HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2023 July 12.

Published in final edited form as:

Nature. 2022 July ; 607(7920): 762–768. doi:10.1038/s41586-022-04916-6.

Immune tolerance of food is mediated by layers of CD4+ T cell dysfunction

Sung-Wook Hong[1,2], **Peter D. Krueger**[1,2], **Kevin C. Osum**[1,2], **Thamotharampillai Dileepan**[1,2], **Adam Herman**[3], **Daniel L. Mueller**[1,4], **Marc K. Jenkins**[1,2,*]

[1]Center for Immunology, University of Minnesota Medical School, Minneapolis, MN 55455 USA

[2]Department of Microbiology and Immunology, University of Minnesota Medical School, Minneapolis, MN 55455 USA

[3]Minnesota Supercomputing Institute, University of Minnesota, Minneapolis, MN 55455 USA

[4]Division of Rheumatology, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455 USA

SUMMARY

Gastrointestinal health depends on the adaptive immune system tolerating the foreign proteins in food[1,2]. This tolerance is paradoxical because the immune system normally attacks foreign substances by generating inflammation. Here, we addressed this conundrum by using a sensitive cell enrichment method to show that polyclonal CD4+ T cells responded to food peptides, including a natural one from gliadin, by proliferating weakly in secondary lymphoid organs of the gut-liver axis due to the action of regulatory T cells. A few food-specific T cells then differentiated into T follicular helper cells that promoted a weak antibody response. Most cells in the expanded population, however, lacked canonical T helper lineage markers and fell into 5 subsets dominated by naïve-like or T follicular helper-like anergic cells with limited capacity to form inflammatory Th1 cells. Eventually, many of the T helper lineage-negative cells became regulatory T cells themselves via an IL-2-dependent mechanism. Our results indicate that exposure to food antigens causes cognate CD4+ naïve T cells to form a complex set of non-canonical hyporesponsive Th subsets that lack the inflammatory functions needed to cause gut pathology and yet have the potential to produce regulatory T cells that may suppress it.

Most people do not mount pathogenic CD4+ T responses to their food despite ingesting about 100 grams of foreign proteins from plants and animals every day[3]. Even though Major Histocompatibility Complex class II (MHCII)-bound peptides from food proteins are

*Corresponding author: jenki002@umn.edu, Requests for reprints and materials generated for this study should be addressed to Marc K. Jenkins.

AUTHOR CONTRIBUTIONS

S.H. and M.K.J. designed experiments. S.H., P.D.K., K.C.O. performed experiments. T.D. produced tetramers. A.H. and D.L.M. performed sequencing data analysis. S.H. and M.K.J. wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

OBTAINING BIOLOGICAL MATERIALS

Reasonable amounts of unique biological materials can be obtained upon request from the corresponding author or from a standard commercial source if one becomes available.

presented[4] to CD4$^+$ T cells with cognate antigen receptors (TCRs), these T cells usually fail to mount an inflammatory response. This oral tolerance has been attributed to death or functional inactivation of food antigen-specific T cells[5], generation of IL-10-producing Tr1 cells[6], TGF-β-activating Th3 cells[7], or Foxp3$^+$ regulatory T (Treg) cells[8–10]. Many of the definitive experiments in this area, however, involved monoclonal TCR transgenic mice, which can generate abnormal immune responses[11,12]. Mechanisms that underlie oral tolerance in individuals with normal T cell repertoires are less clear.

Food antigens induce Th^{lin-} cells

We studied CD4$^+$ T cells specific for a peptide from the wheat protein gliadin to assess the normal response to a genuine food antigen. We identified an I-A^b MHCII-binding peptide (Glp) from gliadin, produced a fluorophore-labelled I-A^b tetramer containing this peptide, and used this reagent to study the cognate T cells in I-A^b MHCII molecule-expressing C57BL/6 mice by flow cytometry after magnetic enrichment[13] (Fig. 1a, b). Mice reared on a gliadin-free diet had about 200 Glp:I-A^b tetramer-binding cells in the secondary lymphoid organs (SLO) (Fig. 1a, c). About 80 % were naïve CD44^{lo} Foxp3$^-$ conventional T cells (Tconv) and 10 % were Foxp3$^+$ Treg cells (Fig. 1a) as observed for other foreign epitope-specific naïve T cell populations[14]. One week after mice were exposed to a gliadin-containing diet, the Glp:I-A^b tetramer-binding population increased to about 2,500 cells (Fig. 1b, c), including 10 % CD44^{lo} naïve Tconv cells, 60 % Treg cells, a 5 % subset expressing CXCR5, a marker of T follicular helper (Tfh) cells[15], and very few cells expressing T-bet or RORγt, the master transcription factors for Th1 and Th17 cells, respectively[16] (Fig. 1d). The CXCR5$^+$ cells functioned as Tfh cells as evidenced by the finding that *Lck^{Cre} Bcl6$^{fl/fl}$* mice, which lack this population[17], failed to generate the weak gliadin antibody response that was observed in wild-type mice after conversion to a gliadin-containing diet (Extended Data Fig. 1). About 25% of the Glp:I-A^b tetramer-binding T cells in mice on a gliadin-containing diet lacked markers of Treg (Foxp3), Tfh (CXCR5), Th1 (T-bet), or Th17 (RORγt) cells (Fig. 1b, d) and expressed the anergic T cell markers folate receptor 4 (FR4) and CD73 (Fig. 1b)[18]. These cells will be referred to as Th lineage-negative (Th^{lin-}) cells.

Mice were fed with other foreign peptides to determine whether the activation program observed in Glp:I-A^b-specific T cells was a general phenomenon. A peptide called 2W[19,20] or peptides from CD4Ag28m from *Toxoplasma gondii* (TOXO)[21] or listeriolysin O from *Listeria monocytogenes* (LLO)[22] were fed to mice by oral gavage on days 0, 2, and 4 and fluorophore-labeled peptide:I-A^b tetramer-binding cells were analyzed in the SLO 2 days after the last feeding. These populations, which each consist of several hundred cells in naïve mice[14], increased to about 2,000 cells in peptide-fed mice (Fig. 1e, f). Each peptide-specific population contained primarily Treg and Th^{lin-} cells with some Tfh cells but few Th1 or Th17 cells (Fig. 1g). The relatively weak expansion induced by peptide feeding was related to a lack of concomitant inflammation as shown by the finding that the 2W:I-A^b tetramer-binding population increased to 60,000 cells (Fig. 1f–h) in mice fed 2W peptide with the mucosal adjuvant, cholera toxin[23]. Although cholera toxin increased the total numbers of each subset (Fig. 1h), including the Th^{lin-} cells, the frequency of Treg and Th^{lin-} cells in the population decreased due to disproportionately large increases in the

frequencies of Th1 and Th17 cells (Fig. 1g). Treg and Th^{lin-} cells remained elevated in peptide-only fed mice 3 weeks after cessation of feeding indicating that these were stable populations (Extended Data Fig. 2a, b). Increasing the number of 2W peptide feedings from 3 to 6 had no effect on the total number of 2W:I-A^b tetramer-binding cells but increased the fraction of Treg cells while reducing the fraction of Th^{lin-} cells (Fig. 1i). Thus, Th^{lin-} and Treg cells dominated the cognate CD4$^+$ T cell populations in mice fed foreign peptides or exposed to a natural protein in the diet in the absence of inflammation, although multiple peptide feedings were needed to drive the Treg cell frequency nearer to that observed for the natural diet.

Th^{lin-} cells arise in the gut-liver SLO

We then determined where in the body cognate T cells became activated following peptide feeding. Treatment with the S1P receptor 1 agonist FTY720, which prevents lymphocyte egress from SLO[24], did not affect the number of 2W:I-A^b tetramer-binding cells in Peyer's patches and mesenteric and hepatic lymph nodes that receive material from the gut-liver axis (GLA SLO)[25], while causing a reduction in the spleen and other lymph nodes (non-GLA SLO) (Fig. 1j). The population in the GLA SLO consisted of 60% Th^{lin-}, 10% Treg, and 15% Tfh cells and had few Th1 or Th17 cells (Fig. 1k, l). A small population of tetramer-binding T cells also appeared in the lamina propria of the small intestine and colon after 3 peptide feedings (Fig. 1k, l). These populations contained a lower frequency of Th^{lin-} and Tfh and a higher frequency of Treg cells than the population in the GLA SLO and also contained some Th1 and Th17 cells. Thus, peptide feeding without an adjuvant causes Treg and Tconv cells to proliferate weakly in the GLA SLO and the Tconv cells to form a major Th^{lin-}, a minor Tfh, and trace Th1 and Th17 subsets. The Th^{lin-} cells migrate to other SLO but inefficiently to the lamina propria, while some of the fewTh1 and Th17 cells accumulate in the lamina propria along with a much larger population of Treg cells.

The Th^{lin-} population is complex

We then assessed the complexity of the Th^{lin-} population. The 2W:I-A^b tetramer-binding Th^{lin-} cells in 2W-fed mice were IL-4 reporter-negative and lacked CD49b, LAG-3, and LAP and thus were not Th2[16], Tr1[6], or Th3[7] cells (Extended Data Fig. 2c). Analysis of a large set of transcription factors, cytokine receptors, and surface proteins revealed that peptide:I-A^b tetramer-binding Th^{lin-} cells from mice fed peptide 3 times were different from naïve, Treg, and Th1 and Th17 effector cells, and related to Tfh and naturally-occurring FR4^{hi} CD73^{hi} anergic cells[18] (Extended Data Fig. 3, Extended Data Fig. 4a, Fig. 2a). Th^{lin-}, Tfh, and anergic cells expressed FR4 and more of the negative regulators CD200, BTLA, and VISTA than Th1 and Th17 effector cells and less RORγt, CD71, CD150, and PSGL1 (Extended Data Fig. 3b, Extended Data Fig. 4a). Th^{lin-} cells expressed more CTLA-4 than Tfh cells, and Tfh cells expressed more CD69 and CXCR5 than Th^{lin-} cells. A more limited analysis showed that cognate Th^{lin-} cells from mice on a gliadin-containing diet had the same protein expression pattern as the cells in peptide-fed mice (Extended Data Fig. 4b).

RNA sequencing was performed on single CD44^{hi} 2W:I-A^b, TOXO:I-A^b, or LLO:I-A^b tetramer-binding cells from mice fed 3 or 6 times with the three peptides alone or 3

times with the peptides plus cholera toxin to assess additional complexity. Naïve CD44^{lo} tetramer-negative cells were included as a control. A dimensional reduction was done by a Principal Components Analysis of the single-cell gene expression data and used to identify 11 Uniform Manifold Approximation and Projection (UMAP) cell clusters among the 4 groups (Fig. 2b, Extended Data Fig. 5a). The cells from the tetramer-negative CD44^{lo} group were almost all in cluster 2 (Fig. 2b) typified by naïve T cell markers *Sell*, *Ccr7*, *Ly6C1*, and *Satb1*[26] (Fig. 2c). The tetramer-binding population from mice fed peptide 3 times with cholera toxin contained *Bcl6*$^{+}$ *Cxcr5*$^{+}$ Tfh cells in cluster 4 (17%), *Rorc*$^{+}$ Th17 cells in cluster 6 (14%), *Tbx21*$^{+}$ *Foxp3*$^{-}$ Th1 cells in one region of cluster 5 (5%), and proliferating cells in the S (*Top2a*$^{+}$, 10%) or G2M phases (*Cenpa*$^{+}$, 6%) of the cell cycle (Fig. 2b, 2c, Table 1, Extended Data Fig. 5b). This group also contained cells in clusters 0 (12%) and 1 (22%) along with a few cells in clusters 3 (5%), 8 (3%), and 9 (3%) (Fig. 2b, Table 1). Cells in cluster 3 and 9 were similar to Tfh cells based on proximity in UMAP space to cluster 4 (Fig. 2b) and cells in clusters 8 and 9 were typified by expression of genes induced by type I interferon receptor-signaling (*Ifit3*[27]) or ongoing TCR signaling (*Nr4a1*[28]), respectively (Fig. 2c).

