Supplementary Materials

Leptin deficiency shifts mast cells toward anti-inflammatory actions and protects mice from obesity and diabetes by polarizing M2 macrophages

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Supplementary Methods

Materials

Human insulin, lipopolysaccharide (LPS), mouse IL-6 and TNF-α were purchased from Sigma Aldrich (St. Louis, MO). Recombinant mouse leptin was purchased from BioVision (Milpitas, CA). Recombinant mouse IL-4, macrophage colony-stimulating factor (M-CSF), and anti-mouse leptin-neutralizing antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Mouse IgG1 antibody was from BD Pharmingen (San Jose, CA). Mouse IgE, JAK inhibitor AG490, IκBα phosphorylation inhibitor BAY11-7082, p38 MAPK inhibitor SB203580, ERK inhibitor PD98059, PI3K inhibitor LY294002, and JNK inhibitor SP600125 were purchased from Sigma Aldrich (St. Louis, MO). STAT5 inhibitor CAS285986-31-4 was from Calbiochem (San Diego, CA). These inhibitors were initially dissolved in DMSO, and then diluted to their final concentrations. In all studies, the final concentration of DMSO was 0.1%.

Patients

Discarded human WAT from obese (body mass index BMI>35, n=17) and lean (BMI<25, n=17) patients was obtained from the Department of Nutrition of Hôtel-Dieu Hospital, Paris, France, according to a approved human study protocol. Obese patients met criteria for bariatric surgery, *i.e.* body mass index BMI ≥ 40 or ≥ 35 kg/m^2 with at least one co-morbidity (hypertension, type II diabetes, dyslipidemia or obstructive sleep apnea syndrome). Obese patients weights were stable (*i.e.* variation of less than 2 kg) for at least 3 months prior to the surgery based on monthly pre-surgery visits and it was their first bariatric intervention. For example, patients switching from gastroplasty to gastric bypass were excluded. Subjects were also excluded if they had acute or chronic inflammatory or infectious disease, viral infection, cancer, or alcohol consumption greater than 20 g per day. Lean subjects were recruited as a control group in the same clinical protocol. These subjects had elective abdominal surgery (inguinal hernia, cholecystectomy, hysterectomy, gastro-oesophageal reflux). They did not have acute inflammatory processes and consented to omental and subcutaneous biopsy in the same location as that of obese subjects. Use of deidentified discarded tissues was approved by the Partners Human Investigation Review Committee.

Immunohistology

Human WAT paraffin sections were stained with antibodies against human leptin (1:100, Abcam, Cambridge, MA), human tryptase (1:100, Dako, Carpinteria, CA), followed by Alex Fluor 568 or 488-labelled secondary antibody to detect leptin and MCs. Paraffin sections from mouse WAT were stained with antibodies against mouse leptin (1:100, Abcam, Cambridge, MA), mouse CD117 (1:20, eBiosciences, San Diego, CA),

mouse CD3 (1:50, Abcam), mouse Mac-2 (1:200, Cedarlane, Burlington, NC), and mouse leptin receptor (1:1000, Abcam, Cambridge, MA), followed by Alex Fluor 568 or 488-labelled secondary antibody to detect leptin, MCs, T cells, macrophages, and leptin receptor in WAT. Immunofluorescent double staining was also performed on mouse liver paraffin sections and bone marrow cells from lean and DIO mice to detect leptin expression on $CD117^+$ MCs. Paraffin sections of mouse WAT and spleen were stained with toluidine blue to detect MCs and immunostained to detect macrophages (Mac-2, 1:25,000, Cedarlane), T cells (CD3, 1:100, Abcam), and neutrophils (NIMP-R14, 1:200, Santa Cruz, Dallas, TX). Researchers blinded to the origin of tissues counted human $tryptase^+$ and mouse $CD117^+$ or toluidine blue-positive MCs, $CD3^+$ T cells, NIMP-$R14^+$ neutrophils, and Mac-2^+ macrophages. Leptin-positive areas in WAT and leptin expression in MCs were quantitated using the Image-Pro Plus software. Adipocyte size was measured using the Image J software.

WAT mast cell isolation and cell sorting

Inguinal WAT were subjected to mechanical dissociation. Visible blood vessels and ganglia were carefully dissected and removed. WAT fragments were digested at 37°C in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.5 mg/ml Liberase TM (Roche Diagnostics, Indianapolis, US) for 30 minutes. After elimination of undigested fragments by 25-μm cell strainers, cells were subjected to centrifugation (630 g, 10 minutes). WAT cells from five mice were pooled for each experiment and incubated for 2 minutes in a hemolysis buffer (140 mM NH_4Cl and 20 mM Tris, pH 7.6) to eliminate red blood cells. Cells were then counted and used for flow cytometry analysis or sorting, before plating or engraftment. Freshly isolated stromal-vascular fraction-derived cells were stained in PBS containing FcγIII/IIR blocking reagent. Extracellular staining was performed using APC- and FITC-conjugated rat anti-mouse monoclonal antibodies against CD117 and FcεR1 and compared with matched isotype controls (eBioscience). Cells were washed in PBS and purified on cell sorter (FACS ARIA II, Becton Dickinson). Purified WAT mast cells were used for RT-PCR and immunoblot with antibodies against mouse leptin (1:500, Abcam, Cambridge, MA) to detect leptin expression or RT-PCR to detect adiponectin to ensure the MC purity.

