Atopic dermatitis-like skin lesions are suppressed in *fat-1* transgenic mice through the inhibition of inflammasomes

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1. Supplementary Methods

Genotyping and phenotyping of fat-1 mice

Heterozygous *fat-1* mice and littermates were bred on the same C57BL/6 genetic background and all mice genotyped. Ear punches and tail tips were incubated with STE buffer (100 mM Tris, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl, pH 7.4) and 0.25 mg/ml Proteinase K for 6 h at 55°C and submitted to a 2-step PCR with Taq polymerase (Clontech, Mountain View, CA, USA) and specific forward (5′-CTGCACCACGCCTTCACCAACC-3′) and reverse (5′-CACAGCAGCAGATTCCAGAGATT-3′) primers. Amplification of a 264 bp band confirmed the *fat-1* genotype. The amount of n-3 PUFAs and n-6 PUFAs was analyzed as described previously (Song et al., 2016).

Electrospray ionization mass spectrometry

Ear tissues were extracted with hexane using a blade type homogenizer (Auto Mill, Tokyo, Japan). The extracts were diluted with methanol (1:9, v/v). Standards for linoleic acid (LA, C18:2), α-linolenic acid (ALA, C18:3), arachidonic acid (AA, C20:4), eicosapentaenoic acid (EPA, C20:5), and docosahexaenoic acid (DHA, C22:6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of LA, ALA, AA, EPA, and DHA were prepared in analytical grade methanol (Merck, Darmstadt, Germany). An optimized multiple reaction monitoring (MRM) method was developed using Ultra-Performance Liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS, Acquity system, Waters, Milford, USA). A UPLC system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters). Chromatographic separations were carried out using revers phase C_{18} column (2.1 × 50 mm, 1.7 μm; Waters) maintained at 30°C. LA, ALA, AA, EPA, and

DHA were separated using a gradient elution with a flow rate of 0.5 ml/min. Mobile phase solvent A was 5 mM ammonium formate (Sigma-Aldrich) in water and solvent B was 5 mM ammonium formate in acetonitrile. The samples were eluted according to the following linear gradient from 70% to 100% B buffer for 10 min. Ions were generated in negative ionization mode using electrospray ionization interface. Peaks were identified by comparison with PUFA standards, and the integrated peak area of each resolved peak was used to calculate the percentage of each PUFA.

Western blot

Ear tissues were homogenized in Mammalian Protein Extraction Reagent (Thermo, Waltham, MA, USA). Homogenates containing 20 μg of total protein were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skim milk, blots were probed with primary antibodies against NLRP3 (Cryo-2), procaspase-1 (casper-1) (Adipogen, San Diego, CA USA), IL-β (3A6), HMGB1 (D3E5) (Cell Signaling Technology, Beverly, MA, USA), TSLP, p65, Sirt1 (SIR11) (Abcam, Cambridge, UK), caspase-1 (14F468), ASC (N-15) (Santa Cruz Biotechnology, Dallas, TX, USA), Ac-p65 (Assay Biotechnology, Sunnyvale, CA, USA), and α-tubulin (G436, Bioworld Technology, St. Louis Park, MN, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Enzo Life Sciences, Farmingdale, NY, USA) secondary antibodies were used for visualization. Signals were detected with a Las-4000 imager (GE Healthcare Life Science, Pittsburgh, PA, USA).