In contrast to peptide plus cholera toxin-fed mice, mice fed peptides alone 3 times had smaller populations of tetramer-binding Tfh (2%), Th17 (4%), and cycling cells (9%) and had some cluster 5 cells (3%) (Fig. 2b, Table 1), which in this case were mostly *Tbx21*$^{-}$ *Foxp3*$^{+}$ Treg cells (Extended Data Fig. 5b). This group also contained small populations of type I interferon-signaled (6%) or TCR-signaling (4%) cells but most of the cells were in clusters 0 (23%), 1 (33%), or Tfh-like cluster 3 (13%) (Fig. 2b, Table 1). Cluster 0 cells were similar to naïve cells based on proximity in UMAP space (Fig. 2b) and appeared to be more quiescent versions of cluster 1 cells with smaller amounts of *Anxa2* and total mRNA (Fig. 2c). Cells in clusters 0, 1, 3, 8, and 9 did not express *Tbx21*, *Bcl6*, *Cxcr5*, *Rorc*, or *Foxp3* (Fig. 2c) and thus were likely contained in the Th^{lin-} population identified by flow cytometry (Fig. 1e). These subsets expressed more of the naïve cell genes *Sell* and *Ccr7* than Th1, Th17, and Tfh cells, while the cells in Tfh-like clusters 3 and 9 had higher expression of *Izumo1r* and the negative regulator *Ctla4* than the other Th^{lin-} subsets. The tetramer-binding population from mice fed peptides alone 6 times was similar to that in mice fed peptide 3 times except that it contained a markedly greater fraction of cluster 5 Treg cells (18%) (Fig. 2b, Table 1, Extended Data Fig. 5b), which were UMAP-proximate to type I interferon-signaled cluster 8 cells (Fig. 2b), and a much lower frequency of blasting cluster 1 cells (4%) (Fig. 2b, Table 1) consistent with conversion into quiescent cluster 0 cells despite prolonged feeding. RNA sequencing showed that the Glp:I-A^{b}-specific T cell population from mice on a gliadin-containing diet also consisted of Treg cells, Tfh-like cells, and a less differentiated naïve-like Tconv subset expressing *Izumo1r* (Extended Data Fig. 6). These results confirm the flow cytometry data showing that the cognate T cell population in mice fed peptide alone consists mainly of Treg and Th^{lin-} cells and that the addition of cholera toxin increases the priming of Tfh, Th1, and Th17 cells and decreases Treg cells without completely eliminating Th^{lin-} cells. Single cell RNA sequencing, however, revealed that the Th^{lin-} population is a complex mixture dominated by Tfh-like and naïve-like subsets.

Th^{lin-} cells are anergic Treg precursors

We then investigated the functional capacity of the Th^{lin-} population. Th^{lin-} cells were worse IL-2 producers than Th1 and Th17 effector cells when challenged in vivo with peptide (Fig. 3a). When transferred into naïve recipients, the Th^{lin-} population generated Th1 cells after systemic priming with peptide and the adjuvant polyI:C[29] but less efficiently than naïve cells (Fig. 3b) and yielded more Th^{lin-} cells and peripheral Treg cells after 3 additional peptide feedings (Fig 3c). Blockade of IL-2, a cytokine known to control Treg cell survival[30], prevented transferred Th^{lin-} cells from becoming Treg cells under these conditions (Fig. 3d). Thus, the Th^{lin-} population was poor at IL-2 production and Th1 formation like anergic T cells and had the potential to become peripheral Treg cells via an IL-2-dependent process. Conversion of Th^{lin-} cells likely accounted for the increase in Treg cells between 3 and 6 peptide feedings as this increase was blocked by IL-2 neutralization (Fig. 3e).

Tregs suppress Th1 but not Th^{lin-} cells

We then determined whether Treg cells were involved in Th^{lin-} cell formation by treating *Foxp3*^{DTR} mice with diphtheria toxin during 2W peptide feeding. These mice lacked Treg cells as expected but had a 2W:I-A^{b} tetramer-binding T cell population after peptide feeding that was 10-times larger than the one in control mice (Fig. 4a, b). Tfh cells increased 20-fold, Th1 cells 100-fold, Th^{lin-} cells 2-fold, and Th17 cells not at all (Fig. 4c). Neutralization of IL-2 inhibited the increase in Tfh and Th1 cells in Treg cell-depleted mice by 50 and 90%, respectively but did not affect the modest increase in Th^{lin-} cells (Fig. 4a–c). These results suggest that Treg cells suppress the formation of cognate Tfh and Th1 cells during peptide feeding, the Th1 cells by limiting IL-2, but are not necessary for Th^{lin-} cells to form.

Treg and Th^{lin-} cells in oral tolerance

Finally, we determined whether the total cognate T cell population in peptide-fed mice had the functional impairments described in the oral tolerance literature[1,2,3]. Indeed, mice fed with 2W peptide generated a poor delayed-type hypersensitivity reaction after immunization with 2W peptide and complete Freund's adjuvant (Fig. 4d). In addition, the T cells in mice fed 2W peptide 3 times expanded 16-fold after immunization with 2W peptide plus polyI:C while T cells in unfed mice expanded 200-fold (Fig. 4e). The cognate T cell population in peptide-fed immunized mice also had a higher frequency of Treg cells and lower frequency of Th1 cells than the population in mice that were only immunized (Fig. 4f). To determine whether these effects were related to a lack of inflammation during feeding, we also tested mice that were fed 3 times with 2W peptide plus cholera toxin and then immunized with peptide plus polyI:C. These mice contained a large population of 2W:I-A^{b} tetramer-binding cells at the time of immunization as expected based on the adjuvant effect of cholera toxin (Fig. 4e). This already large population increased only slightly after immunization with peptide and polyI:C (Fig. 4e), had the lower Th1 cell but not the higher Treg cell frequency of peptide-fed primed mice, and a much higher Th17 cell frequency than the populations in the other immunized groups (Fig. 4f). Thus, the cognate T cell population in peptide-fed mice had defects in immunization-driven clonal expansion and Th1 cell generation, which were not rescued by cholera toxin. Depletion of Treg cells from *Foxp3*^{DTR} mice after

peptide feeding but before immunization increased post-priming Th1 formation but did not completely correct the defect (Fig. 4g). The residual deficit was likely related to the intrinsic impairments of Th^{lin-} cells described above (Fig. 3a, b). Thus, Treg cell suppression and Th^{lin-} cell dysfunction both play roles in oral tolerance to systemic priming.

Others have suggested that food peptide-specific $CD4^+$ naïve T cells first recognize MHCII-bound peptides on tolerogenic $CD103^+$ dendritic cells in GLA SLO[31]. We speculate that this interaction occurs with minimal costimulatory signals[32] or IL-6[33] and in the presence of IL-2-sequestering Treg cells[34]. These conditions likely explain why food peptide-specific $CD4^+$ naïve T cells proliferate poorly and do not become Th17 or Th1 cells, which depend on IL-6[35] and IL-2[36], respectively, and instead form a complex set of non-canonical Th^{lin-} subsets. One scenario that follows from the gene expression relationships shown in Fig. 2 is that food peptide-specific naïve T cells become relatively undifferentiated blasts (cluster 1) shortly after TCR signaling and then differentiate based on microenvironmental cues in the GLA-SLO. Some cells may be activated in areas lacking the Tfh-suppressing cytokine IL-2[20,37,38] and become Tfh-like cells (clusters 3 and 9), while other cells are near sources of IL-2 and become peripheral Treg cells. At the same time, other cells (cluster 8) may be near sources of type I interferon and experience signaling that induces coinhibitory receptors[39], while other cells may lack cytokine cues and revert to quiescent naïve-like cells (cluster 0). We propose that all of these different subsets lack inflammatory functions or suppress them and thus do not trigger pathology under the conditions of low innate immune activation that routinely pertain to the gut during meals. Once a food peptide-specific Th^{lin-} population has been formed, its resistance to Th1 cell formation and delayed-type hypersensitivity reactions even in the face of Th1-polarizing innate immune activation may provide another layer of protection from gut pathology, for example, during enteric infections. The Tfh-like Th^{lin-} cells, which express the anergy markers FR4 and CTLA-4, may be particularly resistant to becoming Th1 cells as suggested by other work showing that anergic T cells express a subset of Tfh genes[18,40]. Should mucosal inflammation occur at the time of first exposure to a food antigen, our results with cholera toxin suggest that some naïve cells become Tfh, Th1, or Th17 cells rather than Th^{lin-} cells, a situation that could contribute to gut pathology. Exposure to food antigens, however, usually induces a complex set of non-canonical hyporesponsive Th^{lin-} subsets that lack the inflammatory functions needed to cause gut pathology and yet have the potential to produce Treg cells that can suppress it.

ONLINE-ONLY METHODS

Mice

Six to eight-week-old C57BL/6 (B6) and B6.SJL-$Ptprc^aPepc^b$/BoyJ (CD45.1) mice were purchased from the National Cancer Institute Mouse Repository. C57BL/6-$Foxp3^{tm1Flv}$/J (*Foxp3*^{RFP}), B6.129(Cg)-$Foxp3^{tm3(DTR/GFP)}$Ayr/J (*Foxp3*^{DTR}), B6.129S(FVB)-Bcl6tm1.1Dent/J (*Bcl6*$^{fl/fl}$), B6.Cg-Tg(Lck-icre)3779Nik/J (*Lck*^{Cre}), Tg(Tbx21-ZsGreen)E3ZJfz (*Tbx21*^{ZsGreen}), and B6.129P2(Cg)-$Rorc^{tm2Litt}$/J (*Rorc*^{eGFP}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). $Il4^{tm1(CD2)Mmrs}$ (KN2) mice[41] were kindly provided by Kristin Hogquist. *Foxp3*^{RFP}, *Tbx21*^{ZsGreen}, and

Rorc^{eGFP} mice were crossed to create *Foxp3*^{RFP} *Tbx21*^{ZsGreen} *Rorc*^{eGFP} mice. SM1 TCR transgenic *Rag*$^{-/-}$ mice[42] were bred in house. *Bcl6*$^{fl/fl}$ mice were bred with *Lck*^{Cre} mice to generate *Lck*^{Cre} *Bcl6*$^{fl/fl}$ mice. Either male or female mice were used for individual experiments and the number in each group was based on past experience. Experiments were performed in an unblinded fashion. All mice were housed in specific-pathogen-free conditions according to University of Minnesota and the National Institutes of Health guidelines. The Institutional Animal Care and Use Committee of the University of Minnesota approved all animal experiments.

Tetramers

Glp (CNVYIPPYCTIAP) was identified as follows. Mice on a gliadin-free diet were immunized with wheat alpha-gliadin in complete Freund's adjuvant. One week later, cells from the draining lymph nodes were divided in into multiple samples, each cultured overnight with one peptide from a set of alpha-gliadin peptides identified by an in-house I-A^b peptide binding algorithm. The next day, an interferon-gamma enzyme-linked immune absorbent spot assay was performed for each sample according to an established protocol[43]. The CNVYIPPYCTIAP peptide gave a strong signal and was chosen for further study. Biotin-labeled I-A^b molecules containing the 2W (EAWGALANWAVDSA), TOXO (AVEIHRPVPGTA), LLO (NEKYAQAYPNVS) or Glp (CNVYIPPYCTIAP) peptides covalently attached to the I-A^b beta chain were produced with I-A^b alpha chains in *Drosophila melanogaster* S2 cells, then purified and tetramerized with streptavidin allophycocyanin (APC) or phycoerythrin (PE) as described previously[13].

