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**Graphical abstract**
Follicular regulatory T cells inhibit the development of granzyme B–expressing follicular helper T cells

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Introduction

Abs with high affinity for antigen (Ag) are produced by B cells that are selected in the germinal center (GC) reaction (1–3). T follicular helper (TFH) cells are critical for this selection process (1–3). TFH cells provide survival and proliferative signals to GC B cells via CD40 ligand as well as the secretion of key cytokines such as IL-4 and IL-10 (1–3). Ab responses are also modulated by T follicular regulatory (TFR) cells, a subset of Foxp3+ regulatory T cells that express CXCR5 and localize to the GC (4–6). Both TFR cells and TFH cells are dependent on the transcriptional repressor protein Bcl6 for their development (3–7). TFR cells are derived from Foxp3+ regulatory T (Treg) cells and have been reported to repress excessive TFH cell and GC B cell proliferation as well as promote the selection of high-affinity B cells (8–11). The suppressor mechanisms used by TFR cells to suppress the GC are not well defined. Furthermore, a few recent studies have challenged the suppressor model of TFR cell function (reviewed in ref. 9).

A mouse model in which the Bcl6 gene is specifically deleted in Foxp3+ T cells (Bcl6-flox/Foxp3-Cre, or Bcl6FC mice), blocking TFR cell development, has been used to precisely target TFR cell development in vivo (7, 12–14). This Bcl6FC mouse model has helped to show that TFR cells can help promote the Ag-specific IgG and GC response, in part by expressing the cytokine IL-10, which can promote GC B cell growth (7, 12). However, the full functions of TFR cells in the GC remain unclear and particularly it is unknown if TFR cells use other mechanisms besides IL-10 to help augment GC B cell responses and Ab production. Here we examined TFH cell gene expression in TFR-deficient mice and have been reported to repress excessive TFH cell and GC B cell proliferation as well as promote the selection of high-affinity B cells (8–11). The suppressor mechanisms used by TFR cells to suppress the GC are not well defined. Furthermore, a few recent studies have challenged the suppressor model of TFR cell function (reviewed in ref. 9).

A mouse model in which the Bcl6 gene is specifically deleted in Foxp3+ T cells (Bcl6-flox/Foxp3-Cre, or Bcl6FC mice), blocking TFR cell development, has been used to precisely target TFR cell development in vivo (7, 12–14). This Bcl6FC mouse model has helped to show that TFR cells can help promote the Ag-specific IgG and GC response, in part by expressing the cytokine IL-10, which can promote GC B cell growth (7, 12). However, the full functions of TFR cells in the GC remain unclear and particularly it is unknown if TFR cells use other mechanisms besides IL-10 to help augment GC B cell responses and Ab production. Here we examined TFH cell gene expression in TFR-cell–deficient and TFR-cell–amplified mouse strains and we find that TFR cells are critical for inhibiting the development of an aberrant TFH cell subset with cytotoxic potential. Our data show that TFR cells control TFH gene expression and point to a mechanism for how TFR cells can promote the GC and Ab response. Our findings also raise the intriguing prospect that TFH cells can control the GC and Ab response by a cytotoxic mechanism.
Results

TFR cells promote stronger GC B cell and IgE responses. To study Ag-specific Ab responses, we used a classic model of food allergy induced by challenge intragastrically (i.g.) with peanut protein plus cholera toxin (PCT) (15). In this model, high levels of peanut-specific IgE are induced and maintained in circulation for weeks after immunization in normal mice. To test the role of TFR cells, we used 3 conditional knockout strains with altered TFR cell development: “Bcl6FC” mice with Bcl6 deletion in Foxp3+ cells and so lack TFR cells (7), “Blimp1FC” mice with Prdm1 (Blimp1) deletion in Foxp3+ cells leading to augmented TFR cells (16), and “DKO” mice with deletion of both Bcl6 and Prdm1 in Foxp3+ cells. All of these mouse strains were grossly healthy with no obvious disease up to 10 weeks of age. As shown in Figure 1A, TFR cells were largely ablated in Bcl6FC and DKO mice but were increased about 2-fold over WT levels in Blimp1FC mice, similar to previously reported observations (16). The lack of TFR cells in DKO mice shows that Bcl6 has a dominant role in promoting TFR cell development over repression by Blimp1. Loss of TFR cells did not affect TFH cell numbers in Bcl6FC mice; however, there was a marked increase in both Blimp1FC and DKO TFH cells (Figure 1B). Blimp1 is required for IL-10 expression by Tregs (17), indicating that Tregs from both Blimp1FC and DKO mice are defective in IL-10 expression. Therefore, the data in Figure 1B suggest that, more than TFR cells, Treg-derived IL-10 controls the expansion of TFH cells. In contrast, GC B cell numbers showed a clear positive correlation with TFR cells in the 4 mouse strains (Figure 1C). There was a 5- to 6-fold lower ratio of GC B cells to TFH cells in TFH-deficient mice compared with TFR-sufficient mice, indicating that TFH cells increase the helper function of TFH cells in the GC (Figure 1, D and E). Analysis of peanut-specific Ab titers revealed that TFR cells were also required for sustained and robust peanut-specific IgE and IgG1 responses in this model (Figure 1F). Overall, these findings support the idea that TFR cells act as helper cells in the GC and promote the Ag-specific IgE response.