RNA isolation and real-time RT-PCR

Total RNA was extracted from ear tissues using TRIzol reagent (Invitrogen). RNA was precipitated with isopropanol and dissolved in diethylpyrocarbonate-treated distilled water. First-strand cDNA was generated with oligo dT-adaptor primers by reverse transcriptase (TaKaRa, Tokyo, Japan). Specific primers were designed using qPrimerDepot (http://mouseprimerdepot.nci.nih.gov, Table S2). Real-time RT-PCR reactions comprised a final volume of 10 μl, containing 10 ng of reverse-transcribed total RNA, 200 nM of forward and reverse primers, and a PCR master mixture. RT-PCR was performed in 384-well plates using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Cell surface staining and intracellular cytokine staining for flow cytometry

Single-cell suspensions prepared from dLNs were stained with anti-CD4-PerCP/Cy5.5 and anti-CD69-FITC antibodies (eBioscience) for 30 min on ice and washed with FACS buffer (2% FBS in PBS) three times. Then, cells were analyzed using an Accuri flow cytometer (BD Biosciences, San Jose, CA, USA). Cells were first stained for cell surface CD4 molecules with anti-CD4-PerCP/Cy5.5 antibody and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate and 1 μM ionomycin in the presence of brefeldin A (eBioscience) for 4 h. Cells were fixed with 4% paraformaldehyde solution and permeabilized with 1× permeabilization solution (eBioscience), followed by staining with anti-IL-4-PE antibody, anti-IFN-γ-FITC and anti-IL-17-FITC. Stained cells were evaluated by flow cytometry analysis (Accuri, BD Biosciences). All antibodies were purchased from eBioscience or Biolegend (San Diego, CA, USA).

Luciferase

Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, HEK293 cells were transfected with 1 μg of *NfkB*- and *Sirt1*-promoter luciferase. One day after transduction, cells were treated with IL-1β and TNF-α, either with or without a 6-h pretreatment with DHA or AA (Cayman, Ann Arbor, MI, USA). After 48 h, cells were harvested in reporter lysis buffer (Promega). Luciferase activity was determined in whole cell lysates using the Promega luciferase assay kit and expressed as relative light units.

2. Supplementary Tables

Table S1. Composition and ratio of n-6 and n-3 PUFAs in wild type and *fat-1* mice

(ng/mg)	C18:2 (LA)	C18:3 (ALA)	C20:4 (AA)	C20:5 (EPA)	C22:6 (DHA)	n-6/n-3 ratio
WT skin	157.3±58.6	5.7±1.9	54.5±18.0	0.8±0.2	10.4±2.6	12.5
fat-1 skin	164.81±53.3**	11.4±2.2	19.2±2.8*	6.3±0.9**	15.1±1.4*	5.6

Values are expressed mean±SEM (n=4). Ratio of n-6/n-3 was calculated from n-6 PUFA (LA and AA) versus n-3 PUFA (ALA, EPA, and DHA). *$p<0.05$, **$p<0.01$ versus WT. LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid

Table S2. Sequences and accession numbers of primers (forward, FOR; reverse, REV) used in real-time RT-PCR