Tissue preparation

Peyer's patches, lymph nodes (cervical, axillary, brachial, inguinal, hepatic, and mesenteric), and spleens were reduced to single cells suspensions by gently mashing the tissue through fine mesh screens. In some cases, mesenteric lymph nodes, hepatic lymph nodes, and Peyer's patches were pooled and used for GLA SLO, and other lymph nodes and spleen were used for non-GLA SLO. Lamina propria cells were prepared as described[44] by removing luminal material from the small intestine and colon and cutting the tissue into small pieces. The tissue fragments were treated with an EDTA solution to remove epithelial cells and washed before digestion with a collagenase solution. The digested tissue fragments were dissociated with a Gentle MACS device and centrifuged through a density gradient. Lamina propria cells were isolated from the buffy coat.

Cell enrichment and flow cytometry

Tetramer-based cell enrichment was performed as previously described[13]. Single cell suspensions from the SLO or lamina propria were incubated for 1 hour at room temperature with PE- or APC-conjugated peptide:I-A^b tetramers and BV650-conjugated CXCR5 antibodies. Cells were enriched using EasySep Mouse PE or APC Positive Selection kits (STEMCELL technologies) according to the manufacturer's instructions. Enriched samples were then stained for 30 minutes at 4°C with fluorophore-conjugated antibodies specific for CD4, B220, CD11b, CD11c, F4/80, CD44, FR4, or CD73. Cell viability was assessed using GhostDye violet 510. For analysis of the expression of transcription factors, stained cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set

(ThermoFisher) according to the manufacturer's instructions. Cells were stained overnight at 4°C with fluorophore-conjugated antibodies specific for Foxp3, T-bet, RORγt, Helios, or Bcl-6. Cells were counted and analyzed by flow cytometry with counting beads on a Fortessa (BD) flow cytometer. Data were analyzed using FlowJo software (TreeStar).

Single cell RNA sequencing

Cells from the SLO of 6 mice fed on days 0, 2, and 4 or days 0, 2, 4, 6, 8, and 10 with 2W, TOXO, and LLO peptides or on days 0, 2, and 4 with the 3 peptides plus cholera toxin were enriched with the relevant tetramers 2 days after the last feeding and tetramer-binding T cells were purified by Fluorescence Activated Cell Sorting. In another experiment, cells were enriched with Glp:I-A^b tetramer from the SLO of 14 mice exposed to a gliadin-containing diet for 1 week or with 2W:I-A^b tetramer from 1 mouse infected 7 days earlier with an attenuated *Listeria monocytogenes* (Lm) strain expressing the 2W peptide[45]. Similar results were obtained for 2W:I-A^b tetramer-binding cells from 2 other *Listeria monocytogenes*-2W-infected mice but results were included from only 1 to keep the number of Glp:I-A^b and 2W:I-A^b tetramer-binding cells in the same range. Tetramer-negative $CD44^{lo}$ naïve $CD4^+$ T cells were also sorted in both experiments. In each experiment, cells from each sample were stained with different Totalseq-A hashtag antibodies (Biolegend), mixed, and loaded into 1 port of a 10X Genomics chip at 1,500 cells/μl. Libraries were prepared for each sample and sequenced using a Novaseq S4 chip (2 × 150bp PE). Features were enumerated using Cellranger (version 3.0, 10X Genomics) and gene expression values were analyzed using Seurat 3.1.1[46]. Single cells from each sample were identified based on hashtags and normalized within each pool using the sctransform method in Seurat[47]. Different clusters were visualized using Uniform Manifold Approximation and Projection (UMAP) dimensional reduction.

Cell transfer

Single cells were collected from pooled spleens and lymph nodes of *Foxp3*^{RFP} *Tbx21*^{ZsGreen} *Rorc*^{eGFP} mice. Cells were enriched with tetramers as described above and stained with antibodies. Th^{lin-} ($CD11b^-$ $CD11c^-$ $CD8^-$ $B220^-$ Live $CD4^+$ $CD44^{hi}$ $Foxp3^-$ $CXCR5^-$ T-bet^- RORγt^-) cells or naïve ($CD11b^-$ $CD11c^-$ $CD8^-$ $B220^-$ Live $CD4^+$ $CD44^{lo}$ $Foxp3^-$) cells were sorted on a FACSAria-II (BD) flow cytometer, respectively. Sorted cells were transferred into naive SM1 TCR transgenic *Rag*$^{-/-}$ or CD45.1 congenic recipients by intravenous injection.

Measurement of protein expression levels

Spleen and lymph node cells from mice expressing different congenic markers and immunized with peptides emulsified in CFA or fed 3 times with 2W, TOXO, and LLO peptides were pooled. Magnetic enrichment with tetramers was performed on the pooled sample and the bound and unbound fractions were split into individual samples, which were stained with fluorophore-labeled antibodies specific for the congenic markers, CD44, Foxp3, CXCR5, and FR4, and 1 of the PE-conjugated antibodies from the LegendScreen panel (Bio Legend, San Diego, CA) or T-bet, RORγt, P2X7, PSGL-1, TCF1/7, FOXO1, Bcl-6, TGF-β receptor I, TGF-beta receptor II, IL-12Rb1, IL-12Rb2, IL-23R, IL-27R, EGR2, Helios antibodies from BD, ThermoFisher, or BioLegend. Tetramer-binding Th1

and Th17 cells were identified in the bound fraction from the peptide and CFA-primed group and tetramer-binding Th^{lin-} cells from the peptide fed group. $CD44^{lo}$ $Foxp3^{-}$ naive, $CD44^{hi}$ $Foxp3^{+}$ Treg, $CD44^{hi}$ $Foxp3^{-}$ $CXCR5^{+}$ Tfh, and $CD44^{hi}$ $Foxp3^{-}$ $CXCR5^{-}$ $FR4^{hi}$ $CD73^{hi}$ anergic cells were identified in the tetramer-negative $CD4^{+}$ T cell population from the unbound samples as shown in Extended Data Fig. 3a. The mean fluorescence intensity (MFI) for each PE-labeled antibody for a given T cell subset was divided by the MFI for that antibody for naïve T cells. These normalized values were used for Principal Component Analysis (PCA) and heatmaps produced with R software.

Treg depletion

In some experiments *Foxp3*^{DTR} mice were treated with diphtheria toxin (0.5 μg/mouse) on days 0, 1, and 3 during 2W peptide feeding on days 0, 2, and 4. In other experiments *Foxp3*^{DTR} mice were treated with diphtheria toxin (0.5 μg/mouse) after 2W peptide feeding on days 0, 2, and 4 and on day 9 were injected with 2W peptide plus polyI:C as described below.

Antigen administration

In most cases, mice were fed with 2W peptide or a mixture of 2W, TOXO, and LLO peptides by oral gavage, 100 μg of each peptide per feeding, on days 0, 2, and 4 or days 0, 2, 4, 6, 8, and 10 and analyzed two days after the last feeding. The 100 μg dose was chosen based on titration experiments indicating that feeding 100 μg of 2W peptide induces maximal expansion of tetramer-binding T cells. Where indicated, mice received 10 μg of cholera toxin (List labs) by oral gavage. In some cases, mice were immunized intraperitoneally with 10 μg of peptides plus 20 μg of polyI:C (Invivogen) or subcutaneously with a mixture of 100 μg each of 2W, TOXO, and LLO peptides emulsified in CFA and analyzed 7 days later. For one set of single cell RNA sequencing experiments, mice were injected intravenously with 10^{7} actA-deficient Lm bacteria engineered to secrete a fusion protein containing an immunogenic peptide called 2W (Lm-2W)[45]. The Teklad global 18% diet (Envigo) contains wheat and was used as the gliadin-containing diet (GCD). Casein-based diet AIN-93G (Envigo) was used as a gliadin-free diet (GFD).

IL-2 neutralization

IL-2 was neutralized in vivo by the intravenous injection of 400 μg of IL-2 (S4B6, BioXcell) monoclonal antibody on days 5, 7, and 9 into mice fed peptides on days 0, 2, and 4 or 4, 6, 8, and 10. T cells in these mice were analyzed by flow cytometry on day 12.

Enzyme-Linked Immunosorbent Assay

Ten-fold dilutions were prepared for each serum sample and added to 96-well plates coated with gliadin (IBL international) for 1 hour at 37° C. Plates were then incubated at 37° C for 30 minutes with horseradish peroxidase-labeled antibodies specific for mouse immunoglobulin G and developed with a 3,3’,5,5’-tetramethylbenzidine (TMB) substrate from Surmodics. A plate reader was used to measure the optical density (450 nm) of each well.

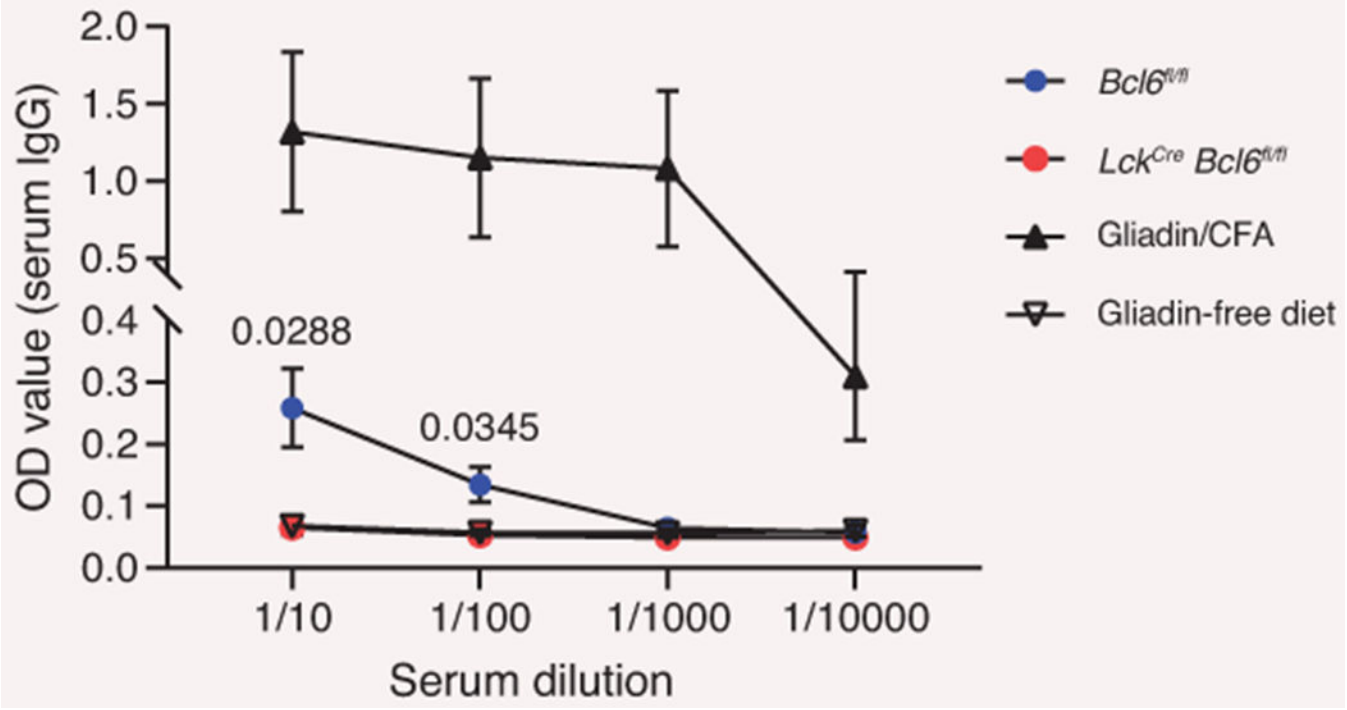
Delayed-type hypersensitivity

Mice that were or were not fed 2W peptide 3 times were injected subcutaneously with 10 ug of 2W peptide emulsified in complete Freund's adjuvant 2 days after the last feeding. Seven days after 2W peptide/complete Freund's adjuvant infection, 1 ug of 2W peptide in 10 ul of incomplete Freund's adjuvant was injected into the right footpads of the primed mice. 10 ul of PBS in IFA was injected into the left footpads as a control. Footpad swelling was measured 24 hours after footpad injection with a micro-caliper. The difference between the thickness of the right and left footpads as taken a measure of the 2W peptide-specific delayed-type hypersensitivity reaction.

