

Supporting information for

**Extracellular adenosine triphosphate skews the T helper cells
balance and enhances neutrophils activation in mice with food
allergy**

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1. DETAILS OF EXPERIMENTAL METHODS

1.1. Preparation of single-cell suspensions

To prepare single-cell suspensions from spleens and mesenteric lymph nodes (MLNs), the separated organs were ground by using a piston rod of a 1 mL syringe and a 70- μ m cytoscreener (258365, NEST Biotechnology). After removing red blood cells with RBC lysis buffer (R1010, Solarbio), the lymphocytes were centrifuged and resuspended with RPMI 1640 medium containing 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin for later experiments. The single-cell suspensions obtained from the lamina propria (LP) of the small intestine were prepared following previous protocols but with some modifications^{1, 2}. In summary, after removing fat tissue, mice small intestines were longitudinally opened and gently washed with cold PBS to remove fecal content. The washed segments were shaken in HBSS containing 5 mM EDTA, 1 mM DTT, and 10 mM HEPES for 30 minutes at 37 °C. Next, the intestinal segments were cut into 1 cm of small pieces and incubated with RPMI 1640 medium containing 10% of FBS, 200 U/mL collagenase VIII (C2139, Sigma-Aldrich), and 0.15 mg/mL DNase I (DN25, Sigma-Aldrich) for 60 minutes at 37 °C in a shaking bath. After filtering, the digested tissues were washed with HBSS and subjected to a 40% and 80% of Percoll density-gradient centrifugation for the isolation of lymphocytes residing in LP. The isolated single-cell suspensions were collected for later experiments.

1.2. Isolation of neutrophils

Neutrophils were extracted from mouse bone marrow based on reported techniques but with some modifications³. Briefly, the treated bone marrow cells were resuspended in complete RPMI 1640 medium and gently dropped onto the 65% and 78% Percoll solutions. Neutrophils were recovered from a monolayer between the two Percoll phases following an 800 g, 35-minute centrifugation. After being verified by flow cytometry, the produced neutrophils were seeded into a 96-well cell culture plate at a density of 1×10^5 cells per well.

1.3. Cell stimulation

2×10^6 of MLN single-cell suspensions obtained from DO11.10 mice were cultured for 72 hours in complete RPMI 1640 medium with the following stimuli: 200 μ g of OVA alone, 200 μ g of OVA mixed with 10 μ M of oATP, or 200 μ g of OVA mixed with different concentrations (10 μ M, 100 μ M, 1 mM, 2 mM) of ATP (R0441, Thermo Fisher Scientific). After 3-day incubation, the stimulated cells were harvested and used for flow cytometry analysis. 1×10^5 of neutrophils from mouse bone marrow were first incubated with 100 ng/ml of LPS (L4391, Sigma) at 37 °C for 3 hours. After that, 10 μ M, 100 μ M, 500 μ M, or 1 mM of ATP were added into the medium and incubated for an additional 21 hours. The supernatant and stimulated cells were then collected for later experiments.

1.4. Flow cytometry

Single-cell suspensions obtained from indicated tissues were incubated with Fixable Viability Stain 780

(565388, BD Bioscience) for live/dead cell discrimination and stained with FITC-conjugated-anti-CD4 (100405, Biolegend), PE-conjugated-anti-CD25 (101904, Biolegend), Brilliant Violet 421-conjugated-anti-GATA3 (653814, Biolegend), PE-Cyanine7-conjugated-anti-T-bet (25-5825-82, eBioscience), PerCP-eFluor 710-conjugated-anti-ROR γ t (46-6981-82, eBioscience), and Alexa Fluor 647-conjugated-anti-FOXP3 (126408, Biolegend) for gating T helper cells (Th1 cell: CD4⁺T-bet⁺, Th2 cell: CD4⁺GATA3⁺, Th17 cell: CD4⁺ROR γ t⁺, Treg cell: CD4⁺CD25⁺Foxp3⁺), or stained with FITC-conjugated-anti-CD45 (103108, Biolegend), eFluor 450-conjugated-anti-CD11b (48-0112-82, eBioscience), and Brilliant Violet 605-conjugated-anti-Ly6G (127639, Biolegend) for discriminating neutrophils. For some experiments, antibodies were changed to PE-conjugated-anti-mouse DO-11.10 clonotypic TCR (551772, BD Bioscience), Brilliant Violet 605-conjugated anti-CD4 (100451, Biolegend), and BB515-conjugated anti-CD25 (564424, BD Bioscience) for avoiding channel overlap. Cell apoptosis experiment was performed by using an APC Annexin V Apoptosis Detection Kit (640932, Biolegend) according to the manufacturer's instructions. All experiments were carried out on a CytoFLEX S flow cytometer.

