known Glycinin G1 epitopes. Further, we found the wheat-responsive TCR recognizes the recently-reported epitope CNVYIPPYCTIAP ($Gliadin_{273\text{-}285}$) (Fig. 1B, fig. S3C) (*6*). In prior work in B6 mice, this epitope was found to be a target of the T cell response to intraperitoneal

injection of gliadin, suggesting some degree of peptide-intrinsic dominance across different immune contexts. Like αZein, gliadin belongs to the water-insoluble prolamin group of seed storage proteins and the epitope is situated within a C-terminal region that is predicted to be unstructured (Fig. 2C).

Conservation of TCR epitopes across the plant kingdom

Immune cross-reactivity has been well documented between allergens (*15-19*), but it is unknown whether tolerogenic epitopes also display cross-reactivity. A computational search for homologous peptides revealed orthologs of the epitope recognized by the soy-specific TCR in the genomes of many other plants. To test for cross-reactivity, we incubated the Gly_TCR_1 T cell hybridoma with seed lysates from 26 plants, along with dendritic cells for antigen presentation. We found that Gly_TCR_1 is robustly activated by seed lysates from numerous plants (Fig. 2D); notable elicitors include other foods such as *Carya illinoinensis* [pecan], *Chenopodium quinoa* [quinoa], and *Sesamum indicum* [sesame seed]. Glycinin is an 11S globulin protein, and homologous 11S globulins could explain the cross-reactive signal in other species (Table S2, e.g., 11S globulin seed storage protein 1-like from quinoa with the sequence EWVSFKTND). Orthologs of this epitope are also conserved in foods we did not assess (e.g. walnut, pistachio, guava) and in trees (e.g., cork oak, white poplar). Curiously plants that are more closely related to soybean – e.g., *Arachis hypogaea* [peanut], *Pisum sativum* [pea]) – were less cross-reactive than more distantly related species.

While Gly_TCR_1 recognizes closely related variants of the soy epitope Glycinin$_{430\text{-}439}$, the αZ_TCRs are more specific. Synthetic peptides that represent other isoforms of 19 kDa αZein with similar sequences elicited no response in the same assay (fig. S3A). Seed lysates from *Panicum miliaceum* and *Setaria italica*, which contained predicted αZein$_{223\text{-}233}$ homologs, mildly activated only αZ_TCR_3 (fig. S3B, Table S3). It is possible that αZein$_{223\text{-}233}$ homologs from other plants are expressed at a low level, different developmental stage, or only under particular environmental/stress conditions. Additionally, processing and presentation of possible epitopes by APCs was not assessed. Thus, weak or lack of signal in this assay does not exclude potential protein cross-reactivity. To directly test activation in response to a homolog, αZ_TCRs were stimulated with equal quantities of a synthetic peptide from a *Panicum miliaceum* homolog or the cognate zein epitope. While these results highlighted the potential for cross-reactivity, they revealed a clear preference for αZein$_{223\text{-}233}$ (fig. S3C). The gliadin$_{273\text{-}285}$ epitope is directly conserved across numerous wheat species and other grasses (Table S4). Thus, taken together, some epitopes are directly conserved (e.g., gliadin), some have close cross-reactive homologs (e.g., glycinin), and some have close homologs but little cross-reactivity (e.g., zein).

αZein$_{223\text{-}233}$-responsive cells are predominantly small intestine Tregs

The T cell epitopes we identified are recognized in the physiological setting of a natural food matrix 'administered' by ingestion—rather than, e.g., a purified protein in drinking water or a food-derived extract administered by injection. Given that the corresponding TCRs were predominantly derived from Tregs, we reasoned that the T cells that recognize αZein$_{223\text{-}233}$,

glycinin$_{430-439}$, and gliadin$_{273-285}$ may represent a previously undescribed population that mediates oral tolerance to food.

To characterize their distribution and functional properties, we obtained an MHCII tetramer loaded with αZein$_{223-233}$, the corn-derived epitope recognized by five of the seven TCRs we mapped. First, we analyzed small intestine lamina propria T cells from young adult (6-12 wk old) mice fed a standard chow diet. αZein$_{223-233}$ tetramer-positive T cells were predominantly Foxp3+ Tregs, and a majority express the transcription factor RORγt (Fig. 3A-B). Within the pTreg population (Foxp3+/Helios- or Foxp3+/RORγt+), on average >2% percent of these cells are tetramer-positive, a strikingly large response against a single food epitope. This result suggests the possibility that a subset of food epitopes dominate immune recognition in the context of tolerance.

A similar distribution of αZein$_{223-233}$ binding cell types was observed in mice from two different vendors (Taconic Biosciences vs Jackson Laboratory), highlighting robustness to differences in microbiome and environmental conditions (fig. S4A). This result suggests that RORγt expression in food-specific Tregs is independent of colonization with segmented filamentous bacteria (SFB, a strong inducer of RORγt+ Th17 cells present in the intestines of Taconic but not Jackson Laboratory mice).