WAT and macrophage flow cytometry analysis

WAT SVF single cell preparation was used for FACS analysis. Briefly, WAT was collected, rinsed in PBS, minced into pieces, and digested in a collagenase buffer (0.7 mg/ml in PBS; Sigma-Aldrich) at 37°C for 45 minutes. Tissue homogenates were passed through a 70-μm mesh to remove undigested clumps and debris. The effluent was centrifuged at 100 *g* for 10 minutes. The dispersed cell preparation was analyzed by FACS. Extracellular staining was preceded by incubation with purified anti-CD16/32 antibodies (FcγRII/III block, 2.4G2) to block nonspecific staining. Cells were stained with PerCPCy5.5-, APC- and Alexa 488-labeled antibodies against CD11c (N418), CD206 (ACRR01), and F4/80 (BM8). All antibodies and all isotype controls were purchased from BD Biosciences (San Jose, CA). Flow cytometry acquisition was performed with a FACS Calibur flow cytometer (BD Bioscience), and data files were analyzed using CellQuest research software (version 3.3, BD Biosciences). M1 or M2 macrophages were identified as $F4/80^+CD11c^+$ or $F4/80^+CD206^+$ cells, respectively.

WAT cytokine level detection

WAT IFN-γ, IL6, IL4, and IL10 levels were detected by ELISA according to manufacturers' instructions (eBioscience). In brief, around 200 mg of WAT tissue was

homogenized in 4 μl/mg tissue homogenization buffer (containing RIPA, 0.5% Triton-X100, and anti-protease tablets from Roche) on ice using Tissuelyser-II (Qiagen) for 5 minutes with a frequency of 30/s. The homogenates were centrifuged at 1,000 g for 10 minutes and the supernatant below the lipid cake was collected. Total protein concentrations of the whole tissue extracts were determined using Pierce BCA Protein Quantitation kit (Thermo Scientific). Each whole tissue extract was diluted to 20 μg total protein in each 100 μl of homogenization buffer and loaded in the 96-well plate and incubated at room temperature for 3 hours for ELISA. Results were presented as nanogram cytokine in each milligram protein.

BMMC culture medium cytokine and chemokine detection

Leptin-induced cytokine and chemokine production from BMMCs were performed for both cytokine array and ELISA analysis. In brief, BMMCs from WT and *ob/ob* mice were suspended in RPMI-1640 with 10% fetal bovine serum (FBS) at 10^6 cells/mL. BMMCs were pretreated with 50 ng/ml of leptin for 12 hours at 37 ^oC and then activated with 1 μM calcium ionophore A23187 (Sigma-Aldrich) for 6 hours. Cell culture media were collected to screen cytokine and chemokine expression using the RayBio® Cytokine Antibody Array (Mouse Inflammation Antibody Array G Series I, RayBiotech, Inc., Norcross, GA). Or BMMCs were treated with only 50 ng/ml of leptin for 24 hours and then cell culture media were collected for ELISA to detect IL6, IFN-γ, IL4, IL10, IL13, MCP-1 and MIP-1α (eBioscience), according to the manufacturers' instructions.

BMMC leptin and leptin receptor expression

BMMCs sorted by FACS from lean and DIO adipose tissue were used for RT-PCR and immunobot (1:500, Abcam) to detect leptin expression. BMMCs from WT and *ob/ob* mice were treated with and without leptin (50 ng/ml) or IgE (50 μg/ml) for 30 minutes, and used for immunoblot to detect the expression of leptin receptor (1:500, Abcam) or for IgE receptor FcεRI by flow cytometry (1:100, eBioscience, San Diego, CA). BMMCs from WT and *db/db* mice were used to detect leptin receptor expression by immunoblot analysis (1:500, Abcam).

RT-PCR and ELISA were used to determine leptin expression in BMMCs. WT BMMCs were stimulated with 7 nM insulin, 100 ng/ml IL6, or 1 nM TNF-α for 48 h, respectively. Cell culture medium were collected for leptin ELISA analysis (Linco Research, St. Charles, MO). Cells were used for RT-PCR.

