Supplemental Information

Natural isoflavone formononetin inhibits IgE mediated mast cell activation and allergic inflammation by increasing IgE receptors degradation

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Supplemental materials and methods

Animals

Female BALB/c mice weighing 20–22 g were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China). Animal experiments were carried out according to protocols approved by the Animal Care and Use Committee of School of Medicine of Shenzhen University and were in compliance with the Guidelines on Animal Welfare of the School of Medicine of Shenzhen University.

Toluidine blue staining

RBLs were seeded in 24-well plates. After being stimulated, or not, with IgE/DNP, the cells were fixed with 4% paraformaldehyde for ~30 min, and then exposed to toluidine blue dye (1%w/v in 0.9% NaCl solution, pH 2.5) for 1 h. The stained cells were washed with PBS three times and then examined with an inverted microscope (Carl Zeiss, Goettingen, Germany).

F-actin microfilament staining

RBLs were fixed with 4% paraformaldehyde for 30 min at room temperature, washed quickly with PBS, and permeabilized in a 0.5% Triton X-100/PBS solution for 5 min. The permeabilized cells were then washed with PBS twice and incubated with 200 μl of fluorescein isothiocyanate (FITC-labeled phalloidin solution (Yeasen, Shanghai, China)) for 30 min at room temperature in the dark. The F-actin labelled cells were observed under a fluorescence microscope (Carl Zeiss, Goettingen, Germany).

PCA model

Mice were randomized into experimentally designated groups (N = 6/group). With the exception of the mice in the naïve control group, 500 ng DNP-IgE was injected into the left ear of each mouse. Drug treatments were administered orally or by intraperitoneal injection after allowing 24 h for infiltration. Two concentrations of FNT (25 mg/kg and 50 mg/kg) were used; and ketotifen (50 mg/kg) served as a positive control treatment for immune response suppression. One hour after drug injection, 20 μg DNP-HSA in 5

mg/mL Evans blue solution was injected into mice via the tail vein. One hour later, the mice were euthanized, their ears were photographed and removed for histology. After measuring ear skin thickness with vernier calipers, half of the ears were fixed in formamide (each ear in 700 μL) and then subjected to extraction at 65 ℃ for 24 h; absorbance at 630 nm was measured with a microplate reader. The other half of the ears were used for H&E and TB staining.

OVA-induced ASA model

A mixture containing 100 μg OVA and 2 mg aluminum adjuvant in normal saline diluent was injected into the abdominal cavity of mice on days 0 and 7 of the experiment. FNT was injected intraperitoneally or given orally on experimental days 9, 11, and 13. On day 14, the mice were injected intraperitoneally with 200 μg OVA to stimulate allergic reaction. The rectal temperatures of the mice were measured immediately within 90 min of OVA injection. Lung tissue was harvested and sliced into 3 μm sections for H&E staining. Blood was obtained from orbital venous plexus and submitted to serum histamine quantitation by ELISA (kits from IBL, Germany).

RNA sequencing

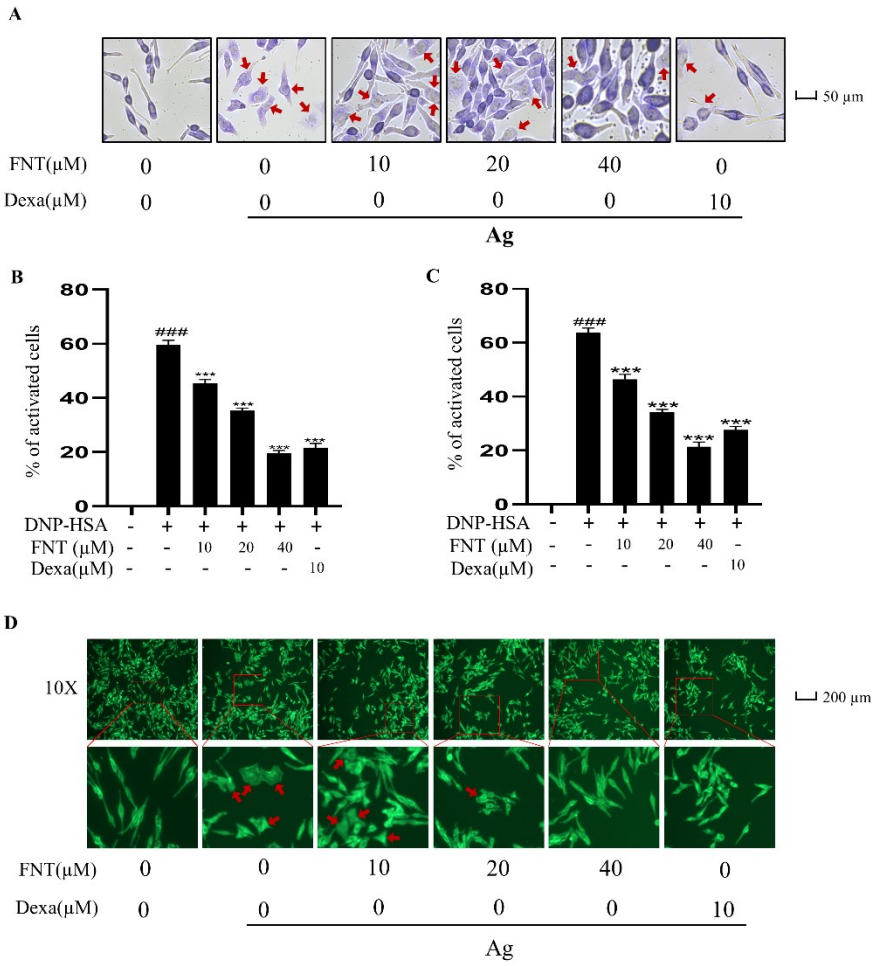
Total RNA quantity and purity were determined with a Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, Santa Clara, CA), high-quality RNA samples (RIN > 7.0) were used to construct a sequencing library. After total RNA was extracted, mRNA was purified from total RNA (5 μg) using Dynabeads Oligo (dT) (Thermo Fisher, CA, USA) with two rounds of purification. Following purification, mRNA was fragmented into short fragments with divalent cations and heating (94 ℃) for ~6 min. The cleaved RNA fragments were reverse-transcribed by SuperScript™ II Reverse Transcriptase (Invitrogen, USA), thus generating cDNAs for the synthesis of U-labeled second-stranded DNAs with *E. coli* DNA polymerase I, RNase H (NEB, USA) and dUTP Solution (Thermo Fisher, USA). An A-base was added to the blunt ends of each strand, preparing them for ligation to indexed adapters containing a T-base overhang for association with A-tailed fragmented DNA. Dual-index adapters were ligated to the fragments, and size selection was performed with AMPureXP beads. After heat-labile