Food-responsive T cells have previously been reported in lymph nodes (*6*). However, we only detected significant levels of αZein$_{223-233}$ responsive T cells in the small intestine lamina propria, not the mesenteric lymph nodes (MLN) or spleen (Fig. 3C, fig. 4B). αZein$_{223-233}$ tetramer positive T cells were also detected in the large intestine (Fig. 3C). Compared to the small intestine, fewer numbers of αZein$_{223-233}$ T cells were detected in large intestine precluding definitive analysis of cell type distribution, though some Tregs were observed (fig. S4C). The abundance and subtype distribution of αZein$_{223-233}$ responsive T cells was stable across a range of ages (fig. S4D-E).

Post-natal development of αZein$_{223-233}$ Tregs

While prior work has established the kinetics of pTreg induction post-birth, the development of food-responsive T cells is less well understood, limiting our knowledge of the window during which tolerance develops. Food tolerance most likely arises either soon after birth, supported by the detection of food peptides in breast milk (*20*), or concomitant with weaning and the introduction of solid foods. To assess these possibilities, dams were randomized during pregnancy to a chow or an amino-acid-defined (AAD, antigen-free) diet. Their pups were fed the same diet until they were sacrificed at 2 or 4 weeks of age. Consistent with previous studies (*21*), we observed an increase in pTregs (Foxp3+Helios- and Foxp3+RORγt+) from 2-4 weeks of age, when solid food consumption begins (Fig. 3D, fig. S5A-B). At 4 weeks of age, Foxp3+Helios- Tregs were higher in mice fed chow than an AAD diet, indicating that a subset of these cells responded to dietary antigens (fig. S5B).

Tetramer-positive T cells were not detected in the small intestine of 2 wk old mice regardless of diet (Fig. 3E) and appeared at 4 wks of age only in the chow-fed mice (Fig. 3F). This finding is consistent with a model in which tolerance to dietary proteins develops alongside the transition to solid food consumption. In other studies, pTregs were detected in the intestine 12 days after antigen exposure, suggesting that initial priming may have occurred around two weeks of age (*9*).

Curiously, IgG antibodies targeting food were higher in the serum of AAD fed mice at 2 weeks of age compared to chow fed mice, and higher in chow fed mice at 4 wks of age (Fig. S5C-D). Since the AAD fed mice were born onto a diet lacking dietary proteins, we suspect that food responsive IgG antibodies in their serum originate from the mother, who consumed chow for most of her life. Maternal antibodies can be shared with offspring both across the placenta and through breastmilk (*22*). Further, while AAD mice showed a decrease in food targeting antibodies from 2 to 4 wks of age, chow fed mice showed an increase during this same time window. These antibodies appear concurrent with the increase in zein-responsive Tregs although a direct link between Tregs and immunoglobulin maturation is not known. IgG responsive to major chow components persists throughout adulthood, but only with continued chow consumption; mice randomized to AAD diet at weaning showed lower food-responsive IgG and a considerable reduction in total IgG in adulthood (Fig. S5E-F). While the role of IgG in tolerance is generally not well understood, there is some evidence that IgG contributes to systemic food tolerance (*23, 24*). Further, IgG responsive to the same dietary components (corn, soybean, and wheat) can be readily detected in adult serum samples from humans. Together, these data show that the first introduction to food is a critical window for development of food-responsive peripheral Tregs which appears to co-occur with an increase in IgG that recognize food components; both arms of immune recognition of food appear to reach a steady state that lasts through adulthood.

αZein-responsive and bacterially induced Tregs emerge through distinct pathways

The identification of RORγt+Foxp3+ Tregs specific for $\alpha Zein_{223\text{-}233}$ suggests a possible parallel between food-responsive and bacterially induced T cells, since commensals such as *Helicobacter hepaticus* (*Hh*) induce the same 'double-positive' Tregs (*25*). Induction of these *Hh* responsive Tregs depends on migration of antigen presenting cells from the intestine to the lymph node in a C-C chemokine receptor type 7 (CCR7) dependent manner (*26*). Corroborating these prior findings, we find that in mice with a CD11c-Cre driven CCR7 deletion ($CCR7^{\Delta CD11c}$), adoptively transferred, small-intestine resident, *Heliobacter hepaticus (Hh)* -specific T cells (Hh7-2) lose the Foxp3+RORγt+ phenotype observed in $CCR7^{fl/-}$ control mice and there is a corresponding increase in Th17 cells (fig. S6A) (*26*). However, $\alpha Zein_{223\text{-}233}$ tetramer positive cells retain the Foxp3+RORγt+ phenotype in $CCR7^{\Delta CD11c}$ mice (fig. S6B). Thus, the requirements for antigen presentation for *Hh* and $\alpha Zein_{223\text{-}233}$ appear distinct.