1.5. RNA isolation and quantitative real-time PCR

About 1 cm of segment was taken from each ileum for RNA extraction, and the total RNA was extracted by using RNAiso Plus (9109, TaKaRa) according to the manufacturer's instructions. The resulting RNA was resuspended in nuclease-free water and stored in aliquots at -80 °C after measuring concentrations by a NanoDrop RNA concentration analyzer (Thermo Fisher Scientific, Waltham, USA). Next, 0.6 μ g total RNA of each sample was retrotranscribed into cDNA by utilizing a Reverse Transcription Kit (R323-01, Vazyme) according to the manufacturer's instructions. Finally, triplicate cDNA samples were subjected to RT-qPCR by using a ChamQ Universal SYBR qPCR Master Mix (Q711-02/03, Vazyme) on an Applied Biosystems QuantStudio 5 (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Primers used in this experiment were listed in Table S1. The levels of gene transcription were normalized to GAPDH and calculated based on the \log_2 - $\Delta\Delta$ Ct method.

1.6. Histology

About 1 cm of clean ileum was collected and fixed in 4% formaldehyde and embedded in paraffin for later staining with hematoxylin and eosin. The immunohistochemistry experiments were carried out by utilizing the myeloperoxidase polyclonal antibody (PA5-16672, Thermo Fisher Scientific) or tryptase monoclonal antibody (19523S, Cell Signaling Technology) according to the manufacturer's instructions. The histopathological scores of the resulting tissues were evaluated based on three aspects according to previous reports ^{4,5}: 1) Inflammation: grade 0: rare granulocytes and isolated cells were observed in the mucosa between villi and crypts; grade 1: few granulocytes scattered in the epithelial layer and lamina propria; grade 2: less than ten granulocytes aggregated in the mucosa; grade 3: two to three solitary groups of up to ten granulocytes aggregation; grade 4: over three solitary groups of granulocytes and larger than ten granulocytes aggregation. 2) Assessment of villi and crypts: grade 0: normal villi/crypt structure without apparent tissue damage; grade 1: low amounts of damage with distinct villi/crypt

structure; grade 2: mucosal components can still be identified but the epithelial layer was separated from the lamina propria; grade 3: the villi/crypt structure began to be disorganized and differentiating between the mucosal components was difficult; grade 4: the villi/crypts structure was chaotic; grade 5: massive villi/crypt damages were observed and broad villi were noticeably destroyed down to the basal layer of the mucosal tissue. 3) Tissue edema: grade 0: not present; grade 1: diffused edema was observed in the lamina propria. The histologic analyses, such as cell count and villi/crypt ratio calculation, were carried out by using ImageJ software following published research ^{6, 7}.

1.7. ELISA

5×10⁶ of splenic single-cell suspensions were cultured in complete RPMI 1640 medium with 200 µg of OVA for 72 hours. Concentrations of mMCP-1, IL-4, IL-5, IL10, IL-13, IL-17A, IL-1β, and IL-6 in cell culture supernatants or serum were determined by using commercial ELISA kits from eBioscience according to the manufacturer's instructions. Serum OVA-specific IgE levels were detected as described previously ⁸.

1.8. 16S rRNA sequence data availability

Luminal contents of ileum from individual mice were collected fresh and stored at -80 °C after snap-frozen by liquid nitrogen. Bacterial DNA isolation and 16S rRNA sequence analysis were performed according to previous report ⁹. The sequence data is available at www.ncbi.nlm.nih.gov. (PRJNA1061658).