BMDM culture

Bone marrow-derived macrophages (BMDMs) were generated from WT and *db/db* mice. Fresh bone marrow cells were cultured on 24-well plates for 7 days in RPMI 1640 medium containing 15 mM of HEPES, 2 g/l of sodium bicarbonate, 1 mM L-glutamine, and supplemented with 20% FBS and 40ng/ml M-CSF. Differentiated BMDM ($1x10^5$) were stimulated with or without LPS (200 ng/ml, 6 hours) or IL-4 (10 ng/ml, 24 hours) and then co-cultured with WT or *ob/ob* BMMCs ($1x10^5$), $CD4^+$ T cells ($1x10^5$), or $CD8^+$ T cells ($1x10^5$) for another 24 hours. Leptin (50 ng/ml) was incubated with WT or *ob/ob* BMMCs ($1x10^5$) for 24 hours and then co-cultured with WT and *db/db* BMDMs. At the end, BMDMs were collected for RT-PCR analysis to detect the expression of iNOS, TNF-α, arginase-1, and IL10.

BMDM expression of iNOS, TNF-α, arginase-1, and IL10 was also analyzed after co-culturing with different cytokine-deficient BMMCs. WT BMDMs ($1x10^5$) were co-cultured

with BMMCs ($1x10^5$) from WT, *ob/ob, Il6*$^{-/-}$*, ifng*$^{-/-}$ and *Il13*$^{-/-}$ mice with or without LPS or IL-4 treatment. BMDM were collected for RT-PCR analysis of iNOS, TNF-α, arginase-1, and IL10.

Real-Time PCR

Real-Time PCR (RT-PCR) was used to determine the expression CD68, MCP-1, CD11c and TNF-α, IL6, IL10, arginase-1, iNOS in WAT stromal vascular fractions (SVF) and BMDMs. WAT SVF and BMDM total RNA was prepared using the Qiagen mini kit according to the manufacturer's recommendations (Qiagen Inc., Valencia, CA).

RNA concentration and quality were evaluated using the Agilent 2100 bioanalyzer (Nano LabChip, Agilent Technologies, Santa Clara, CA). RT-PCR quantified gene expressions on the ABI Prism 7900 sequence detection system (Taqman, Applied Biosystems Inc., Foster City, CA). GAPDH and 18S were used as endogenous controls. Results were expressed as fold of change relative to the control animals or cells as the baselines.

$CD4^+$ T-cell differentiation

To determine the regulatory effect of leptin, WT and *ob/ob* BMMCs on the Th1/Th2/Th17/Treg paradigm *in vitro*, splenocytes from WT mice were prepared and stimulated with leptin (50 ng/ml), WT or *ob/ob* BMMCs ($1x10^5$) for 24 hours, respectively, followed by surface staining with anti-CD3-APC and anti-CD8-FITC and then intracellular staining with PE-conjugated antibodies against IL-17A, IFN-γ, IL-4, or isotype IgG2a control antibody for FACS analysis of Th17, Th1 or Th2 cells. Splenocytes were stained with the Mouse Regulatory T Cell Staining Kit (BD Bioscience) for detection of Treg cells. The proportions of Th1/Th2/Th17/Treg cells were analyzed using flow cytometry. Results are expressed as mean ± SEM of 3 mice from 3 independent experiments.