Immunosuppressive factors and chemokines are altered in food-responsive Tregs

To search for molecular features that are characteristic of naturally-induced food-responsive T cells, we compared the transcriptional profile of αZein-responsive Tregs, adoptively transferred OVA-specific Tregs, and bulk Tregs (all CD4+CD25+ cells of unknown antigen specificity) from chow-fed, AAD-fed, or germ-free (GF) mice. Several immune suppressive agents (e.g., Gzmb and Lag3) emerged in association with food-responsiveness (Fig. 4A-F, fig. S7-8, fig. S9A-D, Table S5). The expression of these immune suppressors in αZein$_{223-233}$ Tregs was independent of expression of RORγt, suggesting heterogeneity in subpopulations defined by this transcription factor and other phenotypic markers (fig. S10). Further, patterns of differential expression of immune suppressors emerge for Gliadin$_{273-285}$ and OVA-responsive Tregs compared to tetramer-negative or host bulk Treg controls (fig. S11). Contrasting with the effect of the AAD dietary perturbation, GF mice showed increased levels of Gzmb (fig. S12), indicating that levels of immune suppressive agents may be tuned in response to specific antigen (food vs bacterial). These data suggest that food-responsive Tregs engage in multiple mechanisms of suppression including binding MHCII (*27*), counteracting inflammatory signaling (*28*), and cell-cell killing (*29*).

In addition to canonical immune suppressors, a set of genes emerged that provides clues into how food-responsive cells traffic and establish a functional niche, including chemokines, chemokine receptors, and matrix metalloproteinases (Fig. 4A, 4G-I; fig. S7B-C; fig. S13; Table S5). Two of the chemokines enriched in the αZein$_{223-233}$ samples, CCL3 and CCL4, are known to be secreted by Tregs to recruit other T cells into proximity for effective suppression (*30*). Interestingly the receptors for CCL3 and CCL4, CCR1 and CCR5, were also upregulated in αZein$_{223-233}$ Tregs compared to bulk Tregs. This may indicate that food-responsive Tregs recruit other Tregs with similar antigen specificity into their niche to coordinate function. CCR5^{hi} Tregs are more immunosuppressive than their CCR5^{lo} counterparts and CCR5$^{-/-}$ mice are defective in oral tolerance, suggesting a link between chemokine-mediated trafficking and immune suppression (*31*, *32*).

Further characterization of αZein$_{223-233}$ T cells by single cell RNA-sequencing revealed a Treg cluster enriched in αZein-responsive cells that was characterized by high expression of IL10, Gzmb, and Lag3, some of the same markers that emerged from bulk analysis (fig. S14-16). These shared transcriptional profiles observed across cells collected from two of our laboratories suggests these genes are robust markers for food responsive Tregs. Further, cloning hybridomas bearing TCRs from unannotated cells in this cluster revealed an additional wheat-responsive TCR (Gli_TCR_2) that also bound to Gliadin$_{273-285}$ (fig. S17). This finding suggests that Gliadin$_{273-285}$ is also an immunodominant tolerogen. Taken together, these data indicate that food-responsive Tregs are uniquely immune suppressive, and the epitopes that they recognize are likely critical for driving tolerance to whole food.

Discussion:

Oral tolerance is a remarkable process of suppressing immune responses toward dietary proteins. However, the antigens that initiate natural induction of Tregs were unknown, limiting our ability

to characterize the initial steps of oral tolerance and mechanisms of Treg immune suppression. Here, we identified food epitopes that are recognized by naturally-induced Tregs after oral introduction of antigens. The antigens identified – αZein, glycinin, and gliadin – are all seed storage proteins, suggesting that this class of proteins is a common source of epitopes for oral tolerance. This expands on the prior observation that seed storage proteins are often the targets of T cell and antibody responses in the context of allergy. These proteins serve as nutrient reservoirs for germination and early seed development; as such, they are protease-resistant and highly abundant, which likely explains their immunogenicity. Cereal grains comprise over half of the world's daily caloric intake, and we anticipate that the proteins identified here will also be common targets of Tregs in humans (*33*).

Our hybridoma TCR mapping and MHC tetramer analysis reveal the immunodominance of a subset of epitopes. Specifically, we repeatedly identified epitopes from the unstructured C-terminal regions of zein and gliadin. It is possible that this portion of prolamin proteins may be selectively proteolyzed or presented on MHC complexes, or have unique access to cognate antigen presenting cells (*34*). Alternatively, TCRs responsive to different antigens are known to be differentially represented in the naïve repertoire (*35*). The relative contributions of antigen presentation or naïve cell frequency are unknown and of interest to future work to refine oral immunotherapy strategies (*36*). It is further unknown how many unique epitopes must be recognized in a food to drive robust tolerance. Since seed storage proteins are abundant in food, it is tempting to speculate that abundance contributes significantly to antigenicity. However, more abundant proteins in a seed are probably more frequently sampled by dendritic cells in our assay. Most of the hybridomas generated were unmapped, and thus cannot be definitively ruled out as food-responders. For example, low abundance seed proteins may be presented at levels that produce an undetectable signal, even if an antigen in the seed was a match for the receptor.