2. SUPPORTING TABLES AND GRAPHICS

Table S1. Primer used in quantitative polymerase chain reaction experiments.

Gene	Primer	Sequence	Gene	Primer	Sequence
<i>T-bet</i>	Forward	CTGCCTACCAGAACGCAGA	<i>GATA3</i>	Forward	TTATCAAGCCCAAGCGAAG
	Reverse	AAACGGCTGGGAACAGGA		Reverse	CCATTAGCGTTCCTCTCCA
<i>RORγt</i>	Forward	ACAAATTGAAGTGATCCCTTG	<i>STAT3</i>	Forward	CCGTCTGGAAAACCTGGATAACTTC
	Reverse	C		Reverse	CCTGTAGGACACTTCTGCTGC
<i>Foxp3</i>	Forward	CCACGGGCACTATCACACAT	<i>FcεRIα</i>	Forward	TGAATGACAGTGGCACCTACCA
	Reverse	TTGCTTGAGGCTGCGTATGA		Reverse	CAGAATCGCCACCAACAATG
<i>FcεRIβ</i>	Forward	AGTGCTGTGTGTTCACTAT	<i>ECP</i>	Forward	ATCCAAGTGGCTTGTGCAGTGAC
	Reverse	TCCAACACTACTGTAAATTGG		Reverse	TAAGGTGTTCTCCTCCGACTGGTG
<i>Ly6G</i>	Forward	TGCGTTGCTCTGGAGATAGA	<i>GAPDH</i>	Forward	CCTGTTGCTGTAGCCGTATTCA
	Reverse	CAGAGTAGTGGGGCAGATGG		Reverse	CCAGGTTGTCTCCTGCGACTT
<i>IL-1β</i>	Forward	GAAATGCCACCTTTGACAGT	<i>IL-6</i>	Forward	TACCACTTACAAGTCGGAGGC
	Reverse	TGGATGCTCTCATCAGGACAG		Reverse	CTGCAAGTGCATCGTTGTTC

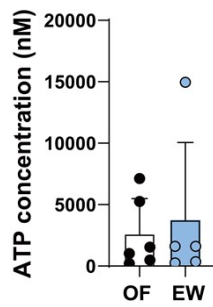


FIG S1. Serum ATP levels of mice fed with OF diet and EW diet.

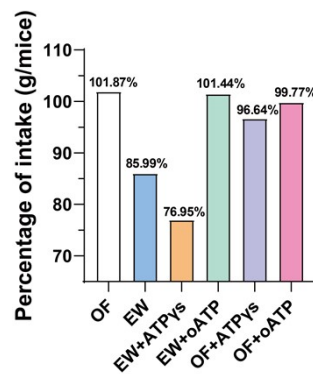


FIG S2. Allergens challenge induced food avoidance. Mice diet intake was recorded daily during oral antigenic challenge. The data was represented as percentage of average diet consumptions (g/mice) compared to consumptions of the week before challenge.