TFR cells inhibit the development of aberrant cytotoxic gene–expressing TFH cells, particularly in the context of an inflammatory environment. To better understand how TFR cells were influencing the ability of TFH cells to help GC B cells, we used RNA sequencing (RNAseq) to profile gene expression in TFH cells from PCT-challenged WT, Bcl6FC, Blimp1FC, and DKO mice (Figure 2 and Supplemental Figure 2A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.128076DS1). TFH gene expression was strongly affected by loss of TFR cells, leading to several hundred up- and downregulated differentially expressed genes (DEGs) for both Bcl6FC and DKO TFH cells (Figure 2A). Gzmb (granzyme B), a gene associated with cytotoxic T cells, stood out as being strongly elevated in TFH cells from both Bcl6FC and DKO mice, strains both lacking TFR cells (Figure 2A). Compared with WT TFH cells, Gzmb was increased 9-fold in Bcl6FC TFH cells and increased 66-fold in DKO TFH cells. Comparing DKO TFH cells to Blimp1FC TFH cells, Gzmb was increased 173-fold in DKO TFH cells. Despite the high number of DEGs, the expression of key TFH genes was essentially normal in Bcl6FC and DKO TFH cells (Figure 2B). To better understand the nature of the DEGs in Bcl6FC and DKO TFH cells, we compared upregulated DEGs from our data set with published gene expression data sets. We found a highly significant enrichment of genes that were at least 2-fold upregulated in effector CD8+ T cells (18) or more than 2-fold downregulated in Stat4−/− Th1 cells (19) within the upregulated DEGs from Bcl6FC and DKO TFH cells (Figure 2C and Supplemental Figure 1). Next, we selected a set of 23 genes that were highly upregulated in Bcl6FC and DKO TFH and also associated with CD8+ effector T cell differentiation (refs. 18, 20, and Figure 2D). About two-thirds (15 of 23) of these genes were both upregulated during CD8+ effector T cell differentiation (Figure 2E) and also positively regulated by Stat4 (Figure 2F). Unexpectedly, the expression of the CD8+ effector genes was nearly identical between WT and Blimp1FC TFH cells, even though Blimp1FC mice have defective Treg cell function and develop inflammatory disease (21, 22). CD8+ cytotoxic effector genes were upregulated to a higher degree in DKO TFH cells than in Bcl6FC TFH cells (Figure 2, C and D), indicating that loss of TFR cells together with loss of Blimp1 in Tregs in the DKO mice promoted a synergistic loss of control in TFH cell differentiation. Blimp1FC and DKO Tregs cannot produce IL-10 due to Blimp1 deficiency and DKO Tregs cannot develop into TFR cells due to Bcl6 deficiency. The deficiency of Treg-derived IL-10 could lead to an inflammatory environment in vivo that contributes to the greatly increased development of cytotoxic-like TFH cells in DKO mice. Additionally, despite significant overlap of the cytotoxic T cell gene program with Stat4 function, the cytotoxic gene program appeared to be a unique non-Th1 differentiation state, as there were no significant increases in Ifng and Tsx21 (Tbet) (Figure 3A), nor was there an enrichment of Tbet target genes in the cytotoxic TFH genes from Bcl6FC and DKO mice (Figure 3B). We then
Figure 1. TFR cells are required for proper GC B cell numbers in a food allergy immune response. WT, Bcl6FC, Blimp1FC, and DKO mice were orally immunized twice with peanut protein plus cholera toxin (PCT). Four weeks after the last PCT immunization (day 36), spleens (SP) were analyzed for the indicated cell populations by flow cytometry. Representative contour dot plots for each cell staining are shown along with graphs showing average percentage of cells as a fraction of parental cell population. (A) Analysis of CD4+FOXP3+PD-1+CXCR5+ TFR cells. Average TFR cells per group are quantitated as a percentage (%) of FOXP3+CD4+ T cells, and absolute number (#). (B) Analysis of CD4+FOXP3–PD-1+CXCR5+ TFH cells. Average TFH cells are quantitated as a percentage of FOXP3–CD4+ T cells, and absolute number. (C) Analysis of B220+CD38–GL7+ GC B cells. Average GC B cells per group graphed as a percentage of B220+ cells and as absolute number. (D and E) Ratio of GC B cells to TFH cells from data in A–C. (F) Titers of peanut-specific IgE and IgG1 by ELISA at day 36. Graphs show the mean ± SEM. P values were calculated by t test, where *P < 0.05, **P < 0.01, ***P < 0.0001. n = 4–6 mice. Data shown are representative of 3 experiments with similar results. ANOVA with Tukey’s post hoc analysis was used to determine statistical significance.
FIGURE 2. TFR cells repress a cytotoxic gene program in TFH cells. WT, Bcl6FC, Blimp1FC, and DKO mice were immunized twice with PCT (n = 4). Four weeks after the last immunization, TFH cells were isolated by FACS, RNA was then prepared and subjected to RNAseq. (A) Volcano plots showing differentially expressed genes (DEGs) for Bcl6FC versus WT (155 genes up, 410 genes down) and DKO versus Blimp1FC (517 genes up, 1041 down). Blue indicates downregulated genes and purple upregulated genes, using FDR < 0.05 after multiple-test correction and fold change (FC) < -1.8 or > 1.8 (linear). Gzmb is specifically marked in both plots. Gzmb is increased 9-fold in Bcl6FC TFH cells compared with WT TFH cells, and Gzmb is increased 173-fold in DKO TFH cells compared with Blimp1FC TFH cells. (B) Heatmap showing expression assessed with RNAseq of 21 hallmark TFH genes sorted alphabetically. Color scale shows log2RPKM of gene expression. (C) Heatmap showing expression assessed with RNAseq of 21 hallmark TFH genes sorted alphabetically. Color scale shows log2RPKM of gene expression. (D) A set of 23 genes was used to create heatmaps based on log2RPKM gene expression for WT, Bcl6FC, Blimp1FC, and DKO TFH cells; (E) naive and effector CD8+ T cells; and (F) WT and Stat4−/− Th1 cells. In E and F, FCs for the paired sets of genes are shown by a single-column heatmap. See Methods for statistical analysis of gene data sets.