Statistical analysis

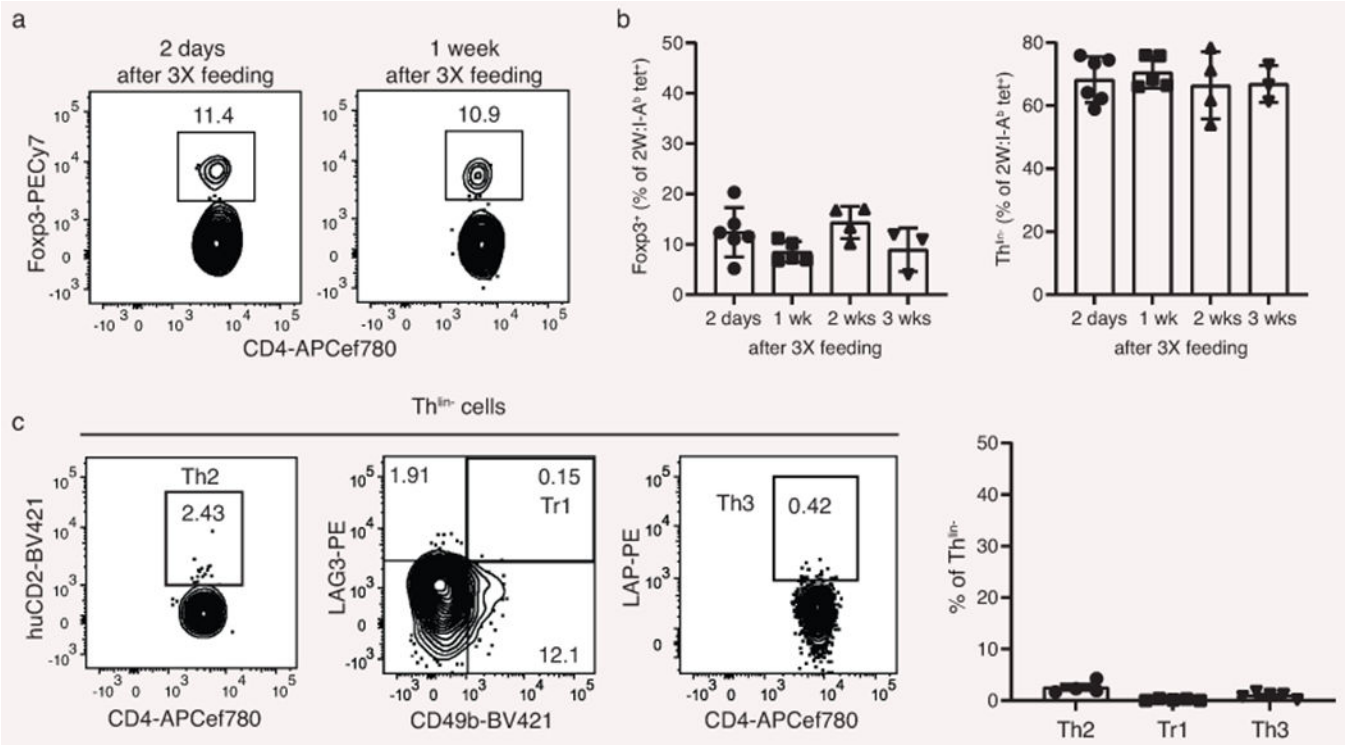
All data were presented as mean ± standard error of the mean (SEM). Data were analyzed by the tests mentioned in the figure legends. Statistical analysis was performed using Prism 9 (GraphPad).

Extended Data



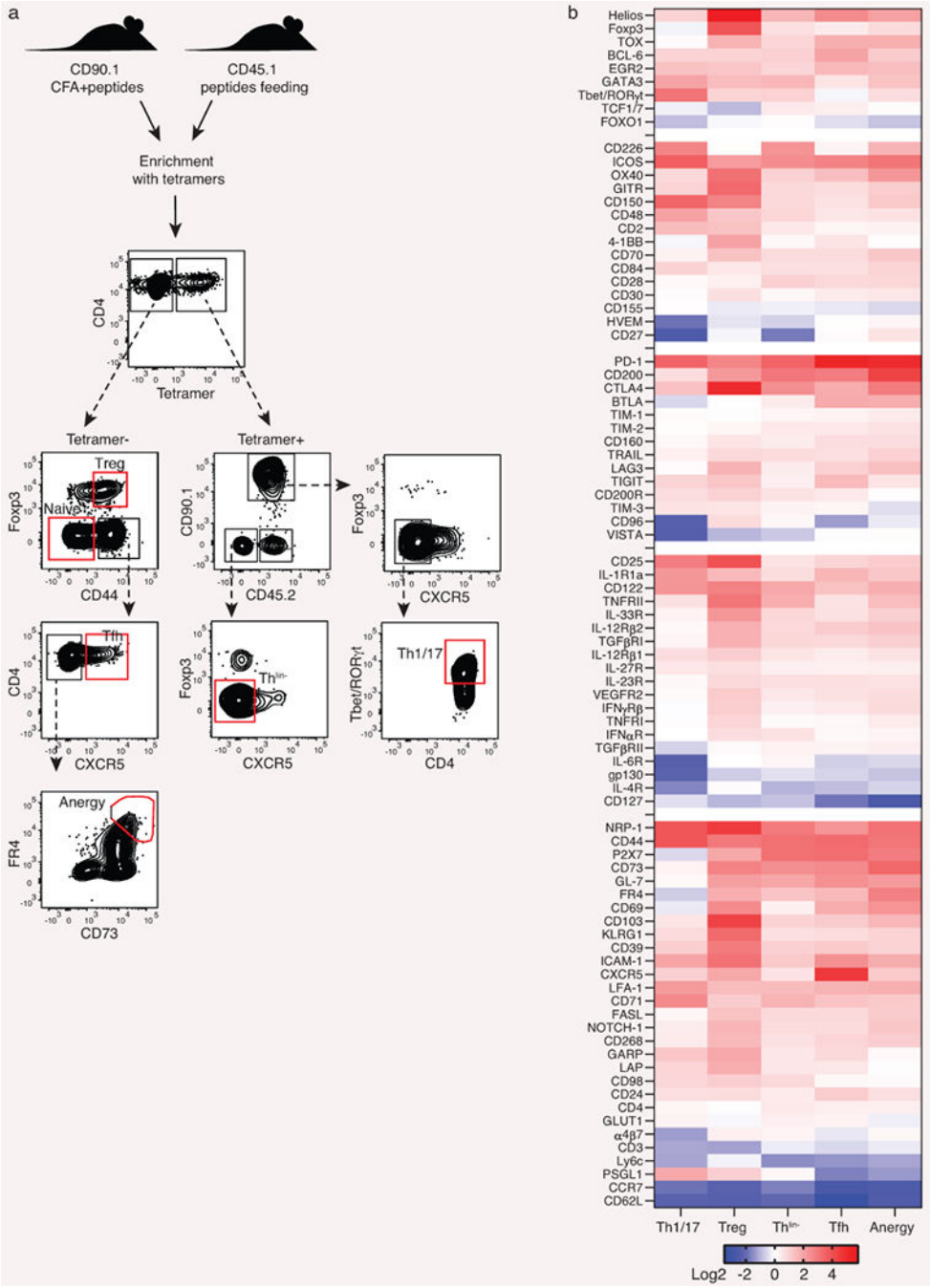
Extended Data Figure 1. Gliadin antibody production after feeding depends on Tfh cells.
Gliadin antibody amounts determined by Enzyme-Linked Immunosorbent Assay in serial dilutions of serum from *Bcl6*$^{fl/fl}$ (n=6 mice) or *Lck*^{Cre} *Bcl6*$^{fl/fl}$ mice (n=6 mice) on a gliadin-containing diet, wild-type C57BL/6 mice on a gliadin-free diet (n=2 mice), or C57BL/6 mice on a gliadin-free diet mice 2 weeks after subcutaneous injection of gliadin in complete Freund's adjuvant (n=2 mice). Wild-type and *Bcl6*$^{fl/fl}$ mice have the capacity to produce Bcl-6 and Tfh cells. *Lck*^{Cre} *Bcl6*$^{fl/fl}$ mice have a T cell-specific defect in Bcl-6 and cannot produce Tfh cells. Values for each dilution from *Bcl6*$^{fl/fl}$ or *Lck*^{Cre} *Bcl6*$^{fl/fl}$ mice were

compared by Student's t-test. The data show that Tfh-sufficient mice on a gliadin-containing diet make small amounts of gliadin antibody, while Tfh-deficient mice do not.



Extended Data Figure 2. Th^{lin-} cells induced by peptide feeding are stable after cessation of peptide feeding and lack markers of Th2, Tr1, and Th3 cells.