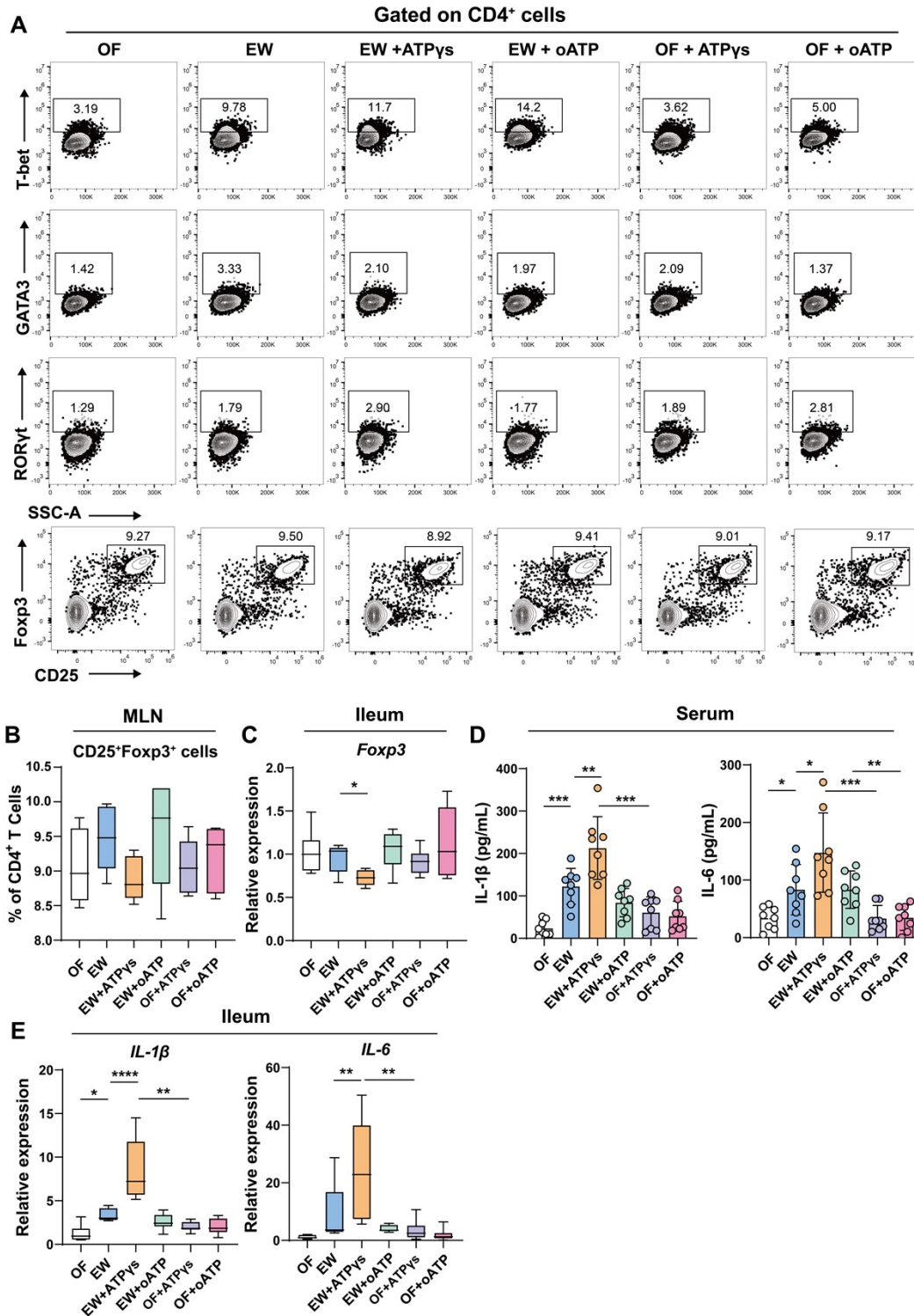


FIG S3. EW diet challenge worsened mice enteritis with skewed T helper cell balance. **A**, Quantification of T helper cells in the MNL. For determination, Th1 cells, Th2 cells, and Th17 cells were gated as live CD4⁺ cells co-expressing T-bet, GATA3, and RORγt, respectively. **B**, Proportion of Treg cells in MLN. Treg cells were determined by the co-expression of CD4⁺ and Foxp3⁺. **C**, Relative expression of ileal *Foxp3* were recorded following qPCR examination. **D**, Serum IL-1β and IL-6 levels were determined by ELISA. **E**, The relative expression of ileal *IL-1β* and *IL-6* were examined by qPCR. Following ATP analogue treatment, the expression of intestinal *IL-1β* and *IL-6* showed a sharp increase. Error bars indicate means ± SDs. Significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

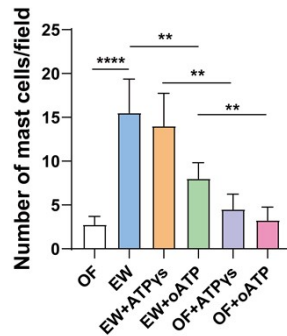


FIG S4. Small intestinal mast cell numbers were recorded within treatment groups. The mast cells resided in the small intestinal mucosa were stained with anti-tryptase and represented as tryptase-positive mast cells per random high-powered field. Error bars indicate means \pm SDs. Significance: * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