Supplementary Figures

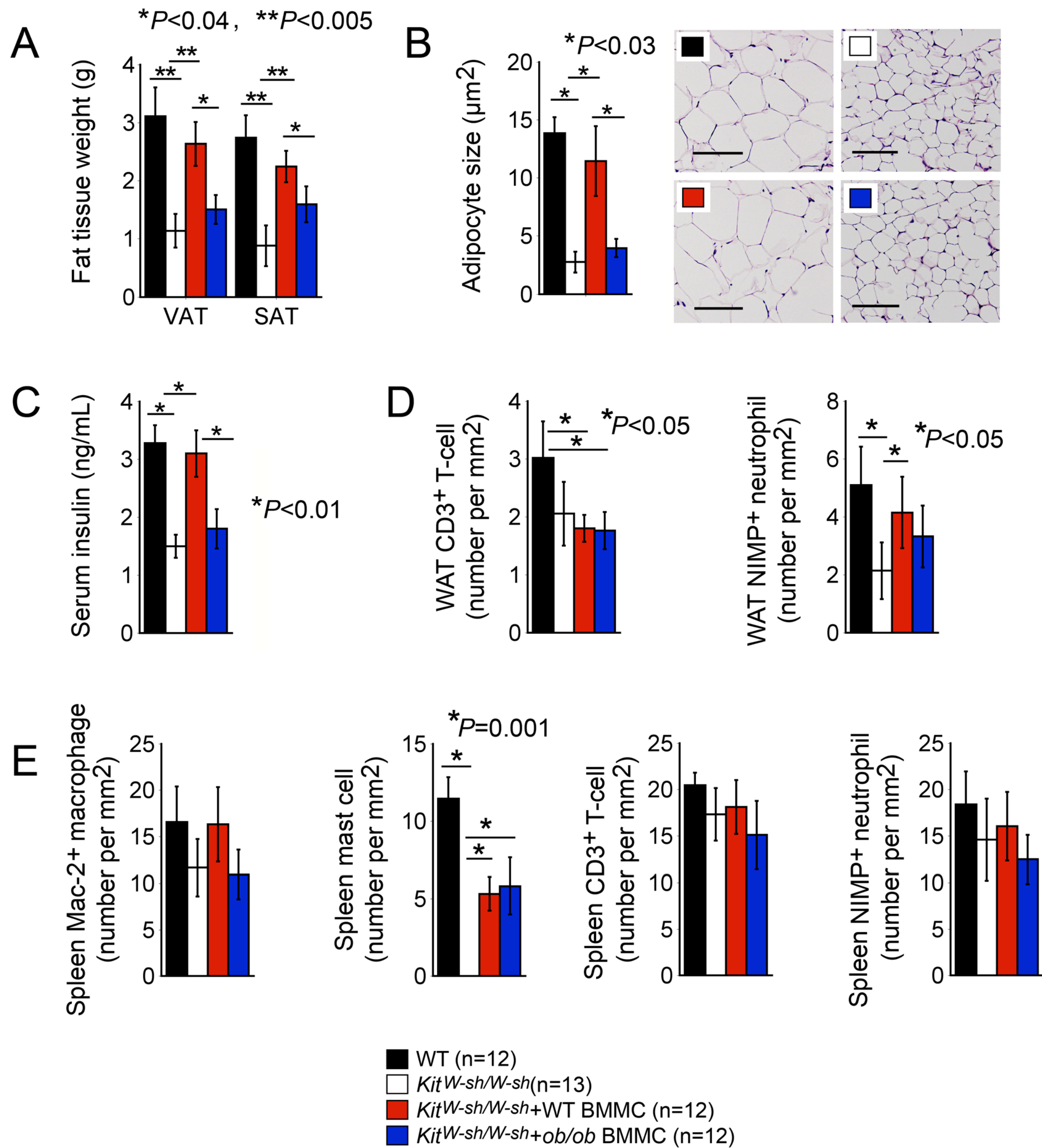

Figure S1, related to Figure 2. Obesity and diabetes in WT and MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice and those receiving BMMCs from WT and *ob/ob* mice. **A**. VAT and SAT weight. **B**. Adipocyte size. **C.** Serum insulin levels. Representative data for panel **B** (scale: 200 μm) are shown to the right. **D.** WAT $CD3^+$ T cells and $NIMP^+$ neutrophils. **E.** Mac-2^+ macrophages, toluidine blue stained MCs, $CD3^+$ T cells, and $NIMP^+$ neutrophils in spleens from different groups of mice as indicated. Number of mice per group is indicated in the parenthesis.