Discussion

In this study, we define a cytotoxic-like TFH cell subset that is repressed by TFR cells and is promoted by proinflammatory signals (virus infection, loss of Treg cell function). These cytotoxic-like TFH cells express high levels of granulocyte B and Eomes proteins and express several other genes associated with CD8+ cytotoxic T cell function. Since the main function of TFH cells is to interact with B cells in the GC, cytotoxic-like TFH cells have the potential to kill B cells in the GC. Our findings therefore open up a possible new regulatory pathway for the GC and high-affinity Ab production. Additionally, TFH cells have been generally considered to repress the GC and Ab response (8–11). However, a recent study showed that TFH cells expressing IL-10 can act as helpers and promote Ag-specific IgG production and the GC response (12). Our data in this study show a potent helper effect of TFR cells on the GC that correlates with suppressed regulatory pathway for the GC and high-affinity Ab production. Additionally, TFR cells have been generally considered to repress the GC and Ab response (8–11). However, a recent study showed that TFH cells expressing IL-10 can act as helpers and promote Ag-specific IgG production and the GC response (12). Our data in this study show a potent helper effect of TFR cells on the GC that correlates with suppressed development of cytotoxic-like TFH cells. As shown in Supplemental Figure 2, injection of WT Tregs capable of developing into TFR cells into DKO mice repressed the abnormal production of cytotoxic-like TFH cells. Thus, TFR cells actively regulate the differentiation of TFH cells into different functional phenotypes. Furthermore, we have revealed what we believe is a new mechanism of TFR cell help in the GC response: inhibiting the development of cytotoxic-like TFH cells.
Our analysis of TFH cells from SIV-infected macaques indicates that the cytotoxic-like TFH cell phenotype that develops in the absence of TFR cells can also develop in a normal (TFR cell sufficient) GC response after virus infection. Granzyme B–expressing circulating TFH cells have been found in humans, and the development of this population is Stat3 dependent (25). However, we were unable to find an enrichment of Stat3 target genes in our TFH cell data (data not shown), suggesting there may be different origins for these circulating granzyme B–expressing TFH cells. Here, we saw a strong correlation of TFH cytotoxic genes with Stat4 transcriptional function. Higher Stat4 activity may indicate that IL-12 is increased in the GC environment in the absence of TFR cells. One key function of Tregs is to downmodulate dendritic cell function, including the secretion of cytokines such as IL-12 (26, 27). Like Tregs, TFR cells may function to suppress dendritic cells, but act in the GC and/or B cell follicle.