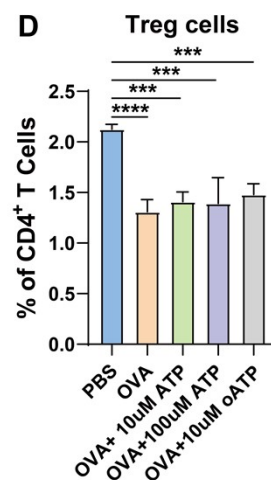
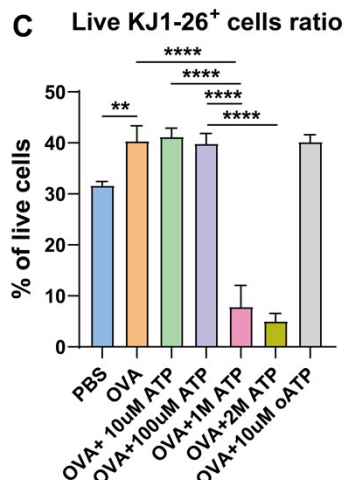
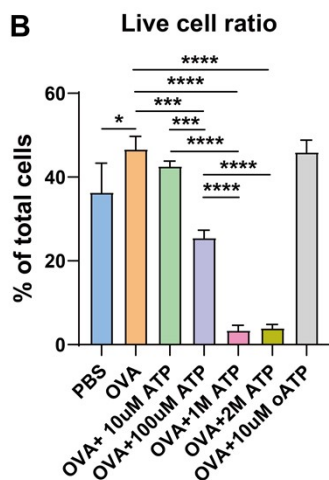
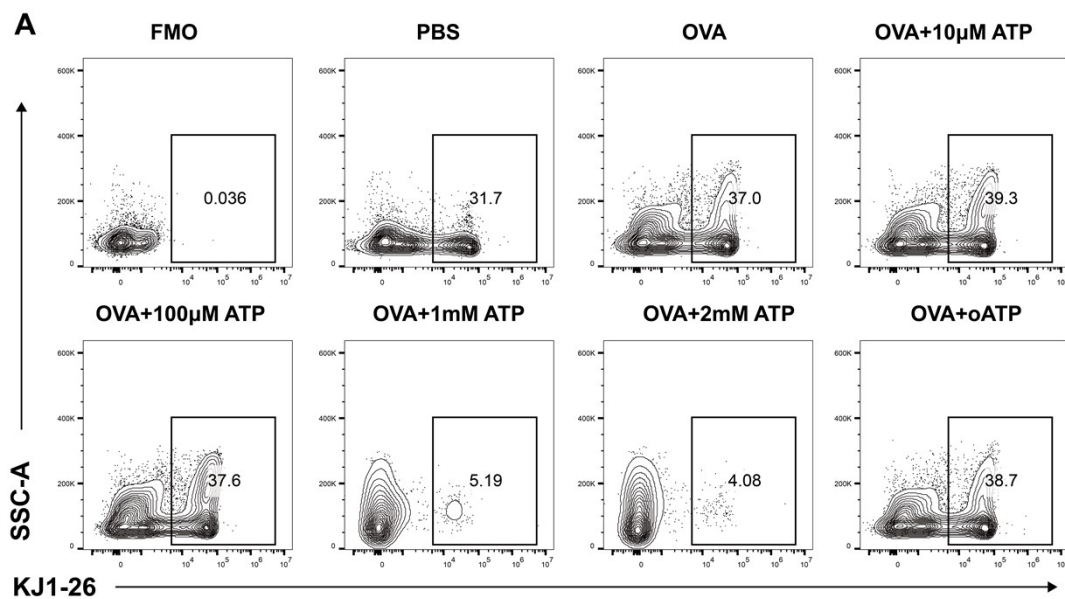