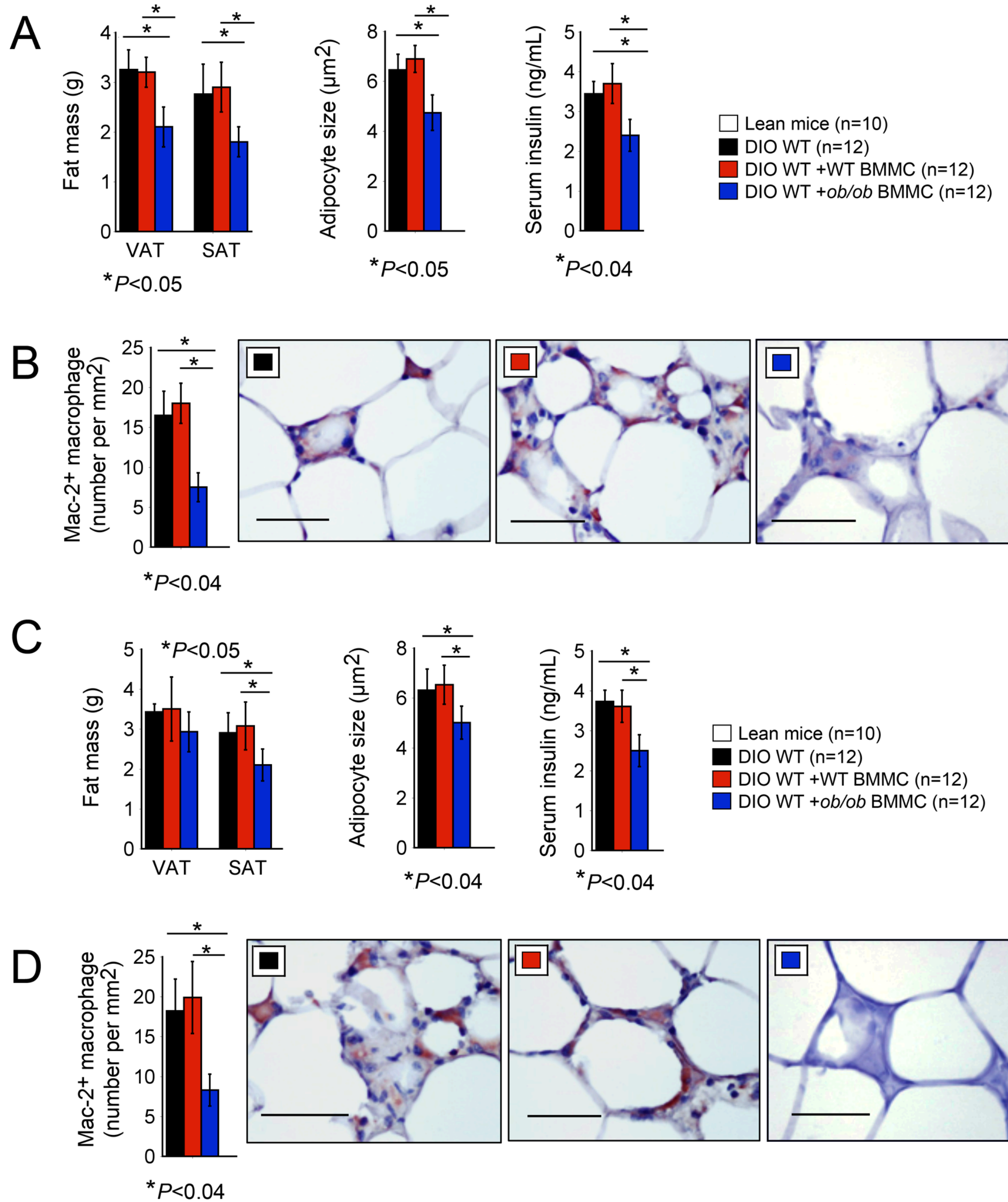

Figure S2, related to Figure 3. Obesity and diabetes in WT DIO mice (**A**, **B**) and those with pre-established obesity and diabetes (**C**, **D**) receiving WT and *ob/ob* BMMCs. **A**. VAT and SAT weight, adipocyte size, and serum insulin levels. **B**. Immunostaining determined Mac2$^+$ macrophage in WAT. **C**. VAT and SAT weight, adipocyte size, serum insulin levels. **D**. Immunostaining determined Mac2$^+$ macrophage in WAT. Number of mice per group is indicated in the parenthesis. Representative data for panels **B** and **D** are shown to the right, scale: 50 μm.