Very recently, granzyme B–expressing TFH cells have been found in GCs in human tonsillitis (28). How these human cytotoxic TFH cells develop is not clear, but they appear to be a defective or exhausted state of TFH cell differentiation induced by chronic infection (28). It is tempting to speculate that defective TFR cell function leads to the development of these human granzyme B–expressing TFH cells, but more research is required for answering this question. However, our results show that this cytotoxic TFH cell

Figure 3. Expression of Th1 genes in TFH cells, Tbet regulation of cytotoxic genes, and cytotoxic TFH genes upregulated after SIV infection. (A) Gene expression plots for Ifng, Tbx21 (Tbet), and Prdm1 (Blimp1) taken from the RNAseq data analyzed in Figure 2. WT (red) TFH cell average mRNA levels were set to 1 and fold changes are shown for Bcl6FC (blue), Blimp1FC (green), and DKO (magenta) TFH cells. The mRNA levels are taken from log2RPKM counts. n = 4. ANOVA with Tukey’s post hoc analysis was used to determine statistical significance. (B) Heatmap of the gene set of 23 CD8+ cytotoxic T cell genes described in Figure 2, D–F, versus published gene expression data for WT and Tbet-KO T cells (GSE105806). (C) Comparison of fold changes in genes from TFH cells from SIV+ infected macaques over uninfected TFH cells from uninfected macaques (published RNAseq data GSE69756). As a group, the 23 CD8+ cytotoxic T cell genes described in Figure 2, D–F, show a statistically significant increase within the SIV+ TFH RNAseq data set over the SIV− TFH RNAseq data set (‘cytotoxic’ column), in comparison with all other noncytotoxic genes expressed in the TFH cells, which on average showed no statistically significant upregulation between the 2 types of TFH cells (‘other’ column). For statistical significance of overlap between 2 sets of genes, the P value was calculated based on hypergeometric distribution.
pathway is conserved between mouse and humans and increases the possibility that TFR cells also control the development of human granzyme B–expressing TFH cells.

Overall, our findings raise the possibility that cytotoxic TFH cells are a normal part of the GC response and that granzyme B+ TFH cells kill GC B cells. These cytotoxic-like TFH cells not only suppress the Ab response, as in the case of PCT immunization, but can fine-tune the types of IgG isotypes within the Ab response as shown with SRBC immunization (Supplemental Figure 3). Increased cytotoxic TFH cells in DKO mice correlated with a significant shift from in the ratio of IgG1 to IgG2b anti-SRBC Ab (Supplemental Figure 3). Cytotoxic TFH cells may also play a role in controlling virus infection in the GC, as we observed higher levels of cytotoxic genes in TFH cells after SIV infection. Augmenting the development of these aberrant TFH cells may be beneficial after virus infection. Cytotoxic CD4+ T cells have been studied previously by many investigators, but were never identified as a TFH cell subset (24). How TFR cells suppress granzyme B–expressing TFH cells and the precise function of granzyme B–expressing TFH cells will be important areas for future research.