FIG S5. High concentrations of ATP treatment led to large cell apoptosis. **A**, FACS plots for live OVA-specific CD4⁺ T cells (KJ1-26⁺ cells) among different levels of ATP stimulation. **B** and **C**, After 3-day incubation, total live cells (**B**) and live KJ1-26⁺ cells (**C**) were recorded. These data suggested that KJ1-26⁺ cells showed stronger anti-apoptosis ability following high dose of ATP treatment compared to other MLN cells. **D**, Treg-cell ratios within different treatment groups. Error bars indicate means \pm SDs. Significance: * P <0.05, ** P <0.01, *** P <0.001,

**** $P < 0.0001$.

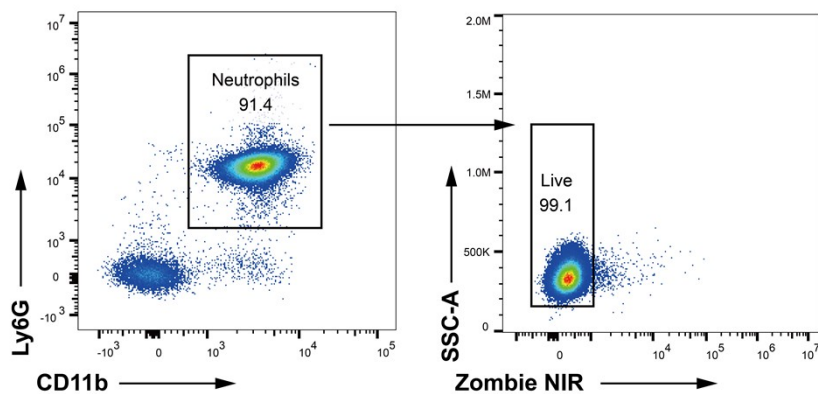


FIG S6. Mice bone-marrow-derived neutrophils were obtained based on the density-gradient centrifugation. Following that, the purity of the resulted cells was verifying by FACS gating on CD11b⁺Ly6G⁺.

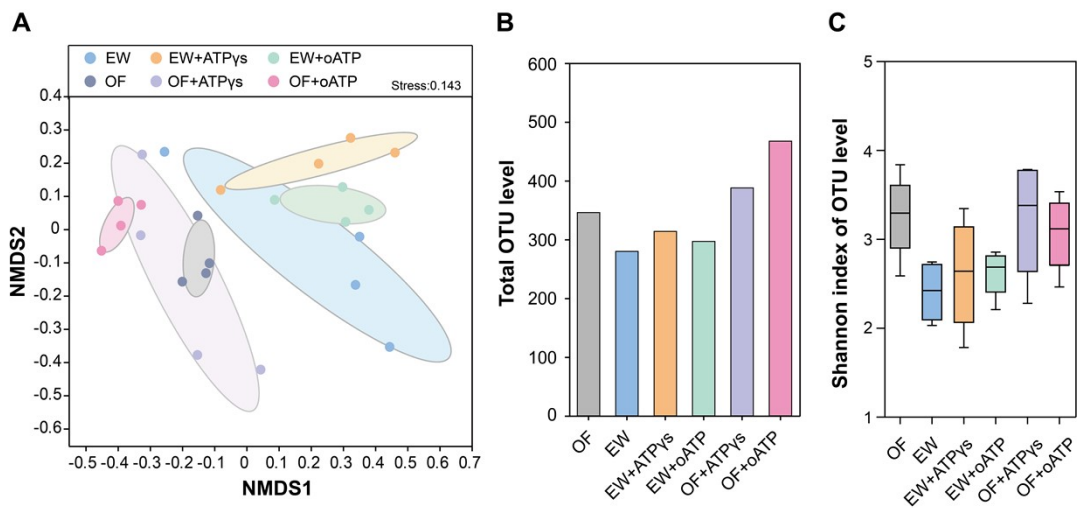


FIG S7. Different diet challenge resulted in distinct gut microbiota composition. **A**, NMDS plot of gut microbiota composition. Mice gut microbiota compositions were quite different after two types of diet feeding. Animals were represented by *dots*, and different treatments were color coded. **B** and **C**, Microbial richness was assessed by total OTU numbers (B) and Shanno index of OUT levels (C).

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