SUPPLEMENTAL INFORMATION

Adipocyte specific IKKβ signaling suppresses adipose tissue inflammation through an IL-13 dependent paracrine feedback pathway

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Figure S1. Adipocyte-specific IKKβ knockout mice, Related to Figure 1 and 2.

(A) IKKβ expression was normal in peripheral tissues of adipocyte-specific IKKβ knockout (Ad-IKKβKO) mice. lkbkb gene expression was normal in muscle, pancreas, liver, brain and splenocytes (n = 4-8 per group). Tissues were frozen in liquid nitrogen and homogenized into QIAzol Lysis Reagent to prepare total RNA and cDNA. WT: wild type mice, KO: Ad-IKKβKO mice. (B) Body weight and body composition were comparable in wild type and Ad-IKKβKO mice. Body weight, lean mass and fat mass were similar in wild type and Ad-IKKβKO mice (n = 10-32 per group). WT and Ad-IKKβKO (8 weeks old male) mice were fed with NCD or HFD for 8 weeks, and body composition was measured at 0, 2, 4 and 8 weeks. (C) Body weight and fat mass of wild type and Ad-IKKβKO mice after normal chow or HFD feeding for 12 weeks. (n = 5-9 per group). sAT: subcutaneous adipose tissue, eAT: epididymal adipose tissue. All data are the mean ± s.e.m., * P < 0.004 compared to NC. (D) Respiratory exchange rate and local locomotive activity were comparable in wild type and Ad-IKKβKO mice. Respiratory exchange rate (RER) and spontaneous locomotor activity in the x, y, and z planes of wild type (WT) and Ad-IKKβKO (KO) mice after normal chow or high fat diet feeding for 12-14 weeks (n = 4-6 per group). All data are the mean ± s.e.m., * P < 0.0004 compared to NC. WT-NC: NCD fed wild type mice, WT-HF: HFD fed wild type mice, KO-NC: NCD fed Ad-IKKβKO mice and KO-HF: HFD fed Ad-IKKβKO mice.
Figure S2. Expression of stromal vascular cells and adipocytes markers and production of inflammatory cytokines in epididymal adipose tissues, Related to Figure 3. (A) Stromal vascular fraction and adipocyte fraction showed enriched expression of cell specific markers in wild type and adipocyte-specific IKKβ knockout (Ad-IKKβKO) mice. T cell (Cd3g) and macrophage (Itgma) markers were enriched in SVF and adipocytes (Lep) marker was enriched in ACF. Wild type (WT) and Ad-IKKβKO (KO) mice were fed normal chow (NC) or high fat diet (HF) for 12-13 weeks (n = 4-7 per group). All data are the mean ± s.e.m., * P < 0.04 compared to NC. WT-SVF: stromal vascular fraction of wild type mice, KO-SVF: stromal vascular fraction of Ad-IKKβKO mice. WT-ACF: adipocyte fraction of wild type mice, KO-ACF: adipocyte fraction of Ad-IKKβKO mice. (B) Secretion of inflammatory cytokines was enhanced in epididymal adipose tissues of high fat diet fed Ad-IKKβKO mice. Secretion of inflammatory cytokines including TNF-α, IL-1β and IL-6 was measured in epididymal adipose tissues of HFD fed wild type and Ad-IKKβKO mice (n = 12-20 per group). Epididymal adipose tissues from HFD fed (12-16 weeks) WT and KO mice were harvested and placed in 24 well plates (100-200 mg tissue/well) with 1 ml cell culture media for 24 h. Media were harvested, and cytokine quantification was analyzed by ELISA. Cytokine concentration is pg/mL per 100 mg tissue. All data are the mean ± s.e.m., * P < 0.0033 compared to WT-HF. WT-HF: HFD fed wild type mice and KO-HF: HFD fed Ad-IKKβKO mouse.