Figure 4. Cytotoxic gene expression in TFH cells and increased GC B cell apoptosis in TFR-deficient mice. Cytotoxic gene expression in TFH cells and increased GC B cell apoptosis in TFR-deficient mice. WT, Bcl6FC, Blimp1FC, and DKO mice were immunized with SRBCs and 9 days later, spleen cells were analyzed by flow cytometry for CD4+FoxP3+CXCR5+TFH cells and staining with (A) anti–granzyme B (Gzmb) Ab, (B) anti–Eomesodermin (Eomes) Ab, or (C) both Gzmb and Eomes Abs. Average Gzmb+ (A), Eomes+ (B), and Gzmb+Eomes+ (C) TFH cells are quantitated and graphed as a percentage of TFH cells, and absolute number. A total of 100,000 cells were analyzed by flow cytometry and TFH cells (0.5%–3%, 500–3,000) were gated for further analysis. n = 4; experiment was repeated 3 times. ANOVA with Tukey’s post hoc analysis was used to determine statistical significance.
Methods

Mice. Foxp3-Yfp-cre only (WT) and Foxp3-Yfp-cre Bcl6-flox (Bcl6FC) mice were previously described (7, 29). Prdm1-flox mice (30) were crossed with Bcl6FC transgenic mice to generate Foxp3-Yfp-cre Prdm1-flox (Blimp1FC) and Foxp3-YFP-cre Prdm1-flox Bcl6-flox (DKO) mice. Male and female mice 6–10 weeks old were used. Mice were bred under specific pathogen–free conditions at the laboratory animal facility at Indiana University School of Medicine (IUSM).

Immunizations. On days 1 and 8, food was withdrawn from mice for 2 hours followed by gavage of 300 μL per mouse with 1.5% NaHCO₃ in water i.g. One hour later, mice were orally immunized i.g. with 1 mg per mouse of peanut extract (Greer Laboratories) together with 10 μg per mouse of cholera toxin (Sigma-Aldrich) (15). For SRBC immunization, mice were injected i.p. with 1 × 10⁹ SRBCs (Rockland Immunochemicals). Mice were sacrificed on indicated days and serum, mesenteric lymph nodes, and spleens were harvested.

Figure 5. Cytotoxic gene expression is enriched in Klrg1⁺ TFH cells. WT, Bcl6FC, Blimp1FC, and DKO mice were immunized with SRBCs and spleens were analyzed for Klrg1 on TFH cell populations by flow cytometry. (A) Analysis of CD4⁺FOXP3⁺CXCR5⁺PD-1⁺ TFH cells for Klrg1 expression 9 days after immunization. Average Klrg1⁺ TFH cells are quantitated and graphed as a percentage of TFH cells, and absolute number. n = 4; experiment was repeated 3 times. Cells (100,000 total) were analyzed by flow cytometry and TFH cells (0.5%–3%, 500–3,000) were gated for further analysis. ANOVA with Tukey’s post hoc analysis was used to test for significant differences. (B) TFH cells from 4 DKO mice (n = 3) were separated into Klrg1⁺ and Klrg1⁻ populations by FACS, and RNA was prepared directly after sorting. RT-qPCR was used to analyze gene expression in Klrg1⁺ and Klrg1⁻ TFH cells. Graphs show relative expression for the indicated genes with the mRNA level in Klrg1⁻ cells set to 1. This experiment was done twice with similar results. Approximately 15,000 Klrg1⁺ and approximately 500,000 Klrg1⁻ TFH cells were sorted per mouse. Student’s t test was used to detect significant differences.
Flow cytometry. Cell suspensions were filtered through a 40-μm cell strainer (Fisherbrand). Cells were washed and diluted in PBS with 1% FBS and were stained with Fc block (BioXCell) for 5 minutes, followed by surface staining for the indicated markers. The following labeled Abs were used: anti-CXCR5 (L138D7), anti–PD-1 (29F.1A12), anti-CD4 (RM4-5), anti-FOXP3 (MF-14), anti-CD38 (90), and anti-B220 (RA3-6B2) were obtained from BioLegend; anti-GL7 (GL7) Ab was purchased from BD Pharmingen. Anti–annexin V Ab was obtained from BioLegend. For intracellular staining, after surface markers were stained, cells were fixed and stained with anti–granzyme B (GB11) and anti-Eomes (Dan11mag) using an eBioscience intracellular kit. All samples were acquired on an LSR2 flow cytometer (Becton Dickinson) and analyzed with FlowJo software.