Leveraging our newly discovered food epitopes, we find that $\alpha Zein_{223\text{-}233}$ tolerance develops rapidly and parallels increased solid food intake. Our data are consistent with prior repertoire level reports that the percentage of Foxp3+ T cells increases significantly in the post-weaning period (*2, 21, 37*). However, we provide deeper insight by monitoring the differentiation of an antigen-specific T cell population. Additionally, the $\alpha Zein_{223\text{-}233}$ responsive T cells phenotyped here show a restricted location and subtype range compared to T cells reported from short-term feedings. The functional consequences of this phenotype for durable tolerance remain unknown. $\alpha Zein_{223\text{-}233}$ responsive Tregs were intestinally localized and nearly exclusively Tregs. In contrast, after 1 week on a gliadin containing diet, gliadin-responsive T cells were observed in the small intestine lamina propria and lymph nodes, and many adopted an anergic phenotype (*6*). In other studies, after short term OVA exposure, only half of adoptively transferred OVA-specific T cells become Foxp3+ Tregs (*2, 9*). The different observations might be due to mode and dose of antigen administration, duration of exposure, time since initial exposure, and location profiled. These Treg populations induced by short-term feeding could be functionally distinct from long-term gut resident Tregs. Thus, identifying and characterizing chow-responsive T cells, is essential to understand the nature of long-standing oral tolerance.

The persistence of αZein$_{223\text{-}233}$ tetramer-positive Tregs in CCR7 $^{\Delta CD11c}$ mice underscores the range of possible antigen presenting cells and mechanisms for Treg induction. In fact, while *Hh* Treg induction strictly depends on CCR7 in CD11c+ cells, CCR7 $^{\Delta CD11c}$ mice are not globally deficient in RORγt+ Tregs, emphasizing the existence of parallel pathways for antigen presentation leading to Treg induction (*26*). Differences between *Hh* and αZein may reflect the nature of the antigen, intestinal region of encounter, or timing of antigen introduction (αZein since birth and *Hh* as an adult). Treg induction to OVA in adult mice is dependent on CCR7 (*38*), suggesting antigen-intrinsic factors or timing of antigen exposure may be the critical determinant of requirements for antigen presentation.

To decipher the acquired phenotype needed to mediate food antigen tolerance, we identified a transcriptional signature of food-responsive Tregs. Food-responsive Tregs displayed elevated levels of multiple immune suppressive agents. Further, αZein$_{223\text{-}233}$ responsive Tregs show an altered chemokine profile, consistent with ability to lure neighboring T effectors cells into closer proximity for efficient suppression. Recently, Lag3, Gzmb, and IL10 were identified as upregulated in intestinal epithelium resident regulatory T cells from chow compared to AAD diet fed mice, suggesting a conserved effect in multiple layers of the intestine (*4*). Our tetramer data corroborates this finding and specifically implicates food-responsive T cells as a source of these differences. This heightened immunosuppressive phenotype may be specific to food responsive Tregs. Notably, transcriptomics analysis of a bacterial responsive Treg population showed no difference in *gzmb*, *il10*, or *lag3* abundance compared to the bulk Treg population (*39*). This profile may be leveraged in the future to expedite further mapping of the whole food-Treg population.

Identifying tolerance epitopes has direct implications for understanding allergies. For example, Glycinin G1 is a known allergen (Gly m 6). However, the epitope we identified does not correspond to a known allergen epitope (from the immune epitope database (IEDB), (*40-47*)), with the caveat that IEDB consists primarily of human epitopes. In addition, the glycinin-responsive TCR is not activated by known Gly m 6 cross-reactive lysates (e.g., peanut lysate/allergen Ara h 3 (*48*, *49*)). In the future, tracking T cell responses to this epitope under inflammatory or homeostatic conditions may reveal vulnerabilities that initiate allergy development. Further, understanding how proteins like αZein, that are not associated with allergy, drive strong Treg responses, could provide a blueprint for programming antigen-specific Treg responses.

Diet is our most intimate interaction with our environment. Correctly recognizing foods as safe creates an anti-inflammatory environment to support nutrient acquisition and prevent allergy. This research advances our understanding of the major dietary antigens recognized by intestinal Tregs. We find that long-term resident food-responsive cells are predominantly Tregs and highly immunosuppressive. Tapping mechanisms of oral tolerance as naturally occurring programming with molecular specificity could enable the use of synthetic approaches for redirecting of allergic and autoimmune states.