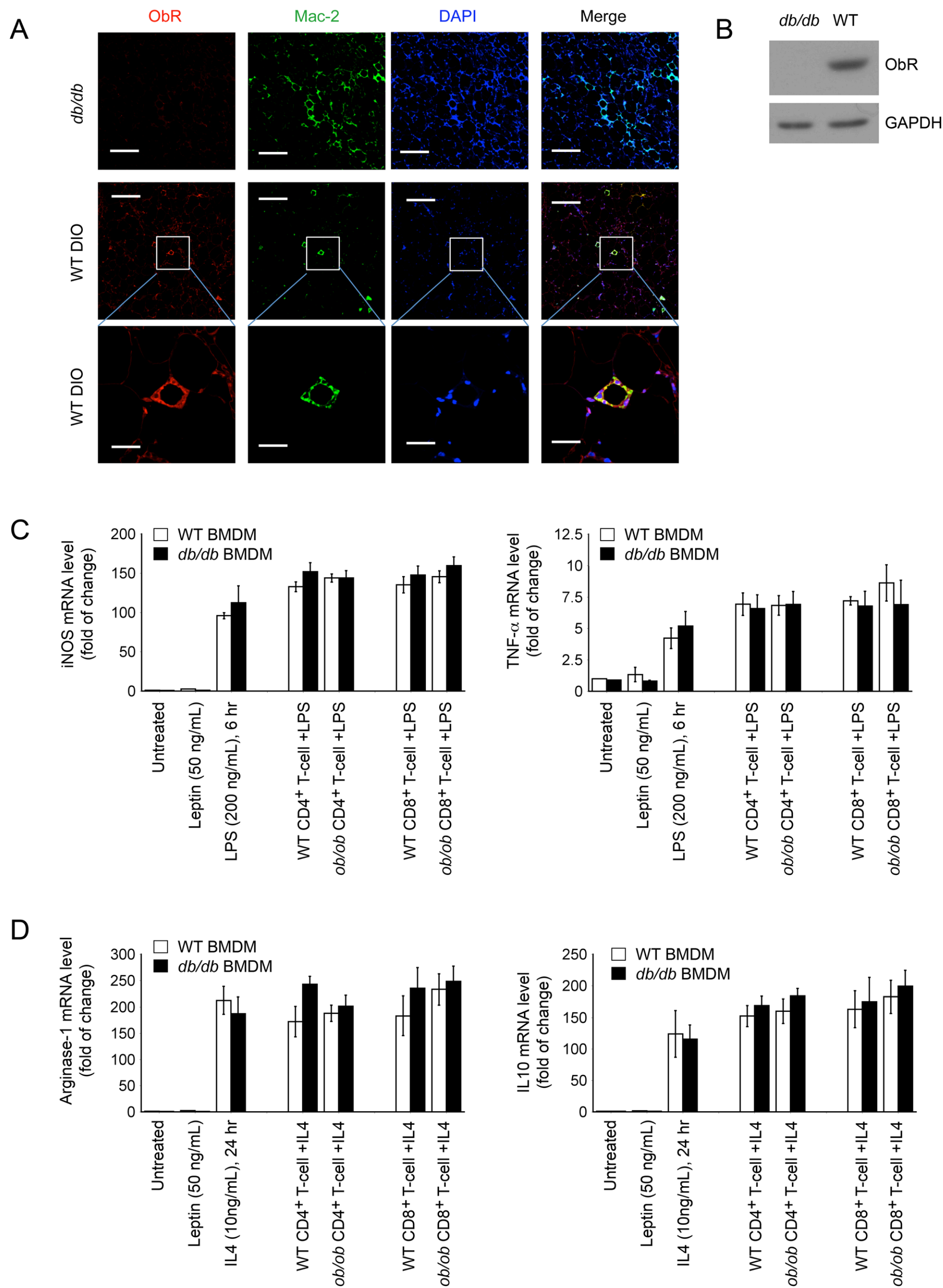

Figure S3, related to Figure 4. Expression of leptin receptor ObR in macrophages and leptin deficiency in T cells does not affect macrophages polarization. **A.** Immunofluorescent double staining of ObR and Mac-2 in WAT from WT DIO mice. WAT from leptin receptor-deficient *db/db* mice was used as negative control to confirm the anti-ObR antibody specificity. **B.** Immunoblot analysis of ObR expression in bone marrow-derived macrophages from WT and *db/db* mice. GAPDH blot was used to ensure equal protein loading. RT-PCR determined the expression of M1 markers iNOS and TNF-α (**C**) and M2 markers arginase-1 and IL10 (**D**) in BMDMs treated with and without leptin, LPS, IL4, $CD4^+$ and $CD8^+$ T cells as indicated.

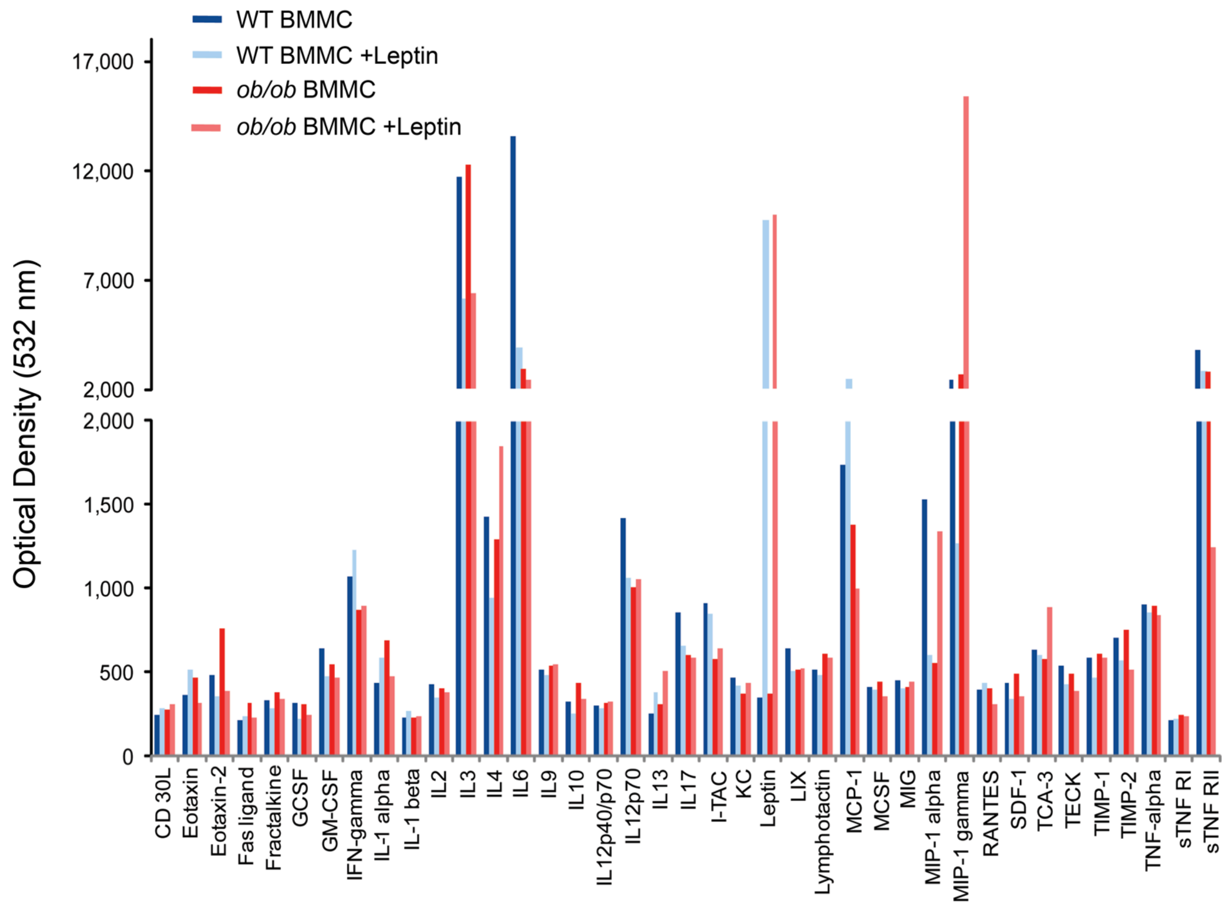

Figure S4, related to Figure 5. RayBio® cytokine antibody array to detect a panel of inflammatory molecules in culture media from WT and *ob/ob* BMMCs treated with and without leptin.