Figure S3. Flow cytometry analysis, Related to Figure 4. (A and B) Determination of M1, M2 and M1/M2 macrophages in epididymal adipose tissue using flow cytometry. (A) FACS profile of M2 (F4/80^+CD11c^-CD206^-), M1 (F4/80^+CD11c^+CD206^+) and M1/M2 (F4/80^+CD11c^+CD206^+) macrophages in SVF of epididymal adipose tissues. (B) Cell numbers of M2 (F4/80^+CD11c^-CD206^-) and M1/M2 (F4/80^+CD11c^+CD206^+) macrophages in epididymal adipose tissues of HFD fed wild type and Ad-iKKbKO mice (n = 6-10 per group). All data are the mean ± s.e.m., * P < 0.0027 compared to NC. (C and D) Determination of CD4^+ and CD8^+ T cells in epididymal adipose tissue using flow cytometry. (C) FACS profile of CD4^+ and CD8^+ T cells in SVF of epididymal adipose tissues. (D) Cell numbers of CD4^+ and CD8^+ T cells in epididymal adipose tissues of high fat diet fed wild type and Ad-iKKbKO mice. All data are the mean ± s.e.m., * P < 0.0027 compared to NC. WT-NC: NCD fed wild type mice, WT-HF: HFD fed wild type mice, KO-NC: NCD fed Ad-iKKbKO mice and KO-HF: HFD fed Ad-iKKbKO mice.
Figure S4. Adipocytes from adipocyte-specific IKKβ knockout mice showed diminished inflammatory responses without changes in adipogenesis and apoptosis, Related to Figure 3. (A) Expression of adipogenesis markers in subcutaneous adipose tissues of high fat diet fed wild type and Ad-IKKβKO mice (n = 4 per group). All data are the mean ± s.e.m., * P < 0.0002 compared to NC. (B) Expression of adipogenesis markers in adipocytes differentiated from stromal vascular fraction of wild type and Ad-IKKβKO mice (n = 4-8 per group). (C) Expression of inflammatory cytokines in adipocytes differentiated from stromal vascular fraction of wild type and Ad-IKKβKO mice after TNF-α and/or IL-1β stimulation (n = 4 per group). TNF-α (20 ng/mL) and IL-1β (10 ng/mL) were treated for 6h. All data are the mean ± s.e.m., * P < 0.00004 compared to control (Ctrl), ** P < 0.0008 compared to WT. (D) Caspase-3 and PARP1 cleavage in subcutaneous adipose tissues of normal chow (NC) and high fat diet (HF) fed wild type (WT) and Ad-IKKβKO (KO) mice. PC: positive control. WT-NC: normal chow diet fed wild type mice, WT-HF: high fat diet fed wild type mice, KO-NC: normal chow diet fed Ad-IKKβKO mice and KO-HF: high fat diet fed Ad-IKKβKO mice.
Figure S5. Cytokine production and macrophage proliferation after IL-13 administration, Related to Figure 5. (A) IL-13 administration suppressed the secretion of inflammatory cytokines in epididymal adipose tissues of HFD fed adipocyte-specific IKKβ knockout (Ad-IKKβKO) mice. Secretion of inflammatory cytokines including TNF-α, IL-1β and IL-6 was measured in epididymal adipose tissues of HFD fed wild type and Ad-IKKβKO mice (n = 8-20 per group) after IL-13 administration. Cytokine concentration is pg/mL per 100 mg tissue. All data are the mean ± s.e.m., * P < 0.0017 compared to WT-HF-PBS or WT-HF-IL-13 and ** P < 0.04 compared to KO-HF-PBS. WT-HF-PBS: HFD fed wild type mice with PBS administration, WT-HF-IL-13: HFD fed wild type mice with IL-13 administration, KO-HF-PBS: HFD fed Ad-IKKβKO mice with PBS administration and KO-HF-IL-13: HFD fed Ad-IKKβKO mice with IL-13 administration. (B-D) IL-13 administration showed similar macrophage proliferation with control HFD fed Ad-IKKβKO mice. (B) FACS profile of macrophage proliferation in SVF of epididymal adipose tissues. Macrophages and eosinophils were determined with F4/80 and Singlec F, respectively. Proliferation of F4/80*Singlec F⁻ macrophages was determined with Ki-67. (C) FACS profile of F4/80*Singlec F⁻ macrophages in SVF of epididymal adipose tissues. (D) Proportion of Ki-67 in F4/80*Singlec F⁻ macrophages. (n = 4-6 per group). All data are the mean ± s.e.m., * P < 0.0001 compared to Isotype IgG. IL-13 (1 µg in 200 µl PBS per mouse) or PBS was administrated for 3-4 weeks to the mice after HFD feeding for 10-11 weeks.
Supplemental Experimental Procedures