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Author contributions:
JEB, KN, MAF, ESS conceptualized the project; JEB, RK, KN, MAF, ESS, DRL designed experiments; JEB, RK, KN, EAS, FMC, RU, GRM carried out the experiments; JEB wrote the paper with edits from ESS, RK, EAS, KN, FMC, RU, DRL, MAF.

Competing interests:
D.R.L. consults and has equity interest in Vedanta, Immunai, Imidomics, Sonoma Biotherapeutics and Pfizer Pharmaceuticals. M.A.F. is a co-founder of Kelonia and Revolution Medicines, a member of the scientific advisory boards of the Chan Zuckerberg Initiative, Stand Up to Cancer, NGM Biopharmaceuticals, and TCG Labs/Soleil Labs, and an innovation partner at The Column Group. The remaining authors have no conflict of interest.

Data and materials availability:
Data are and materials are available upon request.

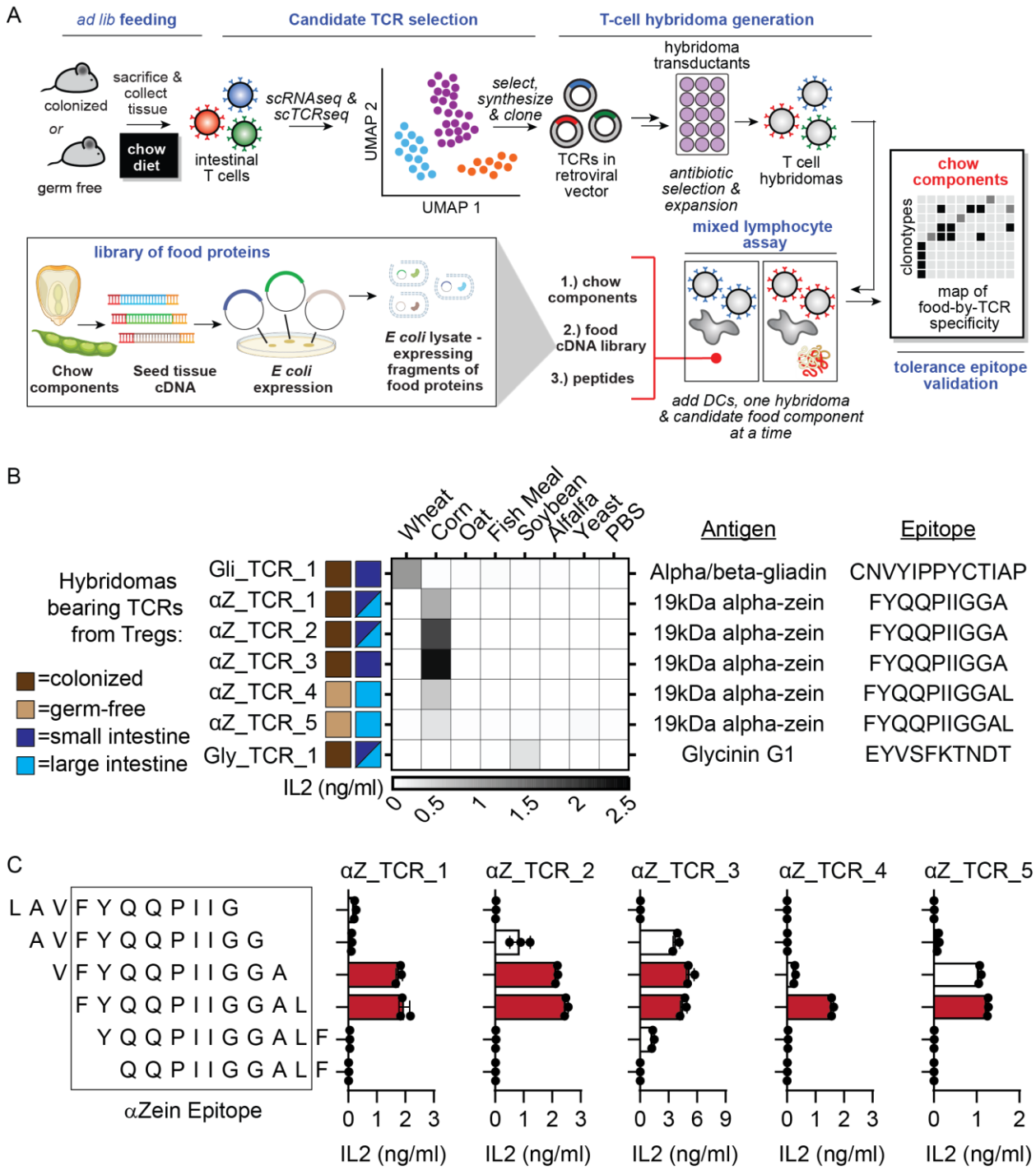

Fig. 1: Epitope-TCR pairs were identified from wheat, corn, and soy.