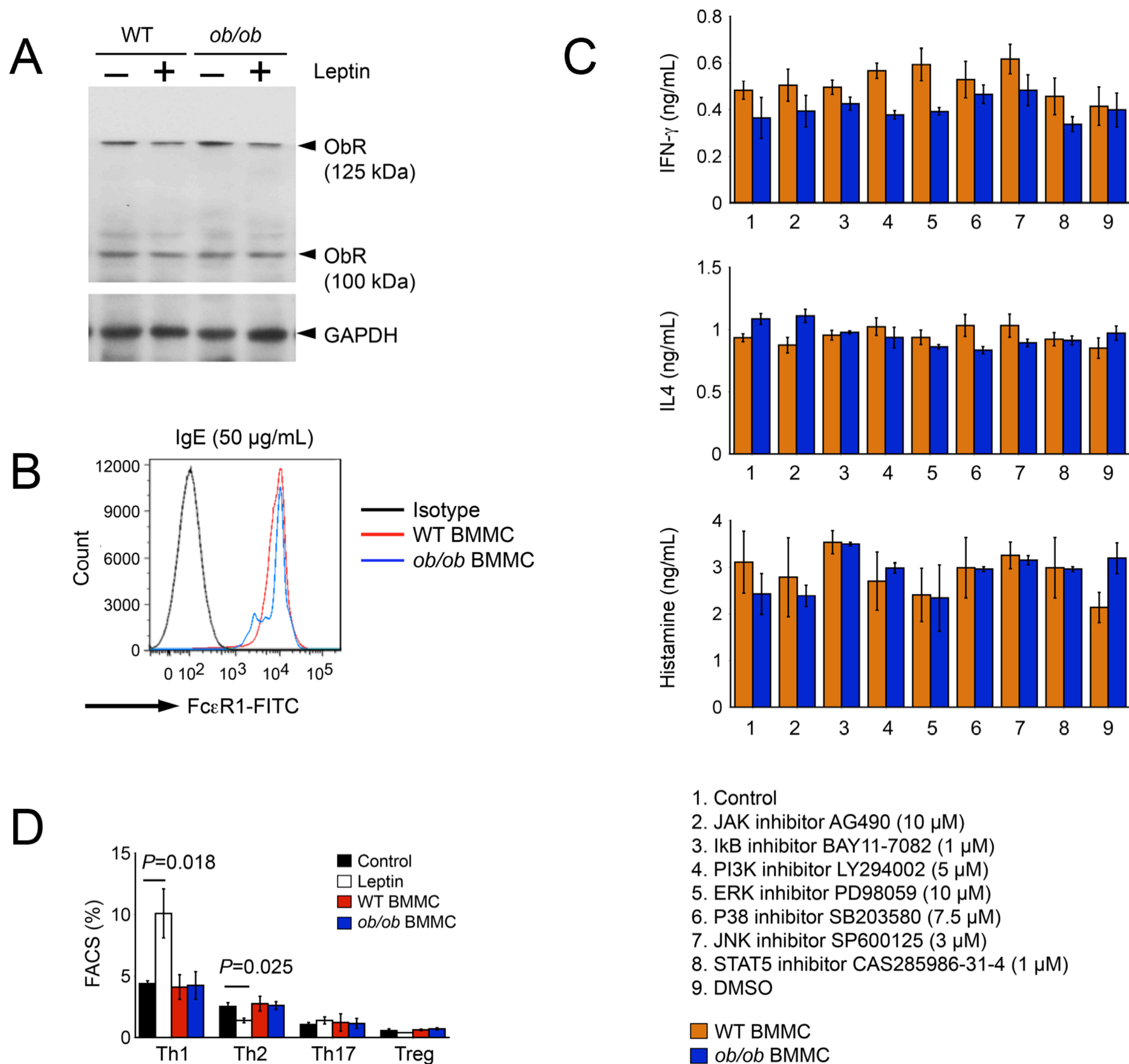

Figure S5, related to Figure 6. A. Immunoblot analysis of ObR in WT and *ob/ob* BMMCs treated with and without leptin. GAPDH blot ensured equal protein loading. **B.** FACS analysis of FcεR1 in IgE-treated BMMCs from WT and *ob/ob* mice. **C.** ELISA determined culture medium baseline IFN-γ, IL4, and histamine levels (without leptin stimulation) in WT and *ob/ob* BMMCs treated with and without different signaling molecule inhibitors or vehicle DMSO. **D.** FACS analysis of Th1, Th2, Th17, and Tregs in naïve CD4^{+} T cells treated with and without leptin, WT and *ob/ob* BMMCs.