UDG enzyme (NEB, USA) treatment of the U-labeled second-stranded DNAs, the ligated products were amplified with PCR with the following conditions: initial denaturation at 95 ℃ for 3 min; eight 15-s denaturation cycles at 98 ℃; annealing at 60 ℃ for 15 s; extension at 72 ℃ for 30 s; and final extension at 72 ℃ for 5 min. The average insert size for the final cDNA library was 300 ± 50 base pairs. We performed 2 × 150 base pair paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 following the vendor's recommended protocol.

Suppmentary Figure 1. FNT suppresses IgE-mediated morphological changes in RBLs. Anti-DNP IgE-sensitized RBLs were pretreated (or not) with FNT for 1 h and then challenged with DNP-HSA (100 ng/mL) for 30 min. (A, B) Representative micrographs of toluidine blue-stained RBLs. (C, D) FITC-phalloidin stained RBLs. Means ± SDs (of 3 independent experiments) are shown; ***$p < 0.001$ vs. activated cells without treatment; ###$p < 0.001$ vs naïve control group.

Suppmentary Figure 2. Stable RBL/FcεRIγ-Flag cells with bicistronic expression of exogenous FcεRIγ-Flag and ZsGreen1 protein. (A) Schematic representation of the gene sequence of RBL/FcεRIγ-Flag cells. (B) Immunoblotting of FcεRIγ, Flag, ZsGreen1 and GAPDH were detected between RBLs and RBLs/ FcεRIγ-Flag cells.

Supplementary Figure 1



Supplementary Figure 2

RBLs

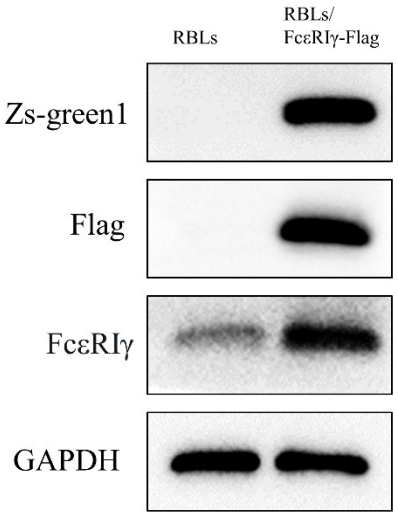


Table S1. Sequences of primers used in real-time RT-qPCR.

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
Rat GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA
Rat IL-13	AGCAACATCACACAAGACC	GGTTACAGAGGCCATTCA
Rat TNF	CCCTGTTCTGCTTTCTCA	GTTCTCCGTGGTGTTCCT
Rat Il-4	GAGGACCAGAACGAGACA	CCAGAAGCGTGACAGAGA
Rat Fcer1A	GGCTGCTGCTCCAATCTTC	GCAATGTCGTCCTTGTAGTAGA
Rat Ms4a2	TGCTCCACACTCCAGACTTC	GCTGCCTCTCACCAGATACA
Rat Fcer1G	GGTGATCTTGTTCTTGCTCCTT	TCACGGCTGGCTATGTCTG
Mouse GAPDH	AAGAAGGTGGTGAAGCAGG	GAAGGTGGAAGAGTGGGAGT
Mouse TNF	CGTGGAACTGGCAGAAGAG	GTAGACAGAAGAGCGTGGTG
Mouse Il-13	CTCTTGCTTGCCTTGGTGGTC	AGGGGAGTCTGGTCTTGTGTGAT
Mouse IL-1β	TGTGTTTTCCTCCTTGCCTCTGAT	TGCTGCCTAATGTCCCCTTGAAT