(A) Workflow to screen TCR hybridomas to find food-responsive TCRs from single cell sequencing data, map the cognate antigens using a cDNA library screen, and map exact epitopes using synthetic peptides. **(B)** 7 hybridomas were mapped to a dietary component, antigen, and epitope. **(C)** Tiling scans with 10-11 amino acid peptides spanning alpha-zein were used to identify minimum antigen epitope. Red bars do not differ significantly from each other ($p > 0.05$) and represent the maximum activation. N=3 replicates/condition.

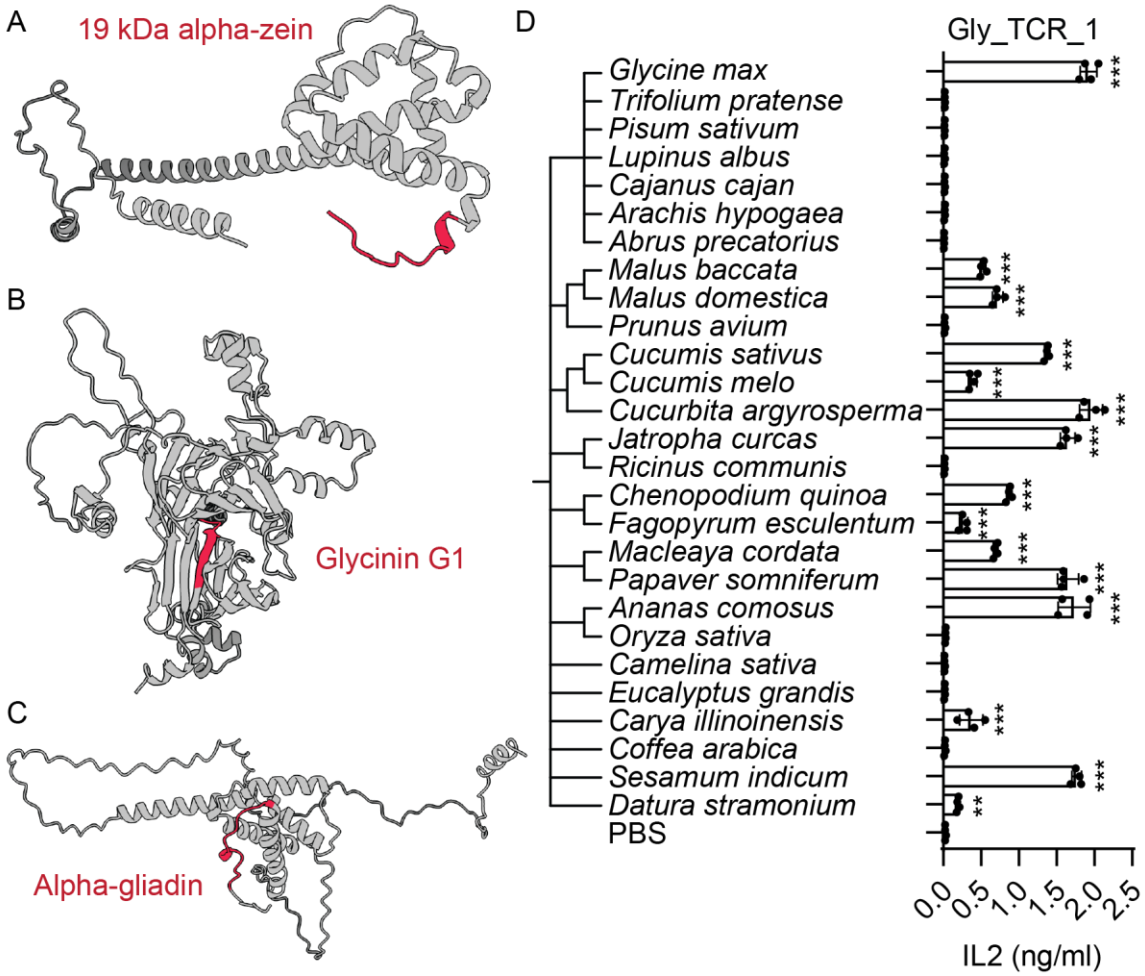

Fig. 2: Identified epitopes and cross-reactivity.
(A-C) Alpha-fold predicted structure of alpha-zein, glycinin G1, and gliadin with identified epitopes highlighted in red. **(D)** Gly_TCR_1 was stimulated with soybean or lysates from seeds containing a putative soybean homolog. * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$ compared to the PBS control.