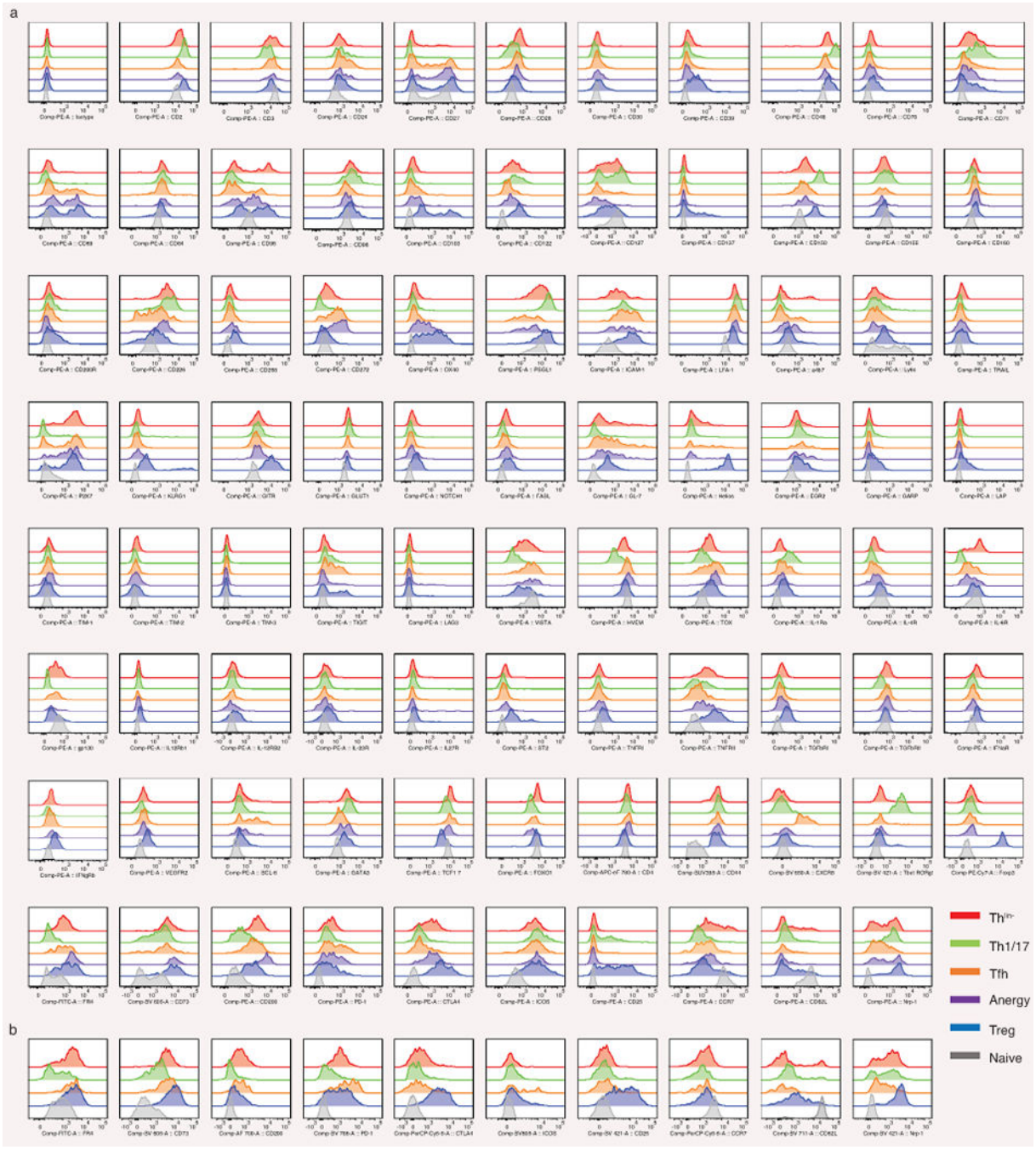
a, Flow cytometry plots of 2W:I-A^b tetramer-binding CD4$^+$ T cells from mice fed with 2W peptide 3 times and analyzed 2 days or 1 week after cessation of feeding, with a gate on Treg cells. **b**, Percentages of Treg (left) or Th^{lin-} (right) cells in 2W:I-A^b tetramer-binding populations from mice fed with 2W peptide 3 times and analyzed at the indicated times after cessation of feeding (n=6 mice for 2 days, n=5 mice for 1 wk, n= 4 mice for 2 wks, n=3 mice for 3 wks). **c**, Flow cytometry plots of 2W:I-A^b tetramer-binding Th^{lin-} cells from *Il4*^{CD2} (left plot) or wild-type (middle and right plots) mice fed with 2W peptide 3 times and analyzed 2 days after cessation of feeding for expression of human CD2 (a marker of IL-4 and Th2 cells in *Il4*^{CD2} mice), LAG-3 (a marker of Tr1 cells), or LAP (a marker of Th3 cells) (n=4 or 5 mice). The scatterplot shows the percentages of cells with these markers in the 2W:I-A^b tetramer-binding Th^{lin-} populations from individual mice.



Extended Data Figure 3. Experimental scheme used to identify CD4$^+$ T cell subsets and a heatmap of protein expression assessed by flow cytometry.

a, Experimental scheme used to identify 2W:I-A^b, TOXO:I-A^b, or LLO:I-A^b tetramer-binding Th^{lin-} cells from CD45.1 mice fed the peptides on days 0, 2, and 4 and analyzed 2 days after the last feeding, 2W:I-A^b, TOXO:I-A^b, or LLO:I-A^b tetramer-binding Th1 and Th17 cells from mice immunized 7 days earlier with the peptides in complete Freund's adjuvant (CFA), or tetramer-negative Treg, naïve, Tfh, and anergic T cells. **b**, Heatmap of the log2 values of mean fluorescence intensity (MFI) for each PE-labeled antibody for a

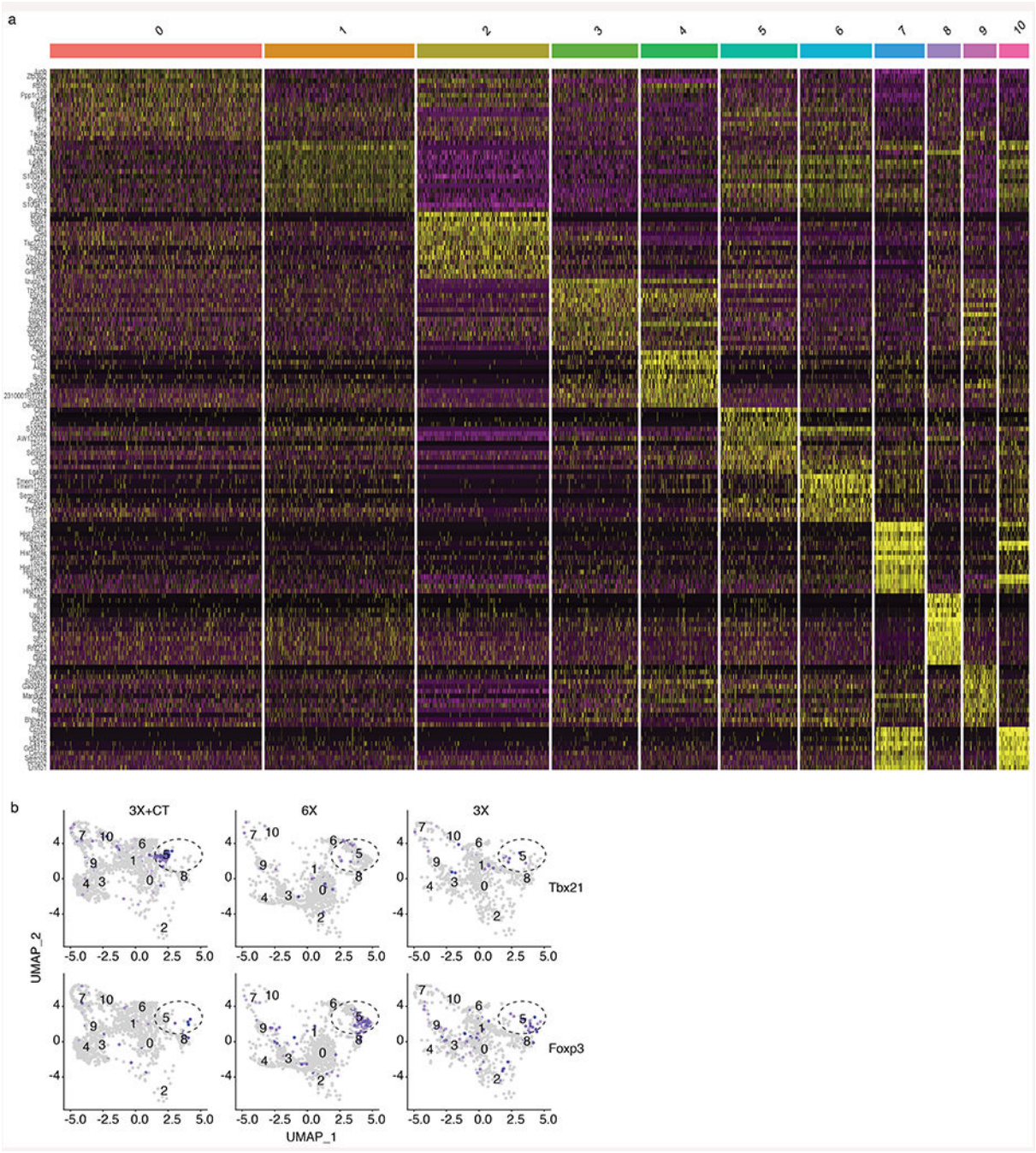
given T cell subset divided by the MFI for that antibody for naïve T cells. These normalized values were used for the PCA shown in Fig. 2a.



Extended Data Figure 4. Flow cytometry histograms of protein expression by CD4$^+$ T cell subsets.

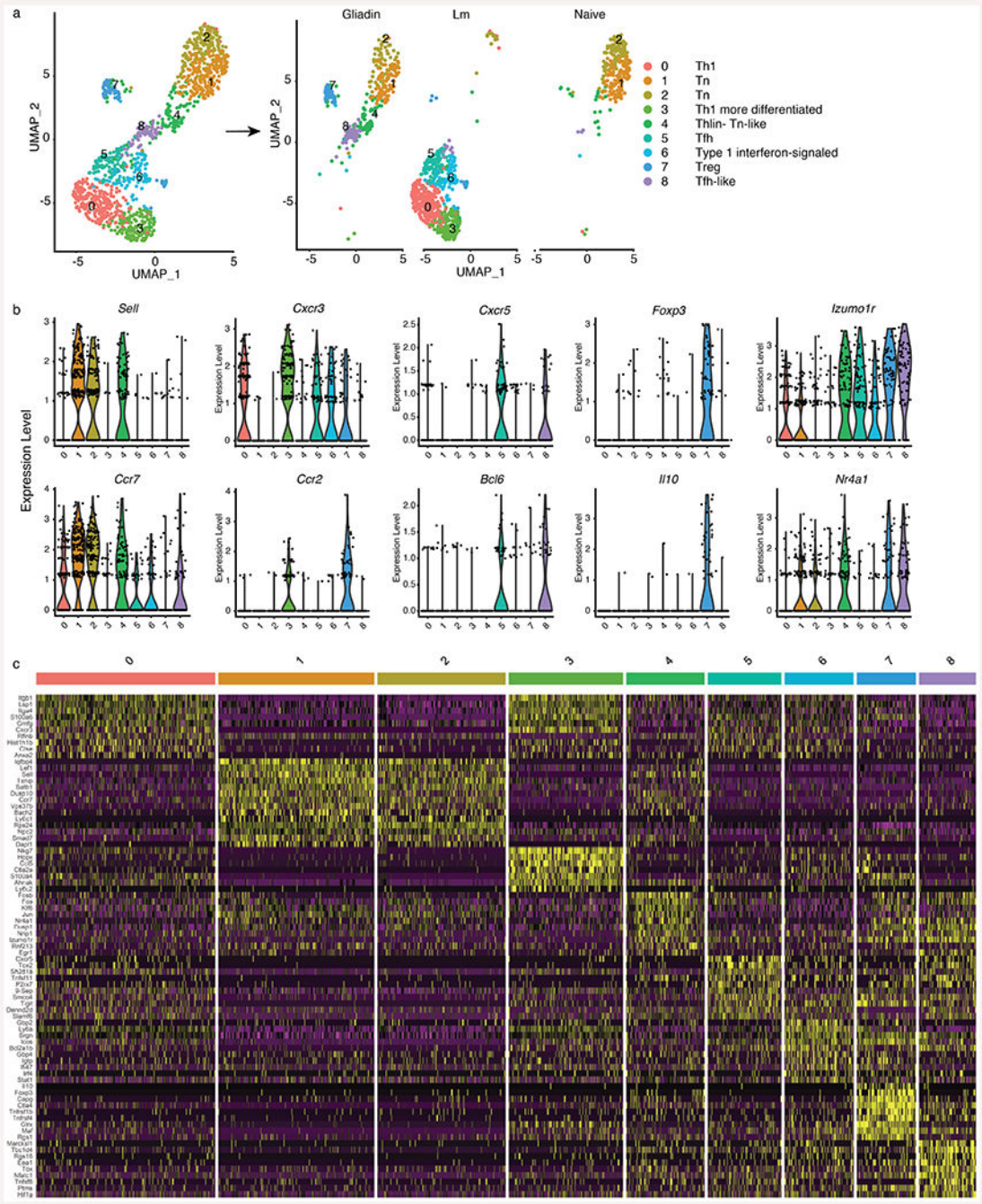
a, Histograms of antibody staining of the indicated proteins on or in the indicated subsets identified in Extended Data Figure 3a. b, Histograms of antibody staining of the indicated proteins on or in Glp:I-A^b tetramer-binding Th^{lin-} cells identified as in Extended Data Figure 3a from mice on a gliadin-containing diet for 1 week. The other subsets were identified

as in Extended Data Figure 3a except that the Th1/Th17 cells were isolated from the non-tetramer-binding CD4$^+$ T cell population.