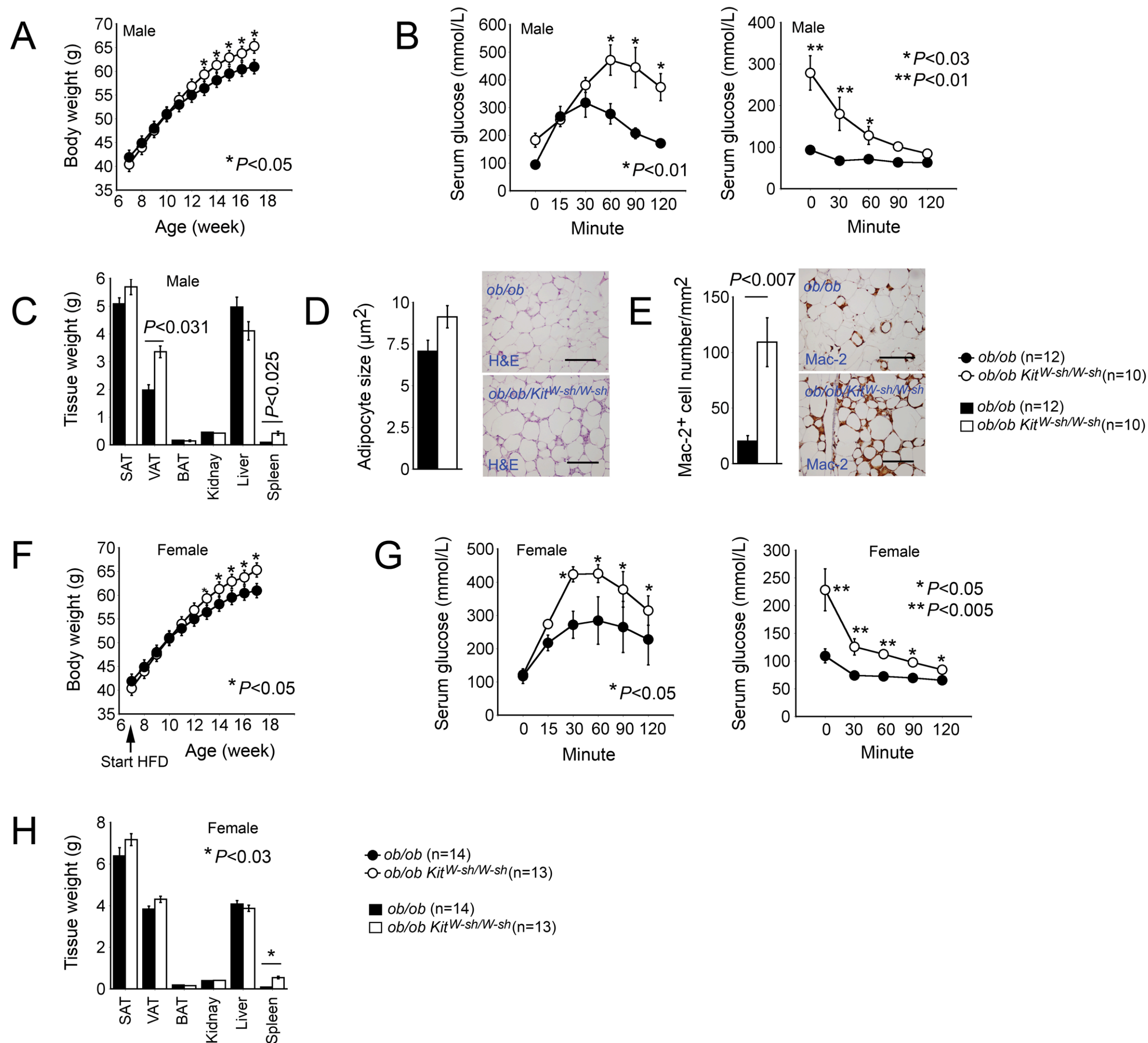

Figure S6, related to Figure 7. Genetic depletion of leptin-deficient MCs exacerbates obesity and diabetes in *ob/ob* mice. Body weight gain (**A**), glucose tolerance and insulin tolerance (**B**), tissue weight (**C**), WAT adipocyte size (**D**), and WAT Mac2^{+} macrophage content (**E**) in male *ob/ob* and *ob/ob* $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Body weight gain (**F**), glucose tolerance and insulin tolerance (**G**), and tissue weight (**H**) in female *ob/ob* and *ob/ob* $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Representative data for panels **D** and **E** are shown to the right. Scale: 200 µm. The number of mice per group is indicated in the parenthesis.