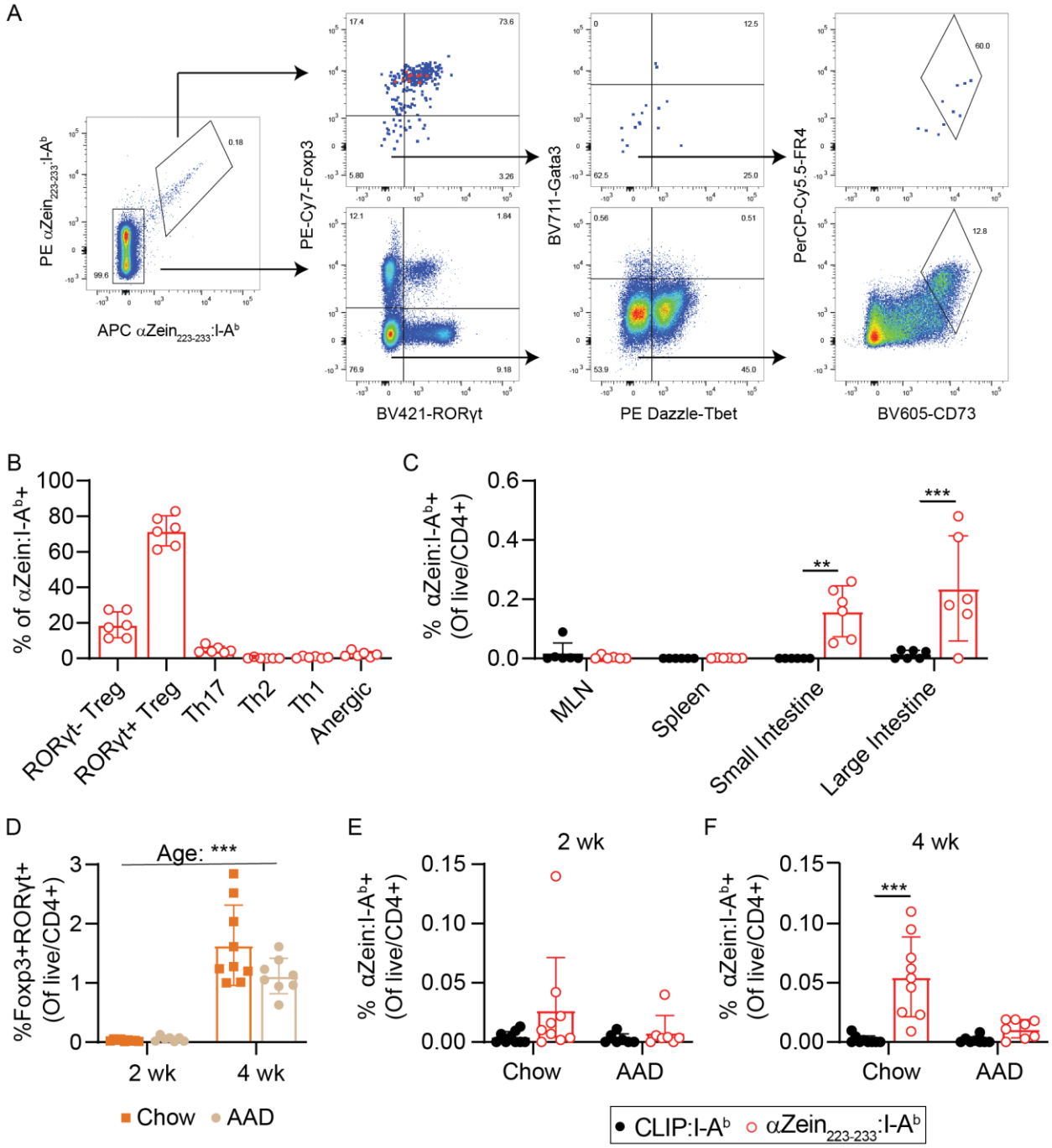

Fig. 3: *In vivo* characterization of αZein223-233 responsive T cells. **(A-B)** αZein223-233 responsive T cells were profiled for T cell subtypes. RORγt- Treg: Foxp3+/RORγt-; RORγt+Treg: Foxp3+/RORγt-, Th17: Foxp3-/RORγt+, Th2: Foxp3-/RORγt-/Gata3+/Tbet-, Th1: Foxp3-/RORγt-/Tbet+/Gata3-, Anergic: Foxp3-/RORγt-/Tbet-/Gata3-/CD73+/FR4+ **(C)** Tetramer positive cells in mesenteric lymph node (MLN), small intestine, large intestine, and spleen. **(D)** Foxp3+/RORγt+ Tregs in mice born onto chow or AAD diets profiled at 2 or 4 weeks of age. Age:*** indicates a significant main effect of mouse age. **(E-F)** Tetramer positive cells in mice born onto chow or AAD diets profiled at 2 or 4 weeks of age. N=6 mice/group in panels B-C. N=7-9 mice in panels D-F. * denotes p<0.05, ** denotes p<0.01 and *** denotes p<0.001.