Ab measurement. Titers of peanut-specific Ab in serum were measured by ELISA, as previously reported (31). For peanut-specific IgE, 96-well Nunc-Immuno plates (Sigma-Aldrich) were coated with IgE Ab (LO-ME-3, Bio-Rad) overnight at 4°C. Wells were blocked with 1% BSA and diluted serum was added and incubated at room temperature for 2 hours. Peanut extract protein was labeled with biotin (Sigma-Aldrich) and added for 1 hour followed by adding poly-HRP streptavidin (Pierce Endogen) for 30 minutes.

Cell sorting, RNA isolation, and gene expression analysis. On day 36 after PCT challenge, CD4+ T cells were isolated from the spleen from mice using a CD4 T cell Macs isolation kit (Miltenyi Biotec). Cells were stained for CD4, CXCR5, and PD-1, and TFH cells were isolated by FACS. For isolation of Klrg1+ cells, mice were immunized with SRBC and TFH cells sorted as above but anti-Klrg1 Ab (2F1/KLRG1, BioLegend) was also used for staining. Total RNA was collected from freshly sorted cells using the RNaseasy Plus Micro kit (QIAGEN). qPCR reactions were performed as previously reported (32).

RNAseq and bioinformatics analysis. RNAseq was performed by the IUSM Center for Molecular Genomics. KAPA mRNA HyperPrep methods were used for mRNA sequencing. Total RNA was evaluated for its quantity and quality using an Agilent Bioanalyzer 2100. For RNA quality, a RIN number of 7 or higher was used. Fifty nanograms of total RNA was used. cDNA library preparation included mRNA purification/enrichment, RNA fragmentation, cDNA synthesis, ligation of index adaptors, and amplification, following the KAPA mRNA Hyper Prep Kit Technical Data Sheet, KR1352 – v4.17 (Roche Corporate). Each resulting indexed library was quantitated and quality accessed by Qubit and Agilent Bioanalyzer. Multiple libraries were pooled in equal molarity. Five microliters of 2-nM pooled libraries per lane were denatured, neutralized, and applied to the cBot for flow cell deposition and cluster amplification, before loading onto the HiSeq 4000 for 75-bp paired-end sequencing (Illumina, Inc.). Approximately 30 million reads per library were generated. A Phred quality score (Q score) was used to measure the quality of sequencing.
More than 90% of the sequencing reads reached Q30 (99.9% base call accuracy). For sequence alignment and gene counts, the sequencing data were first assessed using FastQC (Babraham Bioinformatics) for quality control. Then all sequenced libraries were mapped to the mouse genome (mm10) using STAR RNA-seq aligner with the parameter “--outSAMmapqUnique 60.” The reads distribution across the genome was assessed using bamutils (from ngsutils). Uniquely mapped sequencing reads were assigned to mm10 refGene genes using featureCounts (from subread) with the parameters “-s 2 –p –Q 10.” Quality control of sequencing and mapping results was summarized using MultiQC. Genes with read count per million (CPM) less than 0.5 in more than 4 of the samples were removed. The data were normalized using the trimmed mean of M values (TMM) method. Differential expression analysis was performed using edgeR. False discovery rate (FDR) was computed from P values using the Benjamini-Hochberg procedure. DEGs were determined if their P values were less than 0.05 after multiple-test correction with FDR adjustment and the amplitudes of fold changes (FCs) were larger than 1.8. RNAseq data were submitted to the NCBI Gene Expression Omnibus under accession number GSE133533.

Statistics. Flow cytometry and qPCR data analysis was done using GraphPad Prism software. Graph bars represent the mean ± SEM. Unless otherwise stated, Student’s t test (2-tailed) or ANOVA with Tukey’s post hoc analysis were used to determine significance. Significant differences (P < 0.05) are indicated in the figures. In order to examine the statistical significance of overlap between 2 sets of genes, we calculated the P value based on hypergeometric distribution.

Study approval. Mice were handled according to protocols approved by the IUSM Institutional Animal Use and Care Committee (IACUC).

Author contributions
MMX helped design the study, performed all the biological experiments, analyzed the results, and prepared most of the figures. SF and JW performed bioinformatics analyses of the RNAseq data, integrated results with other published data sets, supported data interpretation, generated figures (Figure 2, Figure 3B, and Supplemental Figure 1), and wrote the methods part of the manuscript. QC performed some of the experiments. HL managed the mouse breeding and genotyping. ALD helped design the study, analyzed the results, and wrote the manuscript.

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