Gene	Sequences for primers	Accession No.
Nos2	FOR: TTCTGTGCTGTCCCAGTGAG	NM_ 010927
	REV: TGAAGAAAACCCCTTGTGCT	
Tnfa	FOR: AGGGTCTGGGCCATAGAACT	NM_013693
	REV: CCACCACGCTCTTCTGTCTAC	
Il1b	FOR: GGTCAAAGGTTTGGAAGCAG	NM_008361
	REV: TGTGAAATGCCACCTTTTGA	
Il6	FOR: ACCAGAGGAAATTTTCAATAGGC	NM_031168
	REV: TGATGCACTTGCAGAAAACA	
Il4	FOR: TCGGCATTTTGAACGAGGTC	NM_011333
	REV: GAAAAGCCCGAAAGAGTCTC	
Il5	FOR: TCACCGAGCTCTGTTGACAA	NM_010558
	REV: CCACACTTCTCTTTTTGGCG	
Il13	FOR: CGCAAGGCCCCCACTAC	NM_008355
	REV: AAAGTGGGCTACTTCGATTTTGG	
Il17	FOR: TCCCCTCTGTCATCTGGGAAG	NM_010552
	REV: CTCGACCCTGAAAGTGAAGG	
Ifng	FOR: TTGGCTTTGCAGCTCTTCCT	NM_008377
	REV: TGACTGTGCCGTGGCAGTA	
Foxp3	FOR: GAAACAGCACATTCCCAGAGTTC	NM_054039
	REV: ATGGCCCAGCGGATGAG	
Gata1	FOR: CAGAACCGGCCTCTCATCC	NM_008089
	REV: TAGTGCATTGGGTGCCTGC	
Gata2	FOR: GCAGAGAAGCAAGGCTCGC	NM_008090
	REV: CAGTTGACACACTCCCGGC	
Ccl12	FOR: CCTGGGTTCCAGCACCAA	NM_011331
	REV: GGCGGGAGTGTGGTATGC	
Cpa3	FOR: GCTACACATTCAAACTGCCTCCT	NM_ 007753
	REV: GAGAGAGCATCCGTGGCAA	
c-Kit	FOR: AGCAATGGCCTCACGAGTTCTA	NM_001122733
	REV: CCAGGAAAAGTTTGGCAGGAT	
Nlrp3	FOR: AAGTAAGGCCGGAATTCACC	NM_145827
	REV: AAAATGCCTTGGGAGACTCA	
Caspase 1	FOR:TGCCGTGGAGAGAAACAAGG	NM_009807
	REV:CCCCTGACAGGATGTCTCCA	
Asc	FOR: GAAGCTGCTGACAGTGCAAAC	NM_023258
	REV: GCCACAAGCTCCAGACTCTTC	
Aim2	FOR: GGAACAATTGTGAATGGGCT	NM_ 001013779
	REV: AGCACCAACACCTCCATTGT	
Pycard	FOR: GCTGGTCCACAAAGTGTCCT	NM_012052
	REV: GAGCAGCTGCAAACGACTAA	
TSLP	FOR: AGGCTACCCTGAAACTGAG	NM_021367
	REV: GGAGATTGCATGAAGGAATACC	
Gapdh	FOR: CGTCCCGTAGACAAAATGGT	NM_008084
	REV: TTGATGGCAACAATCTCCAC	

3. Supplementary Figure

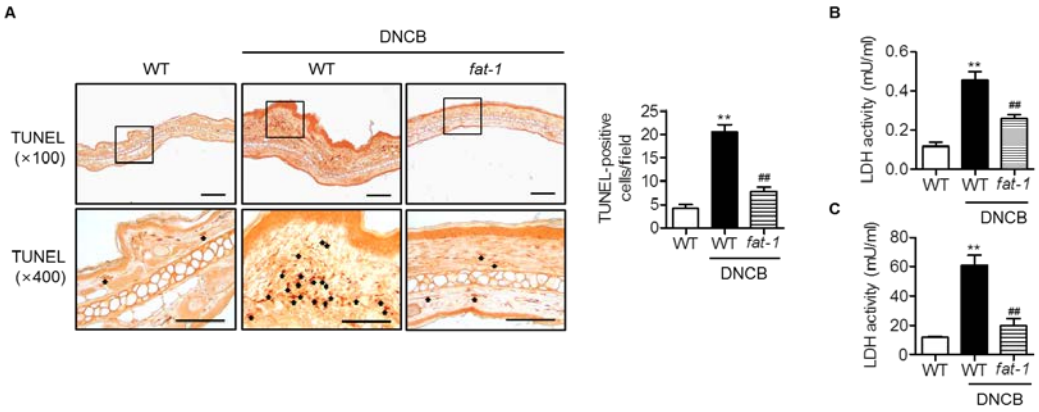

Figure S1. DNCB-induced pyroptosis. (A) At the end of the study, cell death was assessed by TUNEL staining. TUNEL-positive cells were counted and expressed as a cell number in a high power field (n=6). Bars=100 μm. The enzyme activity of LDH in skin tissues (B) and serum (C) were determined by ELISA (n=5). Values are means ± SEMs. $^{**}p<0.01$ vs. WT; $^{\#\#}p<0.01$ vs. WT+DNCB.