Extended Data Figure 5. RNA sequencing data used for definition of clusters of CD4$^+$ T cells from mice fed with peptides.

a, Heat map of signature genes that defined the 11 clusters shown in Fig. 2b with yellow bars indicating larger amounts of mRNA. **b**, UMAPs shown in Fig. 2b with a border around cluster 5 and cells with larger amounts of *Tbx21* (top) or *Foxp3* (bottom) mRNA shown in purple.



Extended Data Figure 6. RNA sequencing data used for definition of clusters of CD4$^+$ T cells from mice on a gliadin-containing diet.

a, Single-cell RNA sequencing analysis of Glp:I-A^b tetramer-binding T cells from mice exposed to a gliadin-containing diet for 1 week (Gliadin), 2W:I-A^b tetramer-binding T cells from mice infected with 2W-expressing *Listeria monocytogenes* (Lm), or naïve CD4$^+$ T cells (Naïve). UMAPs identifying 9 T cell clusters in merged populations (left panel) and in each population (right panel) are shown. **b, c**, Expression levels of signature genes from each cluster are shown as violin plots (**b**) or a heat map (**c**). Note that the total Glp:I-A^b

tetramer-binding T cell population was included in the analysis not just the CD44^{hi} subset. In this analysis, clusters 1 and 2 contained naïve T cells defined by expression of *Sell*, *Ccr7*, and *Ly6C1*. The Lm-induced 2W:I-A^{b}-specific effector cell population, known to contain Th1 and Tfh cells[20], consisted of cells in clusters 0, 3, 5, and 6. Clusters 0 and 3 contained Th1 cells as evidenced by expression of the Th1 markers *Cxcr3* and *Itga4*, with cells in cluster 3 expressing markers of more advanced Th1 differentiation (*Ccr2*, *Nkg7*, and *Ly6C2*)[48–50]. Cluster 5 contained Tfh cells as evidenced by expression of the Tfh markers *Bcl6* and *Cxcr5*. Cluster 6 was defined by cells expressing interferon-induced genes (*Gbp2*, *Stat1*, *Ifi47*, *Ly6A*, and *Igtp*)[27] and may contain Tfh-related cells based on proximity in UMAP space to cluster 5. The Glp:I-A^{b} tetramer-binding population from mice on a gliadin-containing diet contained some naïve cells in clusters 1 and 2 and cells in clusters 4, 7, and 8 that were not found in the Lm-induced effector population. Cluster 7 contained Treg cells based on expression of *Foxp3*, while the cells in cluster 8 were Tfh-like cells based on expression of *Bcl6* and *Cxcr5* but were different from the genuine cluster 5 Tfh cells induced by Listeria infection. The cells in cluster 4 were defined by expression of naïve cell markers *Sell* and *Ccr7* and markers of TCR signaling such as *Nr4a1*, which were also expressed by the Treg cells in cluster 7 and the Tfh-like cells in cluster 8. Glp:I-A^{b} tetramer-binding cells in clusters 4 and 8 also expressed *Izumor1*. Cluster 4 in the Glp:I-A^{b} tetramer-binding population shown in Extended Data Figure 6a likely corresponds to Th^{lin-} naïve-like cluster 0 or 1 in the peptide-feeding experiment shown in Fig. 2b, while cluster 8 in the Glp:I-A^{b} tetramer-binding population likely corresponds to Tfh-like cluster 3 in the peptide feeding experiment. The minor cluster 8 (Th^{lin-} Treg-like type 1 interferon-signaled) and cluster 9 (Th^{lin-} Tfh-like ongoing TCR signaling) subsets that were detected in the peptide feeding experiment (Fig. 2b) were not detected in the Glp:I-A^{b} tetramer-binding population shown in Extended Data Figure 6a, perhaps because fewer cells were analyzed in this case. Thus, the cognate T cell population induced by an antigen in a natural diet and that induced by peptide feeding by gavage contained Treg cells and Th^{lin-} cells with naïve-like and Tfh-like subsets.

ACKNOWLEDGEMENT

The authors acknowledge Jennifer Walter and Charles Elwood for their help with maintaining mice and the University of Minnesota Flow Cytometry Resource for maintenance of flow cytometers used in these studies. This work was supported by NIH grant P01 AI035296.

DATA AVAILABILITY

Data used to produce scatterplots are provided in spreadsheets. Single cell RNA sequencing data can be found in the Gene Expression Omnibus (accession number GSE202423).

REFERENCES

1. Renz H et al. Food allergy. Nat Rev Dis Primers 4, 17098 (2018). [PubMed: 29300005]
2. Green PH, Lebwohl B & Greywoode R Celiac disease. J Allergy Clin Immunol 135, 1099–1106; quiz 1107 (2015). [PubMed: 25956012]
3. Pabst O & Mowat AM Oral tolerance to food protein. Mucosal Immunol 5, 232–239 (2012). [PubMed: 22318493]
4. Liu EG, Yin X, Swaminathan A & Eisenbarth SC Antigen-presenting cells in food tolerance and allergy. Front Immunol 11, 616020 (2020). [PubMed: 33488627]

5. Mowat AM To respond or not to respond - a personal perspective of intestinal tolerance. Nat Rev Immunol 18, 405–415 (2018). [PubMed: 29491358]
6. Battaglia M, Gianfrani C, Gregori S & Roncarolo MG IL-10-producing T regulatory type 1 cells and oral tolerance. Ann N Y Acad Sci 1029, 142–153 (2004). [PubMed: 15681753]
7. Weiner HL, da Cunha AP, Quintana F & Wu H Oral tolerance. Immunol Rev 241, 241–259 (2011). [PubMed: 21488901]
8. Curotto de Lafaille MA et al. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. Immunity 29, 114–126 (2008). [PubMed: 18617425]
9. Hadis U et al. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity 34, 237–246 (2011). [PubMed: 21333554]
10. Kim KS et al. Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science 351, 858–863 (2016). [PubMed: 26822607]
11. Hataye J, Moon JJ, Khoruts A, Reilly C & Jenkins MK Naive and memory CD4+ T cell survival controlled by clonal abundance. Science 312, 114–116 (2006). [PubMed: 16513943]
12. Marzo AL et al. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat. Immunol 6, 793–799 (2005). [PubMed: 16025119]
13. Moon JJ et al. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity 27, 203–213 (2007). [PubMed: 17707129]
14. Nelson RW et al. T cell receptor cross-reactivity between similar foreign and self peptides influences naive cell population size and autoimmunity. Immunity 42, 95–107 (2015). [PubMed: 25601203]
15. Moser B CXCR5, the defining marker for follicular B helper T (TFH) cells. Front Immunol 6, 296 (2015). [PubMed: 26106395]
16. Jiang S & Dong C A complex issue on CD4(+) T-cell subsets. Immunol Rev 252, 5–11 (2013). [PubMed: 23405891]
17. Kotov JA & Jenkins MK Cutting Edge: T cell-dependent plasmablasts form in the absence of single differentiated CD4(+) T cell subsets. J Immunol 202, 401–405 (2019). [PubMed: 30552165]
18. Kalekar LA et al. CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors. Nat Immunol 17, 304–314 (2016). [PubMed: 26829766]
19. Rees W et al. An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. Proc. Natl. Acad. Sci. U S A 96, 9781–9786. (1999). [PubMed: 10449771]
20. Pepper M, Pagán AJ, Igyártó BZ, Taylor JJ & Jenkins MK Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. Immunity 35, 583–595 (2011). [PubMed: 22018468]
21. Grover HS et al. The Toxoplasma gondii peptide AS15 elicits CD4 T cells that can control parasite burden. Infect Immun 80, 3279–3288 (2012). [PubMed: 22778097]
22. Geginat G, Schenk S, Skoberne M, Goebel W & Hof H A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from Listeria monocytogenes. J Immunol 166, 1877–1884 (2001). [PubMed: 11160235]
23. Lycke N The mechanism of cholera toxin adjuvanticity. Res Immunol 148, 504–520 (1997). [PubMed: 9588829]
24. Matloubian M et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427, 355–360 (2004). [PubMed: 14737169]
25. Ohtani N & Kawada N Role of the gut-liver axis in liver inflammation, fibrosis, and cancer: a special focus on the gut microbiota relationship. Hepatol Commun 3, 456–470 (2019). [PubMed: 30976737]
26. Marshall HD et al. Differential Expression of Ly6C and T-bet Distinguish Effector and Memory Th1 CD4(+) Cell Properties during Viral Infection. Immunity 35, 633–646 (2011). [PubMed: 22018471]
27. MacMicking JD IFN-inducible GTPases and immunity to intracellular pathogens. Trends Immunol 25, 601–609 (2004). [PubMed: 15489189]

28. Moran AE et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J Exp Med 208, 1279–1289 (2011). [PubMed: 21606508]
29. Longhi MP et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 206, 1589–1602 (2009). [PubMed: 19564349]
30. Fontenot JD, Rasmussen JP, Gavin MA & Rudensky AY A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat. Immunol 6, 1142–1151 (2005). [PubMed: 16227984]
31. Coombes JL et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 204, 1757–1764 (2007). [PubMed: 17620361]
32. Cong Y, Weaver CT & Elson CO The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. J Immunol 159, 5301–5308 (1997). [PubMed: 9548469]
33. Bromander AK, Kjerrulf M, Holmgren J & Lycke N Cholera toxin enhances alloantigen presentation by cultured intestinal epithelial cells. Scand J Immunol 37, 452–458 (1993). [PubMed: 8469928]
34. Wong HS et al. A local regulatory T cell feedback circuit maintains immune homeostasis by pruning self-activated T cells. Cell 184, 3981–3997 e3922 (2021). [PubMed: 34157301]
35. Bettelli E et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238 (2006). [PubMed: 16648838]
36. Liao W, Lin JX, Wang L, Li P & Leonard WJ Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol 12, 551–559 (2011). [PubMed: 21516110]
37. Ballesteros-Tato A et al. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. Immunity 36, 847–856 (2012). [PubMed: 22464171]
38. Johnston RJ, Choi YS, Diamond JA, Yang JA & Crotty S STAT5 is a potent negative regulator of TFH cell differentiation. J Exp Med 209, 243–250 (2012). [PubMed: 22271576]
39. Sumida TS et al. Type I interferon transcriptional network regulates expression of coinhibitory receptors in human T cells. Nat Immunol 23, 632–642 (2022). [PubMed: 35301508]
40. Trefzer A et al. Dynamic adoption of anergy by antigen-exhausted CD4(+) T cells. Cell Rep 34, 108748 (2021). [PubMed: 33567282]
41. Mohrs K, Wakil AE, Killeen N, Locksley RM & Mohrs M A two-step process for cytokine production revealed by IL-4 dual-reporter mice. Immunity 23, 419–429 (2005). [PubMed: 16226507]
42. McSorley SJ, Asch S, Costalonga M, Reinhardt RL & Jenkins MK Tracking Salmonella-specific CD4 T cells in vivo reveals a local mucosal response to a disseminated infection. Immunity 16, 71–83 (2002).
43. Leehan KM & Koelsch KA T Cell ELISPOT: for the identification of specific cytokine-secreting T cells. Methods Mol Biol 1312, 427–434 (2015). [PubMed: 26044024]
44. Qiu Z & Sheridan BS Isolating lymphocytes from the mouse small intestinal immune system. Feb 28;(132):57281. J Vis Exp (2018).. [PubMed: 29553537]
45. Ertelt JM et al. Selective priming and expansion of antigen-specific Foxp3- CD4+ T cells during Listeria monocytogenes infection. J Immunol 182, 3032–3038 (2009). [PubMed: 19234199]
46. Stuart T et al. Comprehensive integration of single-cell data. Cell 177, 1888–1902 e1821 (2019). [PubMed: 31178118]
47. Hafemeister C & Satija R Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. Genome Biol 20, 296 (2019). [PubMed: 31870423]
48. Bonecchi R et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 187, 129–134 (1998). [PubMed: 9419219]
49. Glatigny S, Duhen R, Oukka M & Bettelli E Cutting edge: loss of alpha4 integrin expression differentially affects the homing of Th1 and Th17 cells. J Immunol 187, 6176–6179 (2011). [PubMed: 22084440]

50. Swarnalekha N et al. T resident helper cells promote humoral responses in the lung. Sci Immunol 6 (2021). 6(55):eabb6808.