Human subject

A total of 16 female subjects participated in adipose tissue biopsies. Adipose tissues were prepared from 11 obese subjects (BMI = 45.4±2.1) during bariatric surgery and from 5 lean subjects (BMI = 24.9±1.3). Adipose tissue samples were immediately washed at least three times with PBS and homogenized into Qiazol Lysis Reagent (Qiagen, Valencia, CA, USA). Before adipose biopsies written consents were obtained from all subjects. All procedures were approved by the Albert Einstein College of Medicine Institutional Review Board.

Quantification of cytokines in culture media and plasma

3T3-L1 adipocytes cell culture media were collected at 24 h after stimulation. For plasma, mice were anesthetized with isoflurane and cardiac puncture was performed. Blood was centrifuged 10 min at 1,000 xg at 4°C. Plasma was collected. For adipose tissue explant culture, adipose tissues from wild type and adipocytes specific IKKβ knockout (Ad-IKKβKO) mice were harvested and placed in 24 well plates (100-200 mg tissue/well) with 1 ml cell culture media for 24 h. Media were harvested. ELISA analysis was used for cytokine quantification (ELISA Kit; Invitrogen, Camarillo, CA, USA).

Stromal vascular fraction and adipocyte fraction preparation

Epididymal adipose tissue was minced into fine (<10 mg) pieces. Minced adipose tissues were placed in HEPES-buffered DMEM supplemented with 10 mg/ml low fatty acid BSA and centrifuged at 1,000 xg for 10 min to precipitate erythrocytes and other blood cells. Samples were treated with 0.05 mg/ml Liberase (Liberase TM Research Grade, Roche Applied Science, Indianapolis, IN, USA) and incubated at 37 °C for 25-35 min with agitation (80 rpm). Samples were passed through a sterile 250-μm nylon mesh. The suspension was centrifuged at 1,000 xg for 5 min. For adipocyte fraction (ACF), upper adipocytes were saved and washed with HEPES-buffered DMEM supplemented with 10 mg/ml low fatty acid BSA. For stromal
vascular fraction (SVF), the precipitated cells were washed with FACS buffer and re-suspended in erythrocyte lysis buffer. The erythrocyte-depleted SVF cells were centrifuged at 500 xg for 5 min. The pellet was re-suspended in FACS buffer.

**Immunohistochemical staining**

Adipose tissue samples were fixed for 24 to 36 h at room temperature in zink-formalin fixative and embedded in paraffin. Paraffin-embedded adipose tissue was sectioned (10-μm) and deparaffinized. Sections were stained with hematoxylin-eosin.