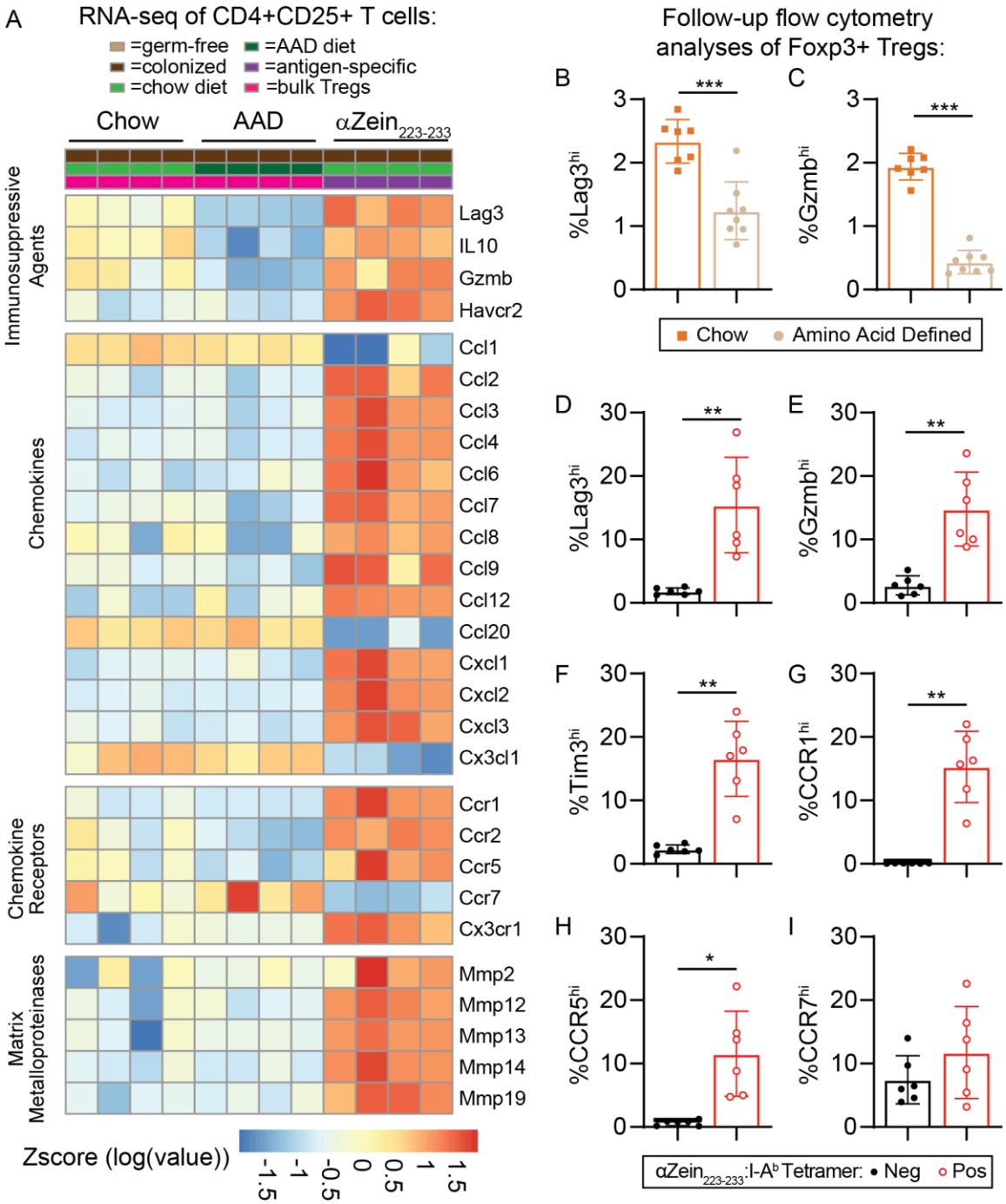

Fig. 4: Bulk RNA-sequencing identified that immunosuppressive markers and chemokines are differentially expressed in food-responsive Tregs.

(A) Heatmap of a subset of differential transcripts between bulk Tregs from chow fed control mice, AAD fed mice, or antigen-specific αZein$_{223-233}$ Tregs. **(B-C)** Lag3 and Gzmb levels in Foxp3+ Tregs from chow or amino acid defined diet fed mice. **(D-I)** Lag3, Gzmb, Tim3, CCR1, CCR5, and CCR7 levels between αZein$_{223-233}$ binding and tetramer non-binding Tregs. N=7-8 in panels B-C. N=6 in panels D-I. * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$.

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