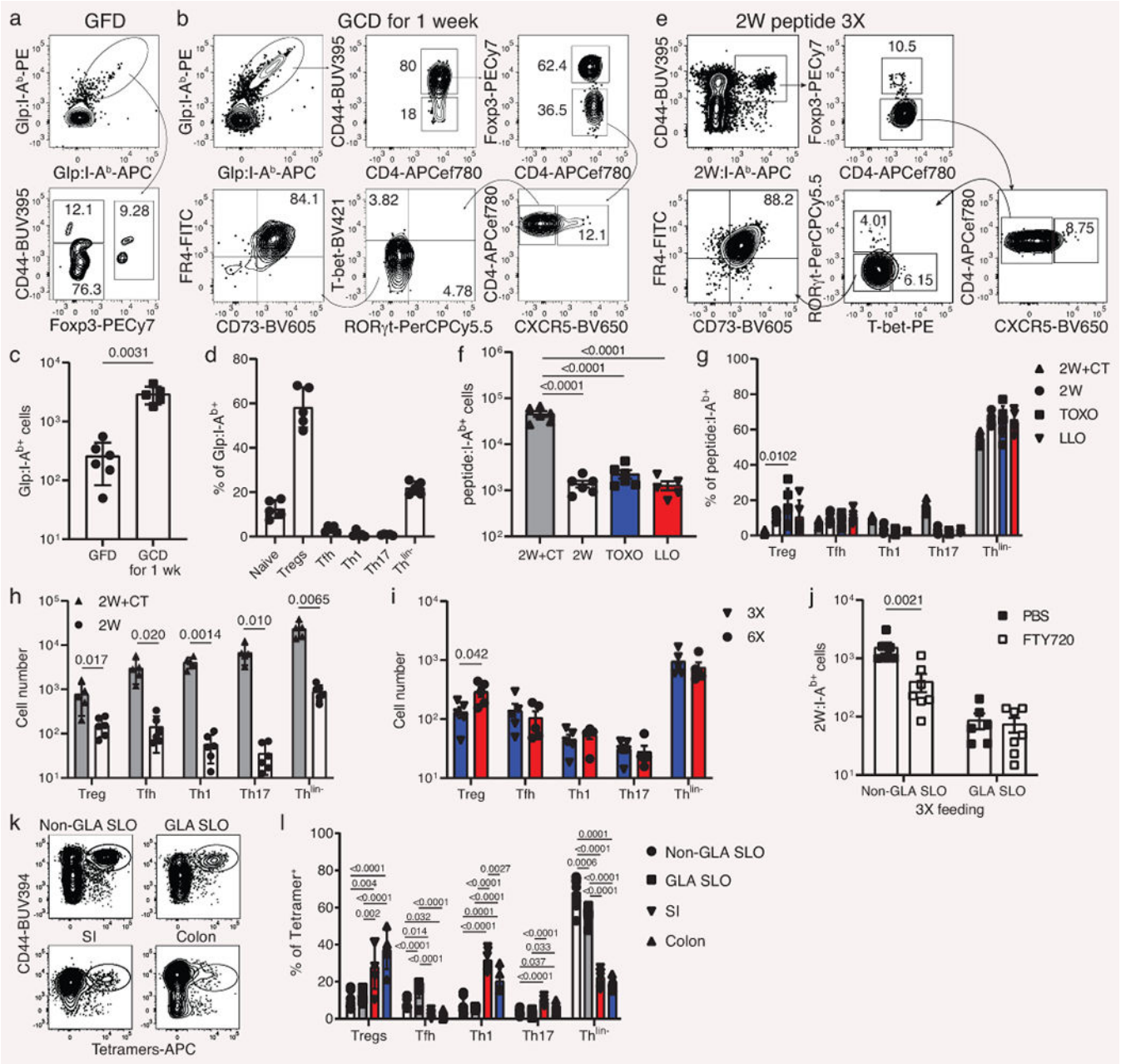


Figure 1. Food antigen-specific T cells mainly become Treg or Th^{lin-} cells in the GLA SLO.

a, b, CD4^{+} T cells from SLO of mice on gliadin-free (GFD) (**a**) or gliadin-containing diets (GCD) for 1 week (**b**). **c, d**, Glp:I-A^{b} tetramer^{+} cell numbers (n=6 mice) (**c**) and frequencies of subsets in the GCD group (**d**) (n=5 mice, 3 independent experiments). **e-h**, (**e**) CD4^{+} T cells from SLO of mice fed with the peptides alone or with cholera toxin on days 0, 2, and 4 and analyzed 2 days after the last feeding (3X protocol) with tetramer^{+} cell numbers (**f**, n=6 mice for 2W+CT, 2W, and TOXO, and n=5 for LLO, 3 independent experiments) and subset frequencies (**g**, n=4 mice for 2W+CT, n=6 for 2W and TOXO, and n=5 for LLO, 3 independent experiments). **h**, 2W:I-A^{b} tetramer^{+} cell numbers in the 2W and 2W+CT groups shown in **g** (n=5 mice for 2W+CT, n=6 in 2W, 3 independent experiments). **i**, 2W:I-A^{b}

tetramer$^+$ cell numbers in the SLO of mice fed on the 3X protocol or on days 0, 2, 4, 6, 8, and 10 (6X) with 2W peptide alone and analyzed 2 days after the last feeding (n=5 mice, 2 independent experiments). **j**, 2W:I-A^b tetramer$^+$ cell numbers in mice fed on the 3X protocol with 2W peptide alone (closed square, n=6 mice, 3 independent experiments) or with FTY720 treatment (open square, n=7 mice, 3 independent experiments). **k**, CD4$^+$ T cells in the indicated tissues from mice fed with 2W, TOXO, and LLO peptides on the 3X protocol. **l**, Frequencies of subsets among the tetramer$^+$ populations shown in **k** (n=9 mice for Non-GLA and GLA, n=5 for SI and Colon, 3 independent experiments). Error bars indicate mean ± SEM. Data in **f** and **l** were compared by one-way ANOVA and post hoc Tukey's test, and in **c**, **h, i**, and **j** by two-tailed Student's t test.

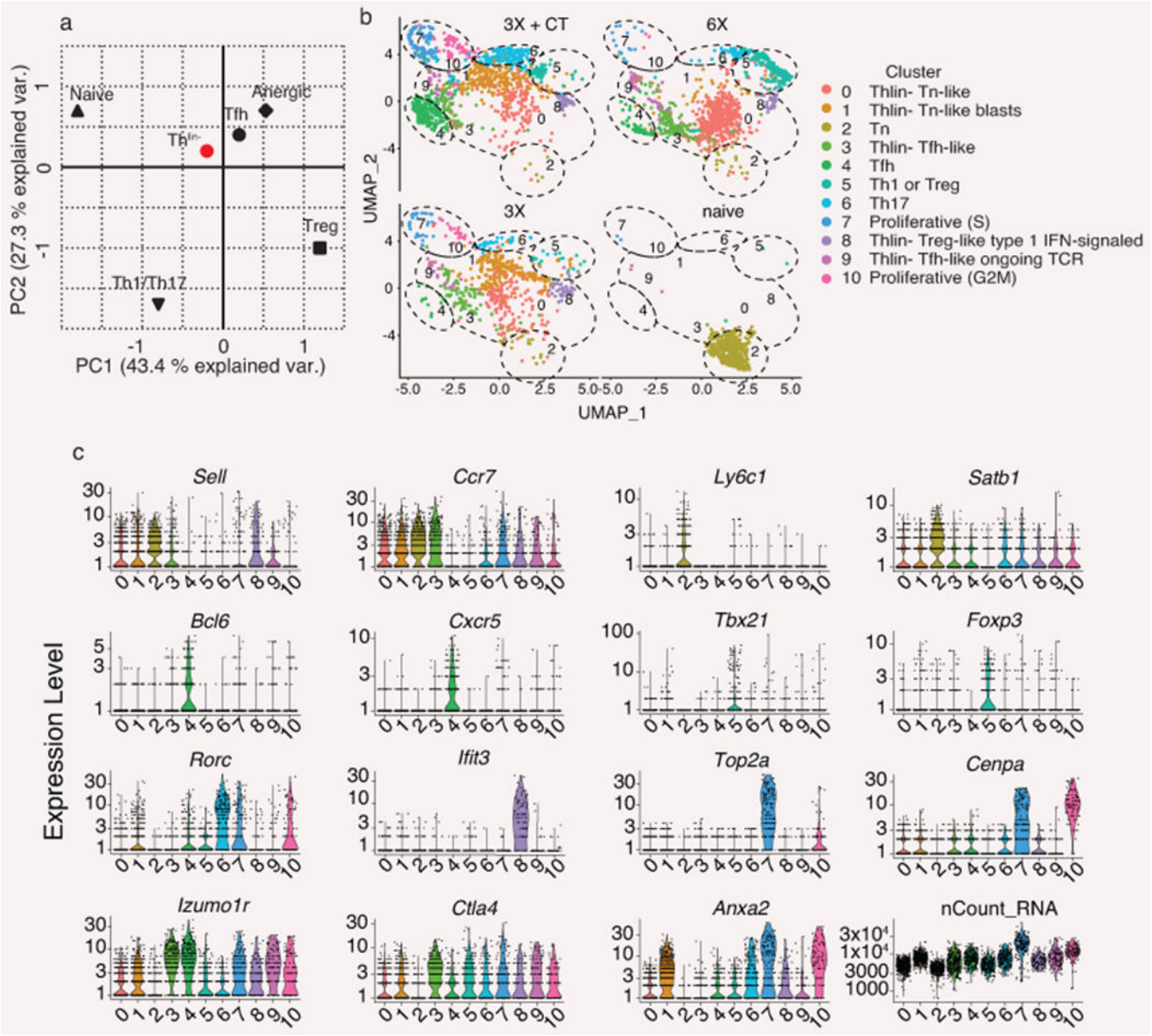


Fig. 2. The Th^{lin-} population is mixture of naïve-like and Tfh-like cells that are related to anergic T cells.

a, Principal Components Analysis plot based on flow cytometry measurements of proteins expressed by naïve, Treg, Anergic, Tfh, Th1 and Th17, and Th^{lin-} cells identified as shown in Extended Data Fig. 3a. **b, c**, Single-cell RNA sequencing analysis of CD44^{hi} 2W:I-A^{b}, TOXO:I-A^{b}, or LLO:I-A^{b} tetramer-binding T cells from the SLO of mice fed on days 0, 2, and 4 (3X) or 0, 2, 4, 6, 8, and 10 (6X) with the 3 peptides alone or days 0, 2, and 4 with the 3 peptides plus cholera toxin (3X+CT) or CD44^{lo} tetramer-negative CD4^{+} T cells (naïve). Tetramer-binding T cells from the fed groups were sorted 2 days after the last feeding. **b**, UMAPs identifying T cell clusters. Ovals delineate individual clusters 2, 4, 5, and 6, or clusters 7 and 10 containing proliferating cells, or the set of Th^{lin-} clusters 1, 2, 3, 8, and 9. **c**, Violin plots showing expression levels of signature genes.