**Western blotting**

Tissues and cells were homogenized in a RIPA lysis buffer containing protease and phosphatase inhibitors. Homogenates were centrifuged for 15 min at 13,000 xg at 4 °C and supernatants were collected. Protein samples (40 μg) were separated on 9% reducing polyacrylamide gels and electroblotted onto nitrocellulose membranes. Immunoblots were blocked with 2% BSA in Tris-buffered saline for 2 h at room temperature and incubated overnight at 4°C with the indicated antibodies in Tris-buffered saline and 0.05% Tween 20 (TBST) containing 1% BSA. Blots were washed in TBST and incubated with IRDye 680LT or IRDye 800CW-conjugated secondary antibodies (1:50,000) for 1 h at room temperature. Membranes were washed in TBST, and antigen-antibody complexes were visualized by Odyssey Imaging System (LI-COR, Lincoln, NE, USA). For AKT phosphorylation, mice were fasted for 6 h and injected with 1 unit of insulin/kg body weight. Tissues were collected after 20 min. Rabbit anti-IKKβ (#8943), rabbit anti-AKT-Thr308 (#9275), rabbit anti-AKT-Ser473 (#4060), rabbit anti-AKT (#4691), rabbit anti-Caspase-3 (#9665), rabbit anti-PARP1 (#9542) were from Cell Signaling (Boston, MA, USA). Mouse anti-GAPDH (#M171-3) was from MBL International (Woburn, MA, USA).  

**Indirect calorimetry**

Metabolic measurement was performed using an Oxymax indirect calorimetry system (Columbus Instruments, Columbus, OH) (Zong et al., 2011). Mice were
individually housed in the chamber with a 12-h light/12-h dark cycle in an ambient temperature of 22–24°C. VO₂ and VC0₂ rates were determined under Oxymax system settings as follows: air flow, 0.6 l/min; sample flow, 0.5 l/min; settling time, 6 min; and measuring time, 3 min. The system was calibrated against a standard gas mixture to measure O₂ consumed (VO₂, ml/kg/h) and CO₂ generated (VC0₂, ml/kg/h). Metabolic rate (VO₂), respiratory quotient (ratio of VC0₂/VO₂), and activity (counts) were evaluated over a 24-h period. Energy expenditure was calculated as the product of the calorific value of oxygen (3.815 + 1.232 × respiratory quotient) and the volume of O₂ consumed.

**Glucose tolerance and insulin tolerance test**

For glucose tolerance test (GTT), control and Ad-IKKβKO mice maintained on a standard chow diet or a high fat diet for 8-9 weeks were fasted for 12 h and injected with 1g D-glucose/kg lean mass. Blood was collected from the lateral vein of the tail prior to and at 15, 30, 45, 60, 90 and 120 min after glucose administration. For Insulin tolerance test (ITT), mice were fasted for 6 h and injected with 1 unit of insulin/kg lean mass. Blood was collected from the lateral vein of the tail prior to and at 15, 30, 45, 60 and 90 min after glucose administration. For the GTT and ITT of IL-13 administrated mice, mice were maintained on a high fat diet for 12-13 weeks with/without IL-13 administration for last 2-3 weeks. Glucose was measured using a Precision Q.I.D glucometer (MediSense, Abbott Laboratories, Abbott Park, IL, USA).

**Total body mass and magnetic resonance imaging**

Total body mass was recorded at 15:00. To determine fat mass, animals were placed in a clear plastic holder without anesthesia or sedation and inserted into the EchoMRI-3-in-1™ System from Echo Medical Systems (Houston, TX, USA).

**Flow cytometry analysis**

Flow cytometry was conducted as previously described (Kwon et al., 2009; Lee et al., 2013). Briefly cells were treated with FcR blocking antibody and then stained with PE-labeled anti-mouse F4/80, APC-labeled anti-mouse CD11c and Biotin-labeled
anti-mouse CD206 followed by streptavidin-eFluor 688 for macrophages. For T cells, cells were stained with PE-labeled anti-mouse CD3, FITC-labeled anti-mouse CD4, and APC-labeled anti-mouse CD8a (BD Pharmingen, San Diego, CA, USA). For macrophage proliferation, cells were stained with PE-labeled anti-mouse F4/80. Cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) and then stained with FITC-labeled anti-human Ki-67 (Amano et al., 2014). 7-AAD was used to exclude dead cells. FACS was conducted using BD FACSCanto II Analyzer (BD Biosciences, San Jose, CA, USA) and data analysis was done with FlowJo (Tree Star, Inc., Ashland, OR, USA).

**Supplemental References**

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