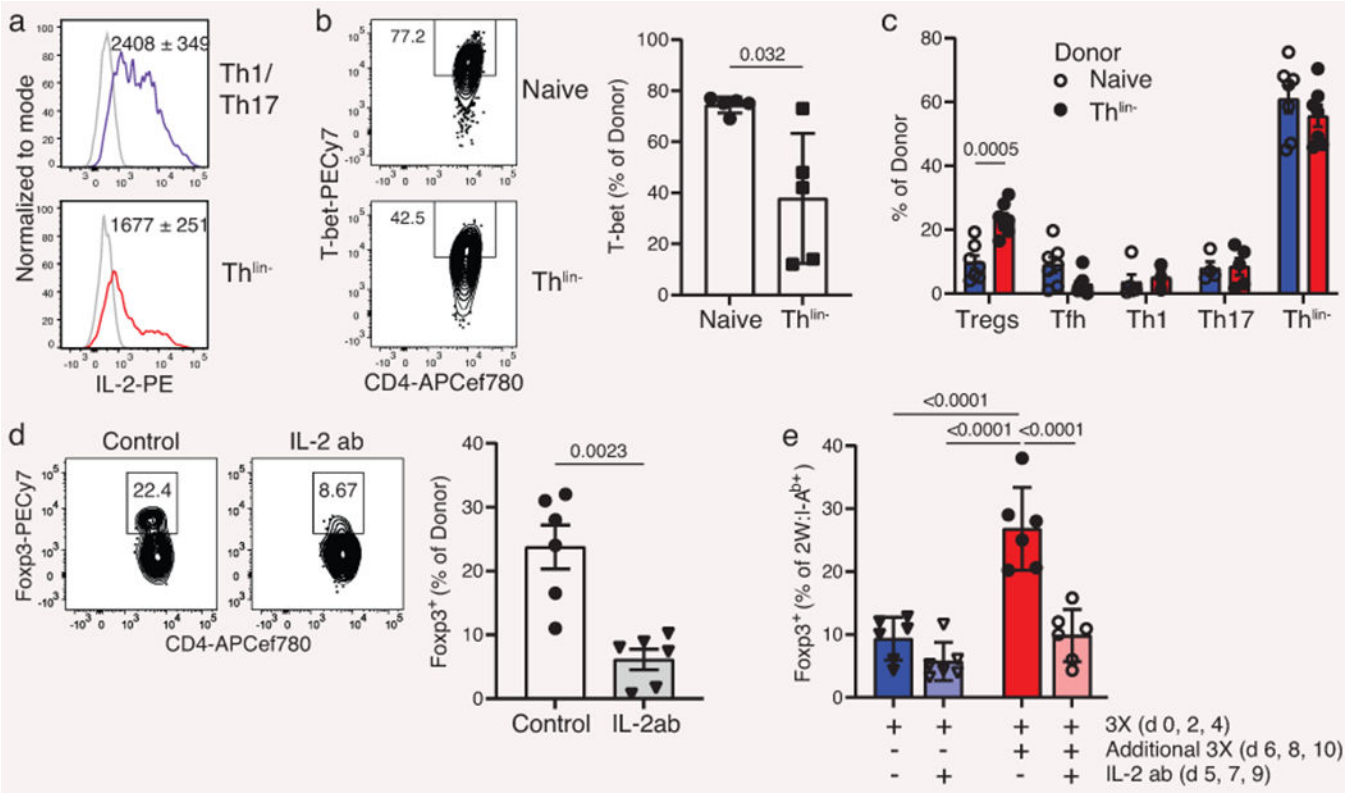


Fig. 3. The Th^{lin-} population is hyporesponsive and generates peripheral T reg cells.

a, IL-2 production by 2W:I-A^b tetramer-binding Th1 and Th17 cells from mice immunized with 2W peptide in complete Freund's adjuvant or Th^{lin-} cells from mice fed 3 times with 2W peptide, 2 hours after intravenous injection of 2W peptide. Mean fluorescent intensity values ± SEM of IL-2 antibody staining are shown. Gray histograms are from CD44^{lo} CD4$^+$ T cells. **b**, CD44^{lo} CD4$^+$ T cells or Th^{lin-} cells sorted from 2W:I-A^b, TOXO:I-A^b, and LLO:I-A^b tetramer-enriched samples from *Foxp3^{RFP} Rorc^{eGFP} Tbx21^{Zsgreen}* reporter mice fed 3 times with 2W, TOXO, and LLO were transferred into CD90.1 SM1 TCR transgenic *Rag$^{-/-}$* mice, which were immunized with polyI:C and peptides and analyzed after 7 days. Frequencies of T-bet$^+$ Th1 cells in recipient mice among tetramer-binding cells from the indicated donors are shown (n=5 mice, 2 independent experiments). **c**, CD44^{lo} CD4$^+$ T cells and Th^{lin-} cells sorted as in **b** were transferred into CD45.1 mice, which were fed 3 times with peptides. Frequencies of subsets among tetramer-binding cells from the indicated donors 2 days after the last feeding are shown (n=5 mice, 3 independent experiments). **d**, Th^{lin-} cells sorted as in **b** were transferred into CD45.1 mice, which were fed 3 times with peptides without (None) or with injection of IL-2 antibody (IL-2 ab) during feeding. Frequencies of Treg cells among donor-derived tetramer-binding cells in the 2 groups are shown (n=6 mice, 2 independent experiments). **e**, 2W peptide was fed and IL-2 antibody was injected as indicated. Treg cell frequencies among CD44^{hi} 2W:I-A^{b+} T cell populations analyzed 2 days after the last feeding (n=6 mice, 2 or 3 independent experiments) are shown. Error bars indicate mean ± SEM. Data in **e** were compared by one-way ANOVA and post hoc Tukey's test, and in **c** and **d** by two-tailed Student's t test.

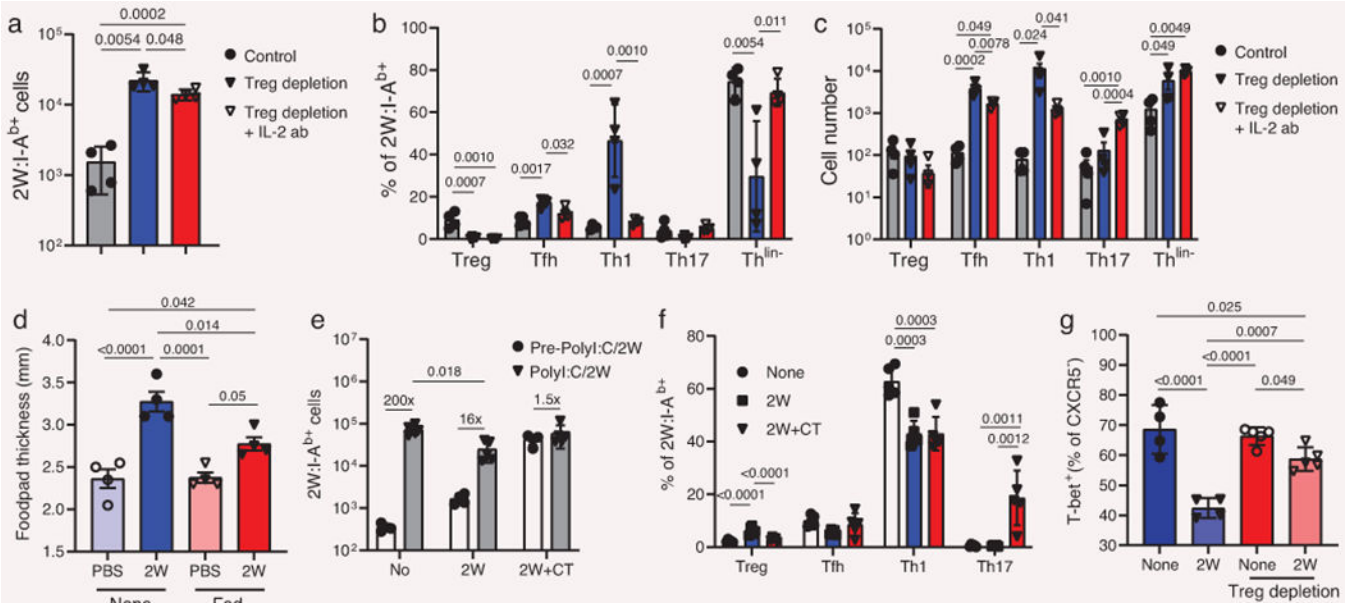


Figure 4. Treg cells inhibit Th1 cell formation during feeding by sequestering IL-2 and after feeding and priming.

a-c, 2W:I-A^{b} tetramer^{+} cell numbers in *Foxp3^{DTR}* mice that were not (gray bars) or were (blue bars) treated with diphtheria toxin (DT) and fed with 2W peptide 3 times or were treated with DT, fed 2W peptide 3 times, and treated with IL-2 antibody during peptide feeding (red bars) and analyzed 2 days after the last feeding (**a**) with the frequencies (**b**) and numbers (**c**) of cells in subsets in each group (n=4 mice, 3 independent experiments). **d**, Footpad thickness of mice fed with nothing or with 2W peptide on days 0, 2, and 4, primed with 2W peptide in complete Freund's adjuvant, and injected with 2W peptide in the footpad 1 day before measurement (n=4 mice, 1 experiment). **e**, 2W:I-A^{b} tetramer^{+} cell numbers in SLO of mice fed no 2W peptide, or on days 0, 2, and 4 with 2W peptide alone or with cholera toxin (CT) and enriched with 2W:I-A^{b} tetramer on day 6 (open bars, n=4 mice, 2 independent experiments), or from mice from those groups primed systemically with 2W peptide plus polyI:C 2 days after the last feeding and enriched with 2W:I-A^{b} tetramer 7 days later (gray bars, n=5 mice, 2 independent experiments). Fold increases in post-peptide/polyI:C priming numbers compared to the pre-priming numbers are shown. **f**, Frequencies of indicated subsets among 2W:I-A^{b} tetramer^{+} cells in the post-peptide/polyI:C primed groups shown in **e** (n=5 mice, 2 independent experiments). **g**, T-bet^{+} Th1 cell frequencies among 2W:I-A^{b} tetramer^{+} cells from naïve or 2W peptide-fed *Foxp3^{DTR}* mice that were (red bars, n=4 mice, 2 independent experiments) or were not (blue bars, n=5 mice, 2 independent experiments) treated with DT before immunization with 2W peptide plus poly I:C and analyzed 7 days later. Error bars indicate mean ± SEM. Data were compared by one-way ANOVA and post hoc Tukey's test.

Table 1.

Frequencies of T cell subsets in fed mice

Cluster	Cell type	3X	3X+CT	6X	Naive
0	Th^{lin-} Tn-like	23.0	12.4	40.9	0.0
1	Th^{lin-} Tn-like blasts	32.7	21.9	4.3	0.0
2	Tn	3.3	0.6	2.6	97.5
3	Th^{lin-} Tfh-like	12.7	5.4	14.1	0.2
4	Tfh	1.6	17.2	5.3	0.0
5	Th1 or Treg	2.7	4.8	17.8	0.7
6	Th17	4.3	14.0	5.4	0.0
7	Proliferative (S)	5.5	10.2	1.4	0.4
8	Th^{lin-} Treg-like type1 IFN-signaled	6.3	3.4	3.1	0.0
9	Th^{lin-} Tfh-like ongoing TCR	3.7	3.3	4.4	0.9
10	Proliferative (G_2M)	3.7	6.4	0.3	0.0

Tn = naïve T cell. Numbers in columns 3-6 present the percentages of the indicated cell types in the single cell RNA sequencing clusters shown in Figure 2b in naïve mice or mice fed peptides 3 times (3X), 6 times (6X), or 3 times with cholera toxin (